- Supporting information -

Materials and Methods

Generation of oxygen-derived species (ozone, singlet oxygen) after UV-C sterilization

UV-C-sterilized OLNDs underwent further analyses to exclude ozone and singlet oxygen generation. To check ozone levels, a photometric assay was performed by using Hach Lange DR5000 UV/V spectrophotometer and LCK310 ozone cuvette test kit, according to manufacturer's instruction. The generation of singlet oxygen was monitored by electron paramagnetic resonance (EPR) spectroscopy (Miniscope 100) by employing 4-oxo-2,2,6,6-tetramethyl-4-piperidone (4-oxo-TMP) as a spin probing agent. Briefly, 100 µl of a 50 mM solution of 4-oxo-TMP in ultrapure water were added to 900 µl of OLND liquid (Phosphate Buffered Saline, PBS) formulation. The suspension was stirred and then irradiated with UV-C for 20 minutes. The EPR spectra were recorded on 50 µl of the suspension withdrawn after 20 min. The experiments were repeated in the absence of illumination. A PBS solution and a suspension of titanium dioxide (Aeroxide P25, 1 mg/ml) were used as negative and positive control, respectively. All experiments were repeated at least three times.

In vitro oxygen release without US

O_2 release by diffusion from OLND, OLNB and OSS liquid or gel formulations was estimated by monitoring O_2 concentration up to 7 h through Hach Langhe LDO oxymeter, displaying an accuracy of 0.01 mg/l. The oxymeter was calibrated in air, waiting for stable temperature and humidity conditions to be reached before each measurement.

In vivo measurement of oxy/deoxy-hemoglobin levels without US (photoacoustic imaging)

The shaved hind limbs of nine anaesthetized mice were topically treated with OLND, OFND or OSS gel formulations. Before, during and after treatment, the subcutaneous levels of oxy- and de-oxy-hemoglobin were monitored by photoacoustic imaging, which was performed with Vevo® LAZR system featuring a hybrid US transducer (central frequency: 21 MHz; spatial resolution: 75 µm), according to manufacturer's instructions. Such an innovative hybrid imaging technique is based on the light absorption and the acoustic transmission properties of a tissue slice interrogated by a computed tomography photoacoustic imager.[1-2] This approach can quantify the density of tissue chromophores such as oxy-Hb and deoxy-Hb and measure some physiological parameters such as blood oxygen saturation and total Hb concentration.[3]

Results

UV-C sterilization of OLNDs does not generate oxygen-derived species (ozone, singlet oxygen).

OLND PBS formulations were sterilized through UV-C exposure for 20 min. Thereafter, the generation of ozone and singlet oxygen were monitored by using a spectrophotometric assay and EPR spectroscopy, respectively. Any significant levels of ozone (not shown) and singlet oxygen (Figure S1) were not detected.

US-untreated OLNDs effectively release high oxygen amounts in a time-sustained manner in vitro.

In vitro oxygen release from OLND liquid (water) and gel (2% HEC) formulations - which did not undergo complementary US administration - was monitored up to 7 h through an oxymeter and

compared with the oxygen levels released from OLNBs and OSS. As shown in Figure S2, both OLND formulations released high oxygen amounts (~14-16 mg/L) for all the observational period. On the contrary, the oxygen levels released by OLNBs and OSS appeared to be high only at the early monitored time-points (up to 21 mg/L and 18 mg/L, respectively); over time, oxygen delivery from OLNBs and OSS rapidly decreased, eventually resulting negligible at the end of the observational period.

US-untreated OLNDs are partially effective in releasing oxygen in vivo.

In vivo oxygen release from OLND gel (2% HEC) formulations - which did not undergo complementary US administration - was monitored for 15 min through photoacoustic imaging and compared oxygen delivery from OFNDs and OSS. As shown in Figure S3, OLND-treated mice displayed an immediate increase of oxy-HB levels; however, such an increase was not sustained over time. Results obtained from OSS-treated mice were similar to those from OLND-treated mice, whereas OFNDs did not promote any significant blood oxygenation, as expected.

Figures

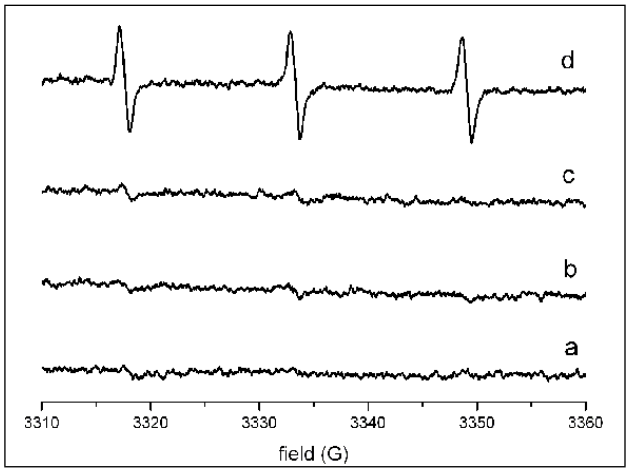

Figure S1. Lack of singlet oxygen generation in OLNDs after UV-C sterilization. OLND PBS formulations were sterilized through UV-C exposure for 20 min. Thereafter, the generation of singlet oxygen was monitored by EPR spectroscopy. EPR spectra were recorded on a) phosphate buffer; b) a buffered suspension of OLNDs in the dark; c) a buffered suspension of OLNDs under illumination with the UV-C lamp; d) a buffered suspension titanium dioxide (positive control). All experiments were performed in the presence of the spin probe 4-oxo-TMP. When singlet oxygen is formed a three line spectra appears. The intensity of the signal is proportional to the amount of singlet oxygen generated. Results are shown as representative images from three independent experiments.

