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Nano-emulsions of fluorinated trityl radicals as sensors for EPR oximetry

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ABSTRACT

This article reports the development and evaluation of two nano-emulsions (F45T-03/HFB and F15T-03/PFOB) containing fluorinated trityl radicals dissolved in perfluorocarbons. Preparation with a high-pressure homogenizer conferred sub-micronic size to both nano-emulsions. In vitro and in vivo EPR spectroscopy showed that the nano-emulsions had much greater oxygen sensitivity than the hydrophilic trityl, CT-03. In vivo experiments in rodents confirmed the ability of the nano-emulsions to follow the changes in oxygen concentration after induced ischemia. Histological evaluation of the tissue injected with the nano-emulsions revealed some acute toxicity for the F45T-03/HFB nano-emulsion but none for the F15T-03/PFOB nano-emulsion. These new formulations should be considered for further EPR oximetry experiments in pathophysiological situations where subtle changes in tissue oxygenation are expected.

1. Introduction

The role played by oxygen in physiopathological processes and therapeutics is of crucial importance. The development of non-invasive tools for monitoring tissue oxygenation may have profound diagnostic and therapeutic implications in oncology and in cardiovascular diseases. Existing methods for estimating pO_2 in tissues include magnetic resonance (MR)-based and non-MR-based techniques. Non-MR-based oximetry methods include polarographic oxygen electrodes, fluorescence quenching, phosphorescence quenching, near infra-red spectroscopy (NIRS), and the use of bioreductive markers selectively trapped in hypoxic regions. MR-based techniques include ^{19}F-NMR spectroscopy/imaging, blood oxygen level-dependent (BOLD) NMR imaging, electron paramagnetic resonance (EPR) spectroscopy/imaging, and dynamic nuclear polarization (DNP) which involves both NMR and EPR (for a review, see [1–3]).

In EPR oximetry, the oxygen content in tissues is measured by the effect of oxygen on the EPR spectrum of a paramagnetic oxygen sensor. These oxygen sensors can be classified into two groups: Particulate paramagnetic materials and soluble paramagnetic materials. The particulate probes include lithium phthalocyanine [4] and naphthalocyanine crystals [5], natural charcoals [6,7], analytical charcoals [8,9], India inks [10,11], and carbon blacks [12]. These materials possess high sensitivity to changes in oxygen concentration (large variation in EPR line width [LW] as a function of the pO_2), in vivo inertness, and a high spin density providing a high signal-to-noise ratio (SNR). However, for imaging applications, it may be interesting to use soluble probes that can diffuse inside tissues and provide information about the spatial distribution of oxygen within a tissue. Among soluble materials, two types of structure are particularly interesting: The nitroxides and the triarylmethyl (trityl or TAM) radicals. Various chemical properties of the nitroxides can be manipulated to modulate intracellular accumulation or tissue selectivity [13,14]. Nitroxides are metabolically converted to the EPR-silent hydroxylamines. Trityls are characterized by a narrower LW and a higher stability compared to nitroxides [15]. Trityls have been used as oxygen/pH probes [16–20], and as contrast agents in EPR [21] and DNP [22,23] imaging. Soluble paramagnetic probes are less sensitive to oxygen changes compared to particulate probes. This limited sensitivity to oxygen can be problematic for evaluating subtle changes in tissue oxygenation. To overcome this limitation, Liu and colleagues proposed encapsulating nitroxides in lipophilic environments [24]. Consistent with the Smoluchowski equation, as oxygen is more soluble in lipophilic environments than in water, an increase in sensitivity can be achieved using these systems. For this proof-of-concept, Liu et al. used nitroxides dissolved in common organic solvents and encapsulated in denaturized albumin microspheres with a size of 2.5 μm. More recently, Kuppusamy's group reported the synthesis [25] and use of a trityl radical (perchlorotriphenyl methyl-triester radical or PTM-TE in HFB). The authors also used bovine serum albumin (BSA) in order to encapsulate a solution of PTM-TE

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dissolved in HMDS [26]. HFB belongs to the class of perfluorocarbons (PFC) that have very high oxygen solubility. PFCs are able to dissolve up to about 50% their volume of oxygen [27].

