International Journal of Pharmaceutics 554 (2019) 87–92

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Preparation and evaluation of trityl-loaded lipid nanocapsules as oxygen sensors for electron paramagnetic resonance oximetry

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ARTICLE INFO

Keywords:
Electron paramagnetic resonance
Oximetry
Hypoxia
Lipid nanocapsules
Trityl
Nitroxide

ABSTRACT

Oxygen is essential in physiology and pathophysiology. Electron paramagnetic resonance (EPR) oximetry, using oxygen sensitive paramagnetic materials, could be attractive for measuring oxygen in tissues. The aim of the present study was to assess the properties of lipid nanocapsules (LNCs) loaded with the nitroxide tempo-benzoate (TB) or tetrathiatriarylmethyl (TAM) radicals. LNCs loaded with the EPR probes were successfully prepared by the phase inversion process leading to nanocapsules of about 60 nm. LNCs protected the TB radical against reduction *in vitro*. The calibration of the EPR line width (LW) as a function of the pO_2 showed a two-fold increase in sensitivity with TAM-LNC compared to hydrophilic trityl radical. The TAM-LNCs were evaluated *in vivo*. Contrarily to unencapsulated TAM, for which a rapid decrease in EPR signal was observed, the half-life of TAM-LNCs administered in muscles or in tumours exceeded an hour. Carbogen-challenges in mice demonstrated that the TAM-LNCs responded well to changes in oxygen environment. However, the apparent pO_2 values acquired were higher than the expected physiological values. These results warrant further investigation in the formulation of stable nano-objects encapsulating EPR oxygen sensitive probes.

1. Introduction

Tissue oxygenation is a key parameter in physiology and pathophysiological conditions, including wound healing, ischemia, peripheral vascular disease and cancer. In oncology, tumour hypoxia is known to decrease the response to radiation therapy (Gray et al., 1953; Mottram, 1931), decrease the efficacy of cytotoxic drugs (Littlewood, 2001; Teicher et al., 1990), and play a pivotal role in malignant progression including metastasis (reviewed in Brown and Giaccia, 1998; Dachs and Tozer, 2000; Lu and Kang, 2010; Semenza, 2008, 2000; Sutherland, 1998). Therefore, appropriate knowledge of tumour oxygenation may help in guiding anti-cancer treatments. An assortment of different techniques has been developed for measuring tumour oxygenation, including electron paramagnetic resonance (EPR) oximetry. EPR oximetry is capable of producing real-time, repeated measurements of partial pressure of oxygen (pO_2) values in a non-invasive manner without consuming O_2. This technique uses paramagnetic spin probes which can be either in solutions or in particulate forms. Interactions between the unpaired electron spin centres (Springett and Swartz, 2007; Swartz et al., 2014) of the probes and oxygen molecules cause a shortening of relaxation times, producing a broadening effect on the EPR line width (LW) of the spin probes. Soluble probes have the advantage of diffusing throughout a tissue and providing estimates of pO_2 values. However, they are also prone to signal decrease due to rapid washout and/or metabolic biotransformation. The encapsulation of EPR spin probes could potentially circumvent these disadvantages (Charlier et al., 2009; Dhimitruka et al., 2016; Frank et al., 2015; Gallez and Mäder, 2000; Glockner et al., 1991; Liu et al., 1994; Sostaric et al., 2007).

The aim of the present study was to explore the possible use of lipid nanocapsules (LNCs) as an encapsulation entity for hydrophobic spin probes including nitroxides and tetrathiatriarylmethyl (TAM) free

Abbreviations: ATCC, American type culture collection; C3H, mouse strain; DMEM, Dulbecco modified eagle medium; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; FSaII, fibrosarcoma murine cell line; LNCs, lipid nanocapsules; LW, line width; mW, milliwatt; MW, molecular weight; MWCO, molecular weight cut off; NaCl, sodium chloride; PDI, polydispersity index; pO_2, partial pressure of oxygen; SD, standard deviation; TAM, trityl tetrathiatriarylmethyl (F15T-03); TB, 4-hydroxy-TEMPO benzoate; TEMPO, 2,2,6,6-tetramethylpiperidine 1-oxyl

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https://doi.org/10.1016/j.ijpharm.2018.11.007
Received 1 July 2018; Received in revised form 8 October 2018; Accepted 2 November 2018
Available online 03 November 2018
0378-5173/