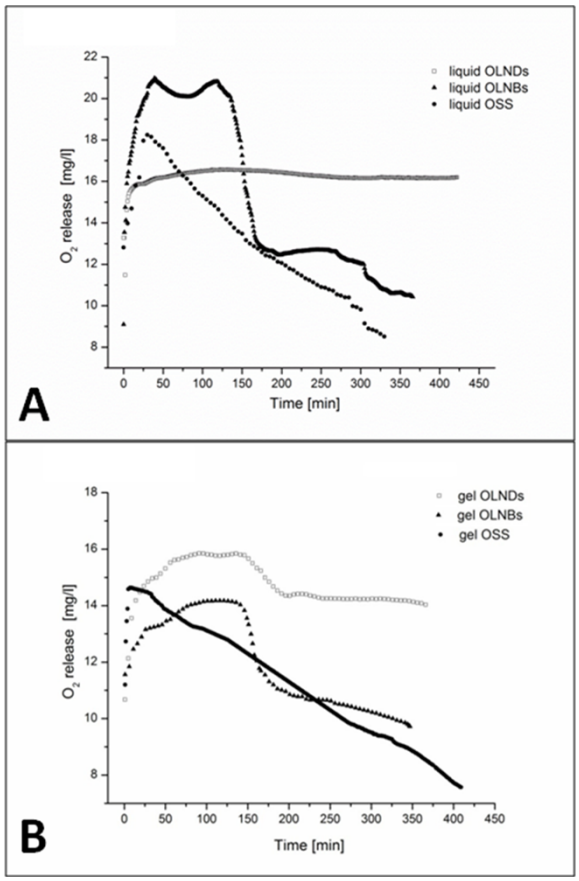

Figure S2. Oxygen release from OLND liquid and gel formulations in vitro. OLND, OLNB and OSS liquid (water) and gel (2% HEC) formulations were monitored up to 7 h through an oxymeter for oxygen delivery by diffusion. Results are shown as a representative image from three independent experiments. Panel A. Release from liquid formulations. Panel B. Release from gel formulations.

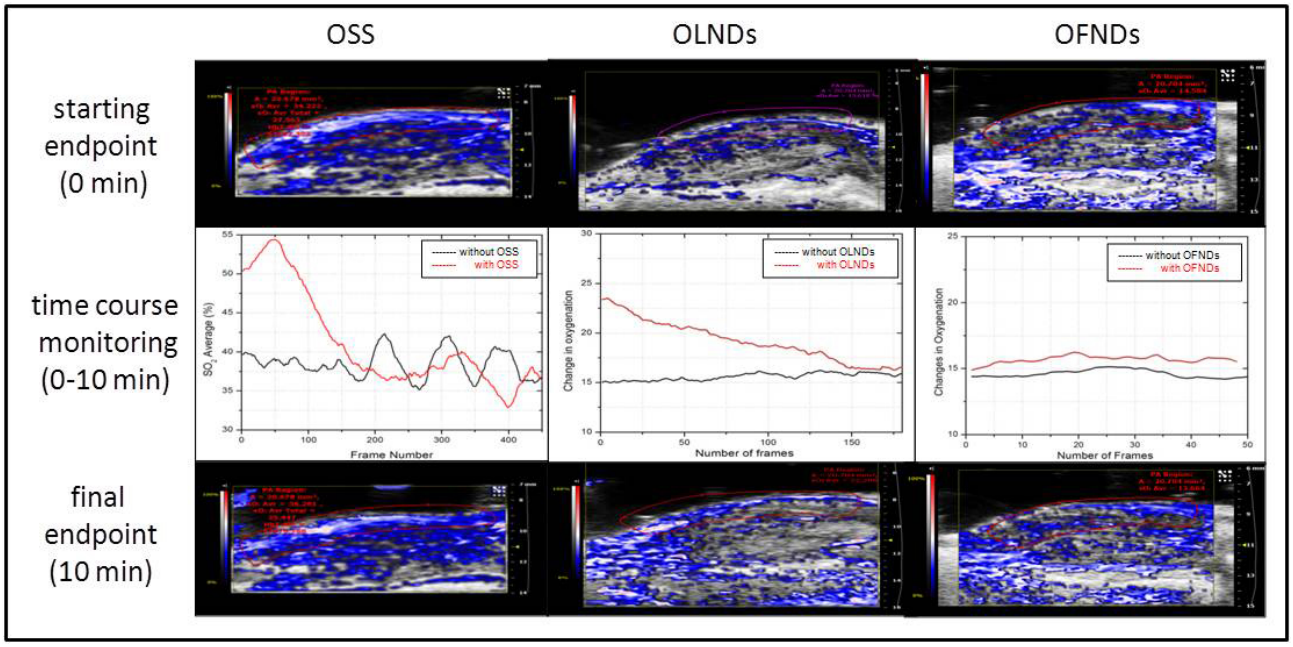

Figure S3. Topical treatment with OLND gel formulation partially enhances oxy-Hb levels in vivo. Shaved hind limbs of anaesthetized