It could be desirable to extend this concept to other PFCs which are biocompatible and already used clinically as blood substitutes (e.g., perfluorooctyl bromide (PFOB)). Moreover, it could be interesting to use a biocompatible nano-capsule as a vehicle, because BSA can generate immunogenic responses [28]. Furthermore, a nano-size formulation is suitable for parenteral administration and decreases uptake by the reticuloendothelial system after intravenous administration. To achieve these goals, we sought to enhance the affinity of the oxygen spin probe for a PFC formulation by attaching a perfluorinated tag on trityl radicals [29]. Introducing a fluorine label on a molecule enhances its fluorophilicity [30]. Two new trityl radicals (Fig. 1), called F15T-03 and F45T-03 (which contain three fluorine chains with 5 and 15 fluorine atoms, respectively), were recently synthesized by our group [29]. These radicals exhibit a single, sharp EPR spectrum. Calibration was performed in PFC solvents according to the respective solubility of the compounds. The oxygen sensitivities of F15T-03 in HFB and F45T-03 in PFOB were 1.87 μT/mm Hg and 1.75 μT/mm Hg, respectively [29]. This sensitivity was markedly increased compared to the hydrophilic trityl, CT-03 (0.064 μT/mm Hg). The aim of the present work was to develop and optimize the preparation of nano-emulsions containing these fluorinated trityls included in PFC solvents. These new oxygen sensor systems were characterized for their size, stability, and oxygen sensitivity in vitro and in vivo; and histological assessment of tissue reaction after administration was also conducted.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Fluka unless specified and were all ⩾99% grade products. F15T-03 and F45T-03 were synthesized as described previously [29]. The hydrophilic trityl, CT-03, was kindly provided by HJ Halpern (University of Chicago).

2.2. Nano-emulsion preparation

F15T-03 and F45T-03 have different solubilities in PFC. F15T-03 was soluble in HFB and not in PFOB, while F45T-03 was soluble in both solvents. The highest concentration of the compounds was chosen according to their solubility in PFC and the need to avoid spin–spin exchange: F15T-03 1 mM in HFB and F45T-03 0.25 mM in PFOB. Solubilization was performed overnight. The solutions were filtered on a 0.2 μm GHP filter (Pall Acrodisc) before further manipulation. The nano-emulsions were prepared using 3-sn-phosphatidylcholine from dried egg yolk (purity ⩾ 50%). 3-sn-phosphatidylcholine (3.120 g) was dispersed for 1 h in 100 ml PBS buffer. Eighteen (F15T-03/HFB nano-emulsion) or 16 (F45T-03/PFOB nano-emulsion) ml of the phospholipid dispersion were collected and further homogenized for 10 min with an Ultraturax (13,500 rpm). Two or 4 ml of the PFC solution (F15T-03 in HFB and FT45-03 in PFOB, respectively) containing the fluorinated trityl were added drop by drop to the phospholipid dispersion. The emulsion was stirred for 30 min with the Ultraturax before being placed in an EmulsiFlex C-5 high-pressure homogenizer (Avestin) working at 20,000 psi for 12–20 min (20–35 passes) (adapted from [34]). Final concentrations were 0.1 μmol F15T-03/0.1 ml HFB/25 mg lecithin and 0.05 μmol F45T-03/0.2 ml PFOB/28 mg lecithin for 1 ml of nano-emulsion.

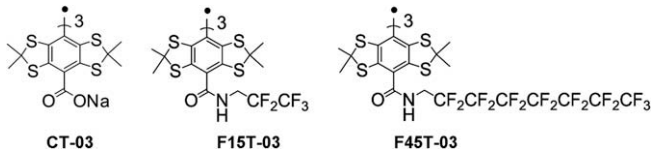

Fig. 1. Chemical structures of hydrophilic trityl CT-03, F15T-03 and FT45-03 fluorinated trityl.

2.3. Size measurement

Particle size was assessed using a Zetasizer Nano ZS (Malvern). The nano-emulsions were diluted (10 times) in PBS buffer to obtain 1 ml for the measurement. Values are expressed as a mean of three measurements ± standard deviation (SD).

2.4. In vitro EPR oximetry

As the oxygen solubility is temperature dependent, all in vitro and in vivo experiment were made at 37 °C. Calibration measurements (EPR LW as a function of the pO_2) were performed at 9.3 GHz with a Bruker EMX EPR spectrometer equipped with a variable temperature controller BVT-3000. The sample (nano-emulsion containing F15T-03 or FT45-03, or aqueous solution of the hydrophilic CT-03) was placed in a gas-permeable Teflon tube (0.625 mm inner diameter; 0.05 mm wall). This tube was folded twice at both ends and placed in a quartz tube open at both ends. Measurements were taken at 310 K and at 21% and 0% oxygen. The oxygen content in the gas was analyzed using a Servomex oxygen analyzer OA540. Typical spectrometer parameters were modulation amplitude less than one third of the peak-to-peak LW, and incident microwave power of 100 μW. The frequency modulation was set at 10 kHz for CT-03, and 100 kHz for the F15T-03 and FT45-03 nano-emulsions. A kinetics experiment was also performed to check that the phospholipid shell allowed equilibrium with the oxygen environment [31]. Air was flushed at 400 l/h into the EPR spectrometer cavity and a first spectrum was acquired. The flushing gas was then changed to N_2 and an EPR spectrum was recorded every minute. After equilibrium, the gas in the cavity was changed back to 21% oxygen.