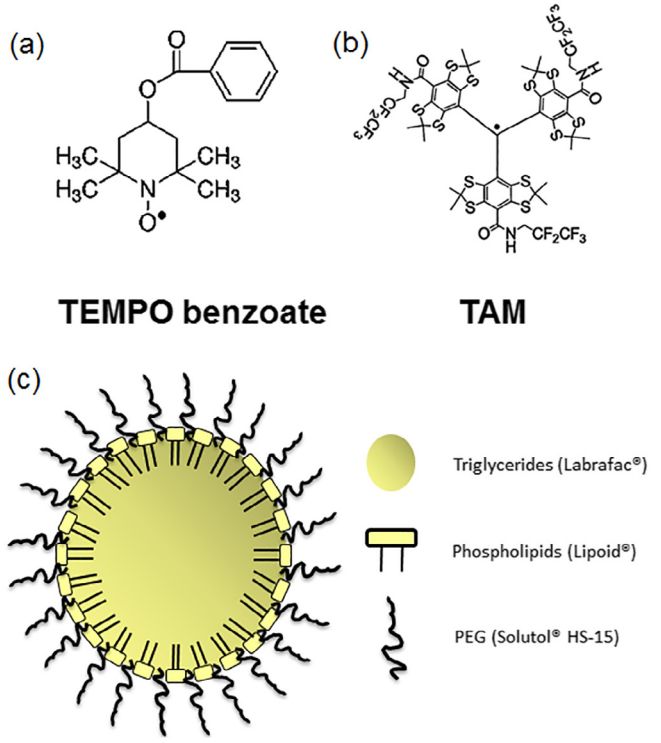

Fig. 1. Chemical structure illustrations of (a) TEMPO benzoate and (b) F15T-03 (TAM), and a schematic diagram of lipid nanocapsules (LNCs) (c).

radicals. LNCs are core-shell based colloidal nano-structures where the core acts as an oily reservoir for loading hydrophobic molecules and/or drugs, and the shell acts as a protective, polymer membrane (Huynh et al., 2009; Sánchez-Moreno et al., 2012). Furthermore, due to this polymeric shell, LNCs are capable of avoiding detection by the immune system thus increasing circulation time (Basile et al., 2012; Hirsjärvi et al., 2013; Hureaux et al., 2009; Mohanraj and Chen, 2006). The encapsulation of lipophilic oxygen sensors in biocompatible, O_2 permeable LNCs (Lemaire et al., 2013) are of particular interest because the sensitivity of spin probes to oxygen can be enhanced by their encapsulation in a lipophilic carrier as the response is greatly increased by the high solubility of oxygen in lipids. Because nitroxides and TAM radicals could be used in *in vivo* EPR oximetry, we sought to encapsulate 4-hydro-TEMPO benzoate (TB) and tetrathiatriarylmethyl (F15T-03, herein referred to as TAM) within LNCs (Fig. 1). The probe-encapsulated LNCs were investigated regarding their physicochemical properties and oxygen sensitivity *in vitro*. For *in vivo* experiments, the performances of TAM-loaded LNCs as oxygen sensors were assessed in muscles and in tumours during carbogen respiratory challenges.

2. Materials and methods

2.1. Materials

Labrafac® WL 1349 (caprylic-capric acid triglycerides) was purchased from Gattefossé S.A. (Saint-Priest, France). Lipoïd S75-3 (soybean lecithin at 69% of phosphatidylcholine) and Kolliphor® HS 15 (mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate) were provided by Lipoïd GmbH (Ludwigshafen, Germany) and BASF (Ludwigshafen, Germany), respectively. The nitroxide 4-hydroxy-TEMPO benzoate (TB) was supplied by Sigma Aldrich Chemie GmbH (Steinheim, Germany), whilst the TAM (tetrathiatriarylmethyl, F15T-03) radical was synthesised as previously described (Driesschaert et al., 2008). NaCl was purchased from Prolabo (Fontenay-sous-Bois, France). Water was obtained from a Milli-Q (Millipore) ultra-filtration system. No metal contaminates such as iron were observed in the EPR spectra.

2.2. Preparation of EPR probe encapsulated lipid nanocapsules

The preparation of nitroxide TB- and TAM-loaded LNCs (henceforth referred to as TB- and TAM-LNCs) was based on a phase inversion method (Heurtault et al., 2002). In brief, the EPR probe was weighed, and dissolved in Labrafac® (oil phase) to reach a final concentration of 10 mM. 1.028 g of Labrafac, containing the respective probe, was mixed with water (2.962 g), NaCl (0.148 g), Kolliphor® HS15 (0.846 g) and Lipoïd (0.075 g), and heated from ambient temperature to 95 °C whilst undergoing magnetic stirring. Subsequently, three heating and cooling cycles from 50 °C to 95 °C were performed. After the third cooling, once the temperature reached 72 °C, a rapid cold dilution was performed by adding 1 mL of 4 °C Milli-Q water.

2.2.1. Size characterisation

The average hydrodynamic size and polydispersity index (PDI) of the probe-loaded LNCs were determined by dynamic light scattering using a Malvern Zetasizer® (Nano Series DTS 1060, Malvern Instruments S.A., Worcestershire, UK) fitted with a 633 nm laser beam (helium–neon laser, 4 mW) at a fixed scattered angle of 173°. The formulations were diluted with pure water (dilution factor: 60) before measurement. Values are expressed as mean of three measurements ± standard deviation (SD).

2.2.2. Osmolarity adjustment of EPR probe loaded LNCs

Dialysis of the synthesised probe loaded LNCs (1 mL) was performed using dialysis tubing (Spectra Biotech Cellulose Ester Dialysis Membrane, MWCO = 100 kDa) for 24 h against pure water (obtained through a Milli-Q apparatus), changing the water hourly for the first 6 h. Then, to reobtain the original volume of LNCs (1 mL), the dilution factor of the dialysed solution was calculated and evaporation, using N_2, was applied under magnetic stirring. Once the initial volume was obtained, evaporation was ceased and 30 µL of a salt solution (320 mg/mL) was added to adjust the osmolarity of the formulation. Osmolarity measurements were performed using a 5520 Vapro vapour osmometer from Wescor (Logan, Utah, USA).

2.3. In vitro EPR measurements

2.3.1. In vitro EPR spectra acquisition

EPR spectra were acquired with a Bruker EMX-Plus spectrometer (Bruker, Rheinstetten, Germany), operating in X-band (9.85 GHz) and equipped with a PremiumX ultra low noise microwave bridge and a SHQ high sensitivity resonator. General settings were as follows: incident microwave power of 2.5 mW for TB and 0.3 mW for TAM; modulation frequency: 100 kHz; sweep width: 50 G for the nitroxide and 10 G for TAM; modulation amplitude: 0.6 G for TB and 0.2 G for TAM; scan time: 10 s.

2.3.2. Ascorbic acid assay

The protective properties of LNCs were investigated with an ascorbic acid reduction assay (Rübe and Mäder, 2005). This was achieved by incubating the TB-LNCs in ascorbic acid, a compound known to reduce the paramagnetic nitroxide into diamagnetic hydroxylamine. Nitroxide-loaded LNC formulations were combined 1:1 with a 2.5 mM ascorbic acid solution, and an aliquot of 100 µL was placed in an open quartz capillary tube (0.5 mm diameter). The non-encapsulated TB (dissolved in dimethyl sulfoxide (DMSO)) was used for comparison by mixing 1:1 with a 2.5 mM ascorbic acid solution diluted with Milli-Q water. EPR spectra were repeated over time to measure the decrease in signal intensity. All measurements were done in triplicate.

2.3.3. In vitro oxygen calibration curve

A calibration curve (LW as a function of pO_2) was obtained for both probe encapsulated-LNCs. LWs were obtained at various oxygen levels to determine the sensitivity of the LW to pO_2. Measurements were performed using the same spectrometer as mentioned in Section 2.3.1, equipped with a variable temperature controller (ER 4131 VT) equilibrated at 310 K. Samples were placed in a gas-permeable Teflon tube (0.625 mm inner diameter, 0.05 mm wall), which was folded and placed in a quartz open ended EPR tube. The oxygen content was varied between 0% and 21% O_2 by using an Aalborg gas mixer. The oxygen content in the mixed gas was measured by a Servomex MiniMP 5200 oxygen analyser (the precision of which is 0.1% with oxygen content).