2.5. In vivo EPR oximetry

Animal experiments were carried out in compliance with national care regulations and the local ethics committee (protocol 2004/UCL/MD/026). NMRI male mice (Janvier) and Wistar rats (Animalerie Facultaire, Université catholique de Louvain) were used for these experiments. The animals were anesthetized with isoflurane before administration of the nano-emulsions into the gastrocnemius muscle (150 μl in mice and 500 μl in rats). In vivo EPR spectra were recorded using an EPR spectrometer (Magnettech, Berlin, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and an extended loop resonator. Local hypoxia was induced by restriction of the blood supply in the muscle; the base of the thigh was reversibly tied with an elastic band to restrict blood flow through the femoral arteries. Measurements ($n = 5$ per group) were carried out during normoxia (3 min after nano-emulsion injection) and 5 min after inducing ischemia. Infra-red lights have been used in order to maintain the temperature of the animals. Parameters for experiments with CT-03 were: center field: 53 mT; modulation amplitude: 0.00315 mT; modulation frequency: 10 kHz; power: 600 μW. For F15T-03 and F45T-03 nano-emulsions, the acquisition parameters were: center field: 53 mT; sweep width: 1.0 mT; modulation amplitude: 0.021 mT; modulation frequency: 100 kHz; power: 23.8 mW.

2.6. Histological evaluation

The effect of the nano-emulsions on the muscle tissue was assessed by histological examination. Thirty microliters of F15T-03/HFB or F45T-03/PFOB were administered intramuscularly. Four or 24 h later, mice were sacrificed and the muscle into which the nano-emulsion had been administered was carefully removed. After fixing the samples ($n = 2$) in 10% formalin for 48 h, they were embedded in paraffin blocks and prepared into histological slices (5 μm thickness). Slices were stained using a classical hematoxylin–eosin procedure. Picture captions were taken using an optical microscope (Nikon Alphashot-2 Y52) (magnification 100×) and a digital photo camera, Nikon CoolPix 4500.

3. Results and discussion

3.1. Size measurement and stability of the nano-emulsions

The homogenizing procedure was critical in order to obtain a stable nano-emulsion. Without high-pressure homogenization, it was impossible to obtain a stable emulsion. After the nano-emulsion had been processed, particle size measurements were performed. The F15T-03/HFB nano-emulsion was characterized by an average diameter of 112 ± 1 nm and a polydispersity index (PDI) of 0.223 on the day of preparation. For the F45T-03/PFOB nano-emulsion, the average diameter was 182 ± 3 nm and the PDI 0.158. This distribution size would allow intravascular administration of these nano-emulsions. The size distribution remained stable for at least one month for the F15T-03/HFB nano-emulsion and two weeks for the F45T-03/PFOB nano-emulsion (data not shown).

3.2. In vitro EPR oximetry

When changing the oxygen environment, the nano-emulsions containing F15T-03/HFB and F45T-03/PFOB had larger variations in LW compared to the hydrophilic CT-03 trityl. The ΔLWs when changing from air to nitrogen were 0.2336 mT (0.0554 mT and 0.289 mT at 0% and 21% oxygen) for the F15T-03/HFB nano-emulsion, 0.0962 mT (0.0548 mT and 0.151 mT at 0% and 21% oxygen) for the F45T-03/PFOB nano-emulsion and 0.010 mT (0.009 mT and 0.019 mT at 0% and 21% oxygen) for the CT-03 aqueous solution. From these data, it is possible to calculate the % increase in LW compared to the values measured at 0% oxygen. The increase in LW were 521%, 275% and 211% for the nano-emulsion F15T-03/HFB the F45T-03/PFOB nano-emulsion and the CT-03 solution, respectively. These data demonstrate that both nano-emulsions have increased sensitivity to oxygen variation.