2.4. In vivo experiments

2.4.1. Animal models

The syngenic FSaII fibrosarcoma murine cell line was provided by ATCC and grown in Dulbecco's modified Eagle's medium (DMEM) high glucose GlutaMAX supplemented with 10% horse serum and 1% antibiotic and antimycotic solution (Gibco, Thermo Fisher Scientific). Animal studies were undertaken in accordance with Belgian and the Université catholique de Louvain ethical committee regulations (2014/UCL/MD/026). Two groups of male C3H mice (Janvier, Le Genest-Saint-Isle, France) were used; one group for intramuscular experiments (n = 17) and another for intratumoural (n = 14). For the tumour model, 2×10^6 FSaII cells in 100 µL serum-free DMEM were inoculated subcutaneously into the hind thigh (gastrocnemius muscle) of mice. Tumour size was measured daily and experiments were completed when tumours reached 8–10 mm in diameter.

2.4.2. Kinetic studies of TAM-LNCs

EPR spectra were acquired *in vivo* with a low-frequency microwave bridge operating at 1.2 GHz L-band EPR spectrometer (Magnettech, Berlin, Germany) with an extended loop resonator possessing an inner diameter of 12.5 mm, and a thickness of 2 mm. Animals were anaesthetised using isoflurane (Forene, Abbot, Queensborough, UK): 3% inhalation mixed with continuous air flow (2 L/min) for induction, and 1.5% isoflurane for anaesthesia maintenance. A circulating water system was used for mouse-body temperature regulation. 50 µL injection of either TAM-LNCs or TAM dissolved in DMSO (10 mM) was injected intramuscularly or intratumourally (for intramuscularly; n = 5 encapsulated TAM-LNCs and n = 2 of unencapsulated TAM dissolved in DMSO, for intratumourally; n = 3 encapsulated TAM-LNCs and n = 2 of unencapsulated TAM dissolved in DMSO) in anaesthetised animals. Prior to the injection of TAM dissolved in DMSO, an occlusion of the femoral artery using a rubber band was performed to enable the measurement of the signal. The animal was placed in a side-way position to enable the access of the experimental gastrocnemius muscle, and the loop resonator was placed over the area of injection. The first EPR spectrum was acquired using the low-frequency EPR spectrometer 2 min after the injection, and this signal intensity was defined as the 100% signal value. All other values were calculated relatively.

2.4.3. Intramuscular and intratumoural EPR measurements using TAM-LNCs during carbogen challenge

Animal anaesthesia and preparation for EPR spectra acquisition with an L-band EPR spectrometer was as previously described in Section 2.4.2. 50 µL of TAM-LNCs were injected intramuscularly (n = 10) or intratumourally (n = 9) into the anaesthetised animal. An EPR spectrum was recorded whilst the animal was breathing air (basal pO_2). Another EPR acquisition was performed after a 20 min carbogen (95% O_2 and 5% CO_2) challenge.

2.5. Statistical analysis

For the *in vitro* characterisation of probe loaded-LNCs, the data are presented as mean ± SD from triplicate experiments. For *in vivo* pO_2 measurements with TAM-LNCs, the paired Student's *t*-test was implemented, wherein a p value of < 0.05 was considered significant.

Table 1
Characteristics of EPR probe-loaded LNC preparations.

Preparation	Particle size (nm)[1]	PDI[1]	LW_{air} (G)
TB-LNCs	60.8 ± 0.2	0.195 ± 0.007	1.92[2]
TAM-LNCs	57.4 ± 0.9	0.165 ± 0.015	0.71

[1] Mean ± SD (n = 3).
[2] Measured on the central peak of the nitroxide.

3. Results and discussion

3.1. Probe loaded-LNCs characterisation

The phase inversion process was exploited to obtain lipophilic EPR probes encapsulated within the lipidic core of the LNCs. The physicochemical properties of different types of the LNC preparations are presented in Table 1. The size of TB-LNCs and TAM-LNCs were 60 nm and 57 nm, respectively with a PDI under 0.2. The size of the probe loaded-LNCs was comparable to previously studied standard blank LNCs which showed good stability stored at 4 °C from 12 to 18 months (Heurtault et al., 2003; Thomas and Lagarce, 2013). Both probe loaded-LNCs types exhibited an EPR signal demonstrating the effective inclusion of TB and TAM in the LNCs (Fig. 2). Of note, our attempt to prepare TEMPO loaded-LNCs was not successful as no EPR signal was recorded after the preparation. This could be due to the lower lipophilicity of TEMPO (log P = 1.40) compared to TB (log P = 3.10) (Kim et al., 2016). Interestingly, the intensity of the three EPR lines in the EPR spectrum of TB-LNCs was not equivalent, with a reduced height of the third line (Fig. 2), indicative of motion restriction of TB within the LNCs. As expected the LW of the TAM-LNCs (equilibrated in air at room temperature) was narrow (0.71 G, under air) compared to the LW of the TB-LNCs (1.92 G, measured on the central peak of the nitroxide). To further ascertain the entrapment of the probes inside the LNCs, we used an ascorbic acid assay on TB-LNCs. It is well established that ascorbic acid rapidly reduces nitroxides in solution, and similarly it was found for unencapsulated TB (Fig. 3): the signal intensity of TB completely

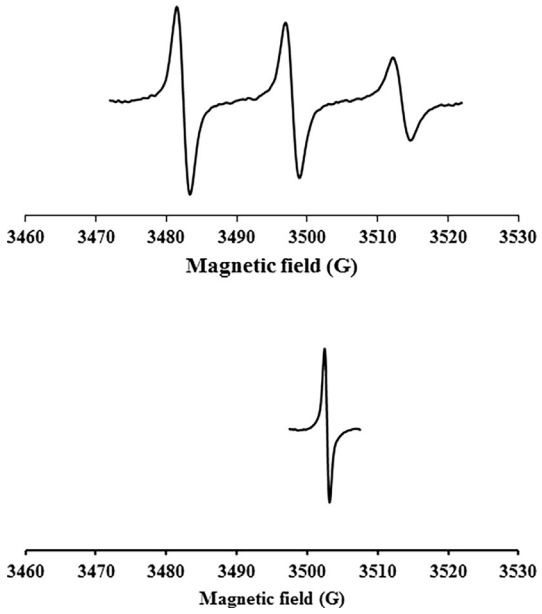

Fig. 2. EPR spectra acquired *in vitro* (under air) of TB-LNCs (top) and TAM-LNCs (bottom).

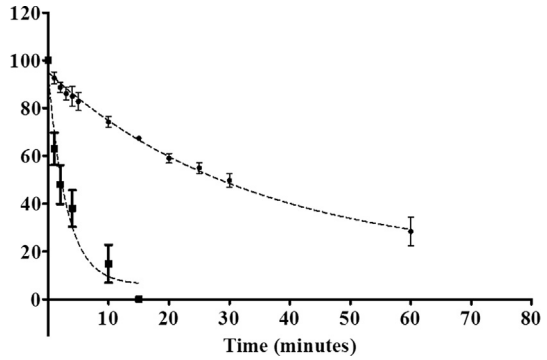

Fig. 3. Evolution of EPR signal intensity over time of unencapsulated TB (squares) or TB-LNCs (circles) in the presence of ascorbic acid. Results are expressed as relative to the initial signal intensity (mean ± SD) (n = 3) wherein the dotted line indicates the trend.

disappeared within approximately 10 min in the presence of ascorbic acid. Conversely, the signal intensity of TB-LNCs decreased much slower over time (Fig. 3) indicating that the TB was not directly exposed at the surface of the LNCs. Of note, we confirmed that there was no change in size and PDI when LNCs were exposed to ascorbic acid indicating that the integrity of LNCs was not affected by the presence of ascorbic acid. This is consistent with an encapsulation of TB into the lipidic core of the LNCs, with a slow exchange of a small fraction at the interfacial surface leading to a slow reduction of the nitroxide. TAM-LNCs were not tested using this protocol as trityls are not sensitive to the presence of ascorbic acid.