Interestingly, the LW equilibrium values in air were comparable for F15T-03 when dissolved in HFB or encapsulated in the nano-emulsions. However, the LW values obtained for F45T-03 were larger when this compound was dissolved in PFOB [29] than when it was encapsulated in PFOB (0.334 mT in PFOB versus 0.151 mT in the nano-emulsion). Importantly, the calibration (LW as a function of pO_2) remained stable over time (more than one week, data not shown). The kinetics experiment clearly showed that the phospholipid shell around the dispersed PFC nano-droplets was totally permeable to gaseous oxygen exchange. As shown in Fig. 2, the equilibrium of the nano-emulsion of F15T-03 with the gas environment was achieved within less than 5 min when changing from air to nitrogen and vice-versa.

3.3. In vivo EPR oximetry

After intramuscular administration of hydrosoluble CT-03 trityl or F15T-03 or F45T-03 nano-emulsions, the animals were placed in our L-band EPR spectrometer. At this frequency, EPR can be carried out with a depth of penetration of about 1 cm, which is optimal for studies in rodents. The LWs (means ± SD) observed in normoxic muscle were 0.0134 ± 0.0011 mT for the CT-03 solution, 0.092 ± 0.0044 mT and 0.079 ± 0.0025 mT for F15T-03 and F45T-03 nano-emulsions, respectively. These values correspond to pO_2 values (means ± SD) of 70.3 ± 18.2 mmHg for the CT-03 solution, 25.0 ± 3.0 mm Hg and 40.8 ± 4.1 mm Hg for F15T-03 and F45T-03 nano-emulsions, respectively. These values are consistent with previously published data recorded in muscles of rodents anesthetized with isoflurane [32,33]. The differences between the two nano-emulsions are not significant in term of pO2. The apparent discrepancy could come from the inflammatory reaction that has been observed with the use of the F15T-03/HFB nano-emulsion. The important standard deviation using CT-03 can be attributed to the limited resolution accuracy when measuring very small LW.

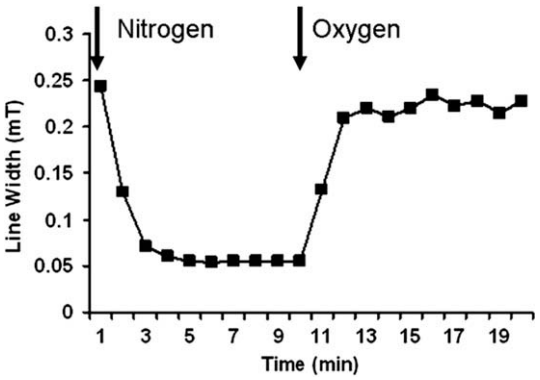

Fig. 2. Kinetic of fast oxygen exchanges in nano-emulsions of F15T-03. Nitrogen was flushed into the chamber for 10 min starting from time 0 (first arrow); at 11 min oxygen was flushed (second arrow).

The variation in LW after interruption of blood flow was significantly lower with the hydrophilic CT-03 than with the nano-emulsions. The hydrophilic CT-03 solution had a ΔLW of 0.0038 ± 0.0023 mT, compared to 0.0208 ± 0.0096 mT and 0.0194 ± 0.0071 mT for F15T-03 and F45T-03, respectively (Fig. 3). This corresponds to a fivefold increase in sensitivity to oxygen variations in vivo. This increase in oxygen sensitivity is appreciable when monitoring subtle modulations of oxygen in vivo. However, a disadvantage of using nano-emulsions is the decrease in signal intensity obtained in vivo compared to the hydrophilic compound, CT-03. This is the result of three different phenomena: (1) an intrinsically larger LW even at low pO_2 (the signal intensity is inversely proportional to the square of the EPR LW); (2) the need

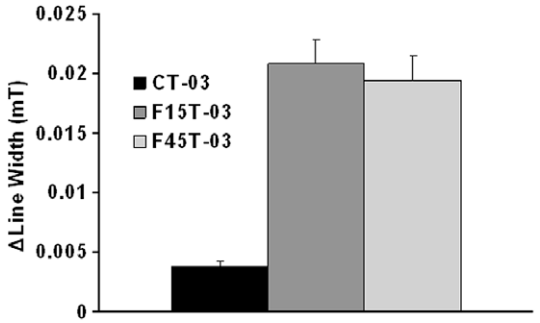

Fig. 3. In vivo ΔLW of measurement at normoxia and after leg ligation in mice injected with the trityl CT-03 (black columns), or the F15T-03/HFB nano-emulsion (dark gray columns), and rats injected with the F45T-03/PFOB nano-emulsion (light gray columns).