3.2. In vitro EPR oximetry with TB- and TAM-LNCs

The EPR LW (peak-to-peak) of TB- and TAM-LNCs increased linearly with pO_2. From nitrogen to air, the TB-LNCs LW (mean ± SD) increased from 1.621 ± 0.001 to 1.846 ± 0.011 G (presenting only a 14% increase in LW) whilst TAM-LNCs increased from 0.511 ± 0.003 to 0.711 ± 0.002 G (presenting a 39% increase in LW) (Fig. 4), indicating that TAM-LNCs were more sensitive to change in O_2. The slope of the TAM-LNCs calibration curve (1.25 mG/mmHg) was about 2-fold increased by comparison with the sensitivity of the hydrophilic trityl CT-03 (0.64 mG/mmHg) (Driesschaert et al., 2008). This increase in oxygen sensitivity is consistent with the higher solubility of oxygen in lipophilic phases as predicted by the Smoluchowski equation (Liu et al., 1994). Of note, the sensitivity observed in LNCs was lower compared to previously developed nanocapsules containing F15T-03 in the perfluorocarbon hexafluorobenzene (14 mG/mmHg) (Charlier et al., 2009). Concerns about the biocompatibility of hexafluorobenzene (Mignion et al., 2013) led us to consider other excipients such as LNCs based on Labrafac as the lipid core. While the oxygen sensitivity for TAM-LNCs remained limited, it was an improvement over hydrophilic trityls and deserved further consideration for *in vivo* applications. Thus, due to the characterisation and oximetry results obtained for the probe encapsulated-LNCs, TAM-LNCs were used for *in vivo* studies as it is more stable, has a narrower LW at lower O_2 concentrations, and has a better signal-to-noise ratio as compared to the nitroxide TB-LNCs.

3.3. In vivo measurements using TAM-LNCs

A low-frequency (1.2 GHz) EPR spectrometer was used for *in vivo* experiments as EPR spectra can be recorded in tissues at a depth of

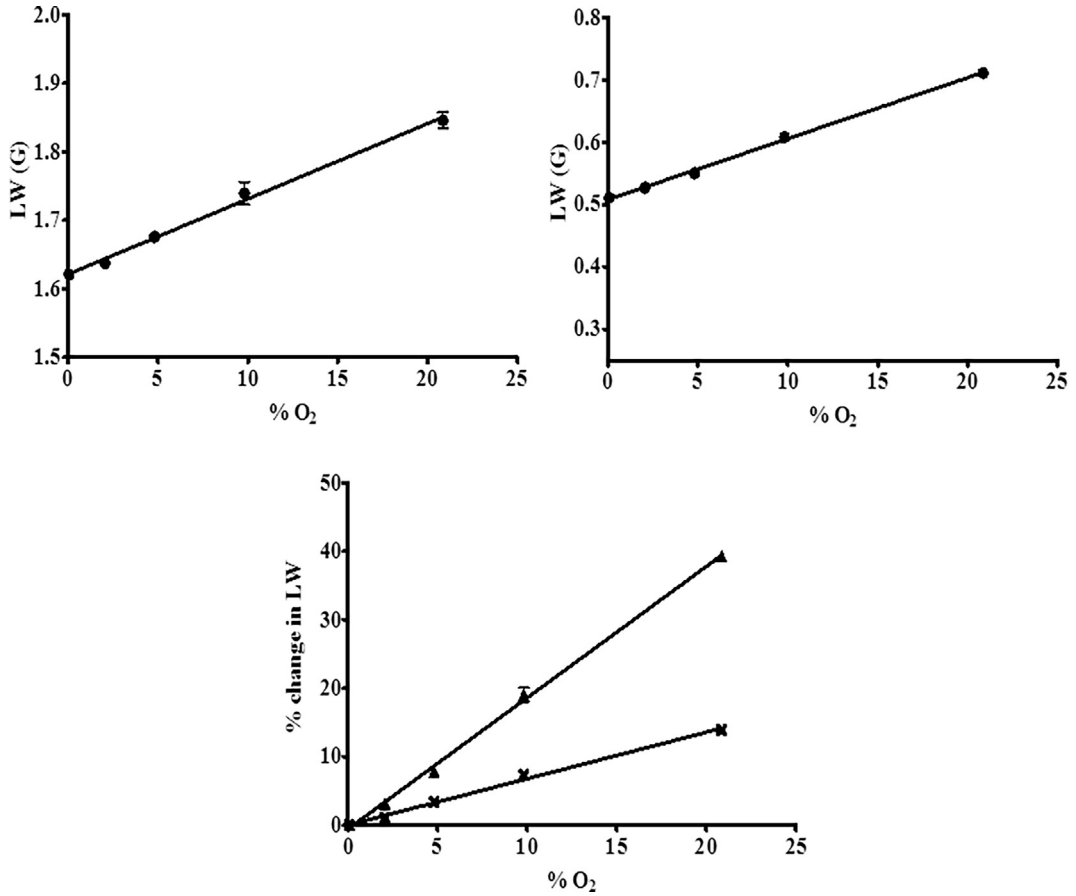

Fig. 4. Calibration of the EPR LWs recorded with TB-LNCs (top, left) and TAM-LNCs (top, right) as a function of % O_2. Relative sensitivity to O_2 as % change in LW as a function of O_2 concentration (bottom) shown with both TB-LNCs (cross) and TAM-LNCs (triangle). Curves show an R^2 of 0.987 and 0.994, respectively.

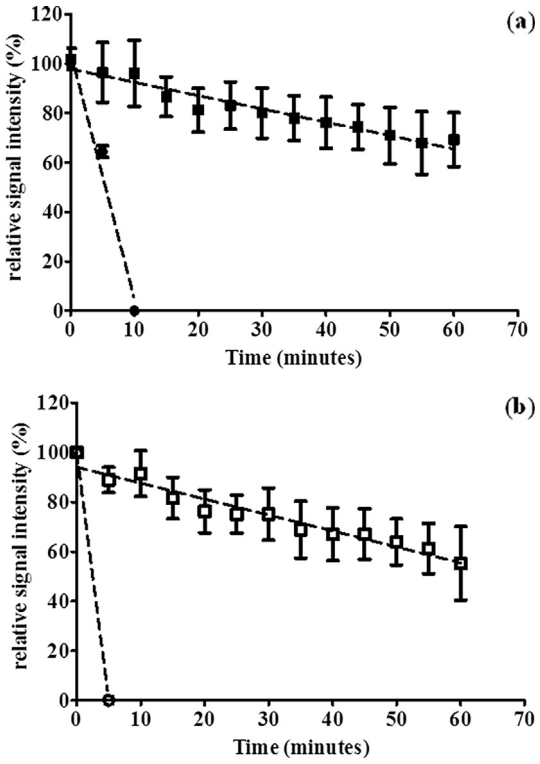

Fig. 5. Evolution of the EPR intensity recorded in muscles or in tumours after administration of unencapsulated TAM or TAM-LNCs, wherein (a) indicates the injected muscle group with unencapsulated TAM shown as closed circles (n = 2) and TAM-LNCs shown as closed squares (n = 5), and (b) indicates the injected tumour group with unencapsulated TAM shown as open circles (n = 2) and TAM-LNCs shown as open squares (n = 3). Results are expressed as relative to the initial signal intensity (mean ± SD).

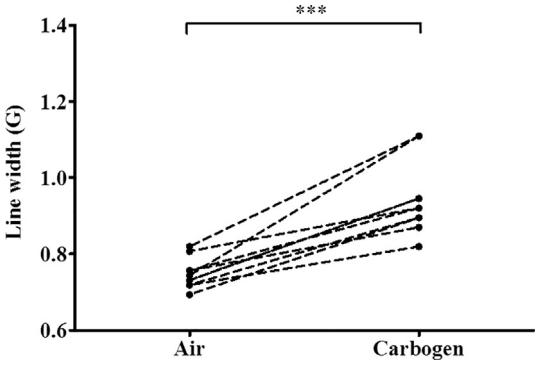

Fig. 6. *In vivo* EPR LW from TAM-LNCs injected in the muscle (n = 10) when mice were breathing air or carbogen. *P* value of < 0.0001 indicated with *** as determined by the paired Student's *t*-test.

approximately 1 cm. After intramuscular or intratumoural administration of unencapsulated TAM, the EPR signal decreased very rapidly (in approximately 10 min) (Fig. 4). By contrast, 1 h after administration, the EPR signal intensity of TAM-LNCs was only reduced by 25% in the muscle and 35% in the tumours (Fig. 5). Due to this slow clearance of the EPR probes, it was possible to assess the sensitivity of TAM-LNCs to variations in tissue oxygenation. After administration of TAM-LNCs in the muscle, EPR spectra were acquired whilst the animals (n = 10) were breathing 21% oxygen, then after a carbogen breathing challenge of 20 min. The LWs (mean ± SD) measured in the animals were 0.748 ± 0.039 G in mice breathing air and 0.943 ± 0.095 G in mice breathing carbogen (Fig. 5). The difference was statistically significant ($p < 0.0001$). TAM-LNCs were also injected into subcutaneous FSaII