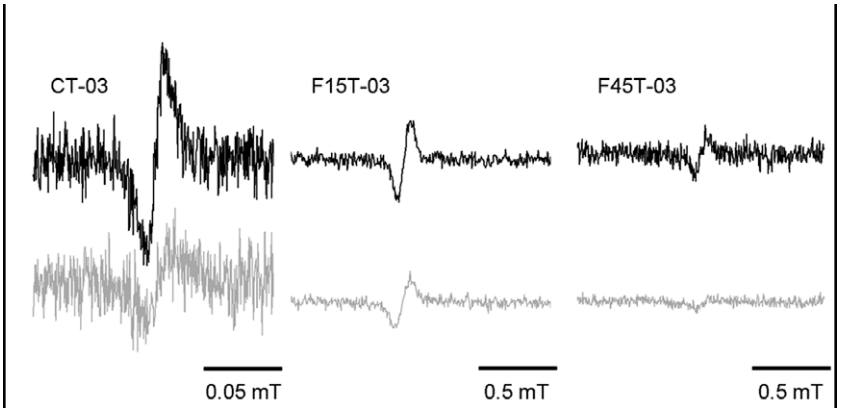

Fig. 4. In vivo spectrum of measurement at normoxia (gray) and after leg ligation (black). Left: CT-03; center: F15T-03/HFB; right: F45T-03/PFOB.

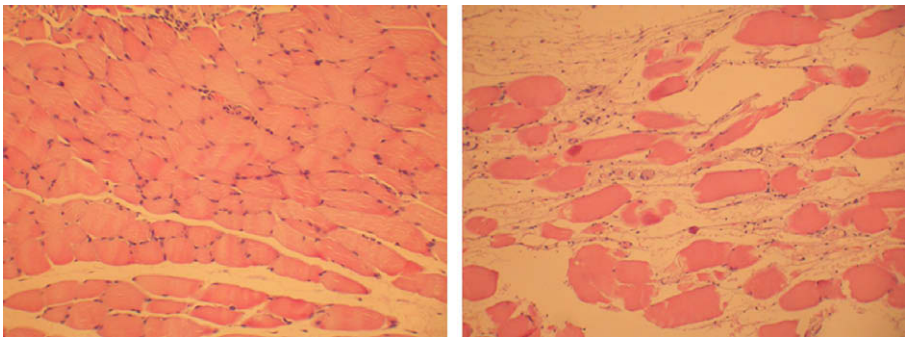

Fig. 5. Histological sections of muscles after administration of F45T-03/PFOB at 4 h (left) and F15T-03/HFB at 4 h (right).

to avoid a spin–spin exchange in the starting PFC solution (concentration < 5 mM); (3) the dilution factor when preparing an emulsion of PFC in water solutions. This difference in signal intensity is illustrated in Fig. 4.

3.4. Reactivity in tissues and histology

After intramuscular administration, the F15T-03/HFB emulsions rapidly produced edema in the muscle, but there was no visible reaction with the F45T-03/PFOB nano-emulsions. To better characterize this phenomenon, a preliminary histological examination was carried out. No inflammation was detected either at 4 or at 24 h after injection (Fig. 5, left panel) in mice which had received the F45T-03/PFOB nano-emulsion. This is in accordance with the considerable amount of work investigating the use of this PFC as a blood substitute [34]. The situation was different in the group that received F15T-03/HFB. As shown in Fig. 5 (right panel), an inflammatory process was observed after administration of this compound, characterized by considerable edema, eosinophilia, abnormalities in the muscle cell structure, and lack of cell nucleus. This situation persisted 24 h after administration of the nano-emulsion.

4. Conclusions

We have developed new systems that incorporate fluorine trityls in nano-emulsions containing PFC. These compounds were evaluated for their performance as EPR oxygen sensors. Stable formulations were achievable using high-pressure homogenization, and nano-emulsions were usable for at least two weeks after preparation. A large increase in sensitivity to oxygen was observed both in vitro and in vivo when using these nano-emulsions compared to hydrophilic trityls. When comparing the two nano-emulsions, the F15T-03/HFB nano-emulsion had the highest sensitivity in vitro to oxygen variation. Moreover, its higher solubility (up to 1 mM in HFB) allowed preparation of a nano-emulsion detectable in vivo with a high signal-to-noise ratio. However, in vivo this compound induced an inflammatory reaction and edema. The F45T-03/PFOB nano-emulsion, while being apparently less efficient, is still five times more sensitive to oxygen than hydrophilic trityls. This formulation did not induce any inflammatory response in preliminary tests, and should be considered further as a system to monitor subtle changes in tissue oxygenation in vivo.

Acknowledgments

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