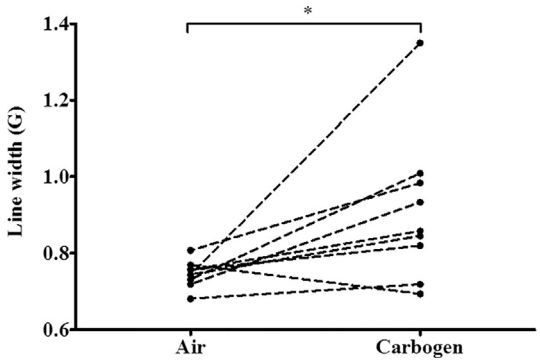

Fig. 7. *In vivo* EPR LW from TAM-LNCs injected in the FSaII fibrosarcoma (n = 9) when mice were breathing air or carbogen. *P* value of 0.03 indicated with * as determined by the paired Student's *t*-test.

tumours (n = 9) The LWs (means ± SD) observed in the animals were 0.745 ± 0.035 G during air breathing, and 0.912 ± 0.196 G after carbogen challenge (individual changes in LWs are represented in Fig. 7). These values indicate a sensitivity of the EPR probe to the changing O_2 environment within the tumour, as indicated by the significant increase in LW (difference between means of −0.167 G, $p = 0.03$) following the application of carbogen. We observed a larger variation in the response to carbogen in tumours compared to the muscles. This observation is consistent with the heterogeneity in perfusion occurring in tumours, wherein the vascular network is well hierarchized in muscles. While the TAM-LNCs were sensitive to variations in oxygen changes in muscles and in tumours, it should be noted that the transposition of the LWs into pO_2 values led to non-physiological estimates. As an example, measurements in muscles while mice were breathing air provide estimates of pO_2 close to 150 mmHg which are higher than the expected physiological values. This is in contrast with pO_2 estimates that are routinely obtained in tissues when using the same EPR system and other EPR oxygen sensors such as charcoal particles and lithium phthalocyanine (Diepart et al., 2009; Dinguizli et al., 2006; Jordan et al., 1998). The higher sensitivity of these particulates probes to changes in oxygenation may potentially explain the lower uncertainty and better accuracy when trying to estimate the pO_2 *in vivo* using EPR. Another factor could be the potential instability of the LNCs *in vivo* that may lead to change in the viscosity around the trityls. It has been recently reported by Frank et al. that changes in viscosity may profoundly affect the LWs of trityl radicals (Frank et al., 2015) (Fig. 6).

4. Conclusion

LNCs loaded with EPR probes TB and TAM were successfully prepared by the phase inversion process leading to nanocapsules of about 60 nm. Experiments performed with TB-LNCs in the presence of ascorbic acid demonstrated that the encapsulation provided resistance against the reduction into hydroxylamine. The calibration of the EPR LW as a function of the pO_2 showed a two-fold increase in sensitivity with TAM-LNCs compared to hydrophilic trityl radical. The TAM-LNCs were evaluated *in vivo*. Contrarily to unencapsulated TAM, for which a rapid decrease in EPR signal was observed, the half-life of TAM-LNCs administered in muscles or in tumours was longer than one hour. Carbogen challenges in mice demonstrated that the TAM-LNCs responded well to changes in oxygen environment. However, the apparent pO_2 values acquired were higher than the expected physiological values, which could be due to the instability of LNCs *in vivo* and potential changes in micro-viscosity surrounding the oxygen sensor. These developments and results warrant further investigation in the formulation of stable nano-objects encapsulating EPR oxygen sensitive probes.

Acknowledgements

The authors would like to thank the NanoFar Erasmus Mundus program, the “Fonds du Patrimoine” (Secteur des Sciences de la Santé, UCLouvain) and comité départemental de Maine and Loire de la Ligue Contre le Cancer (France) for providing the funding for this project.

Conflict of interest

The authors report no conflict of interest.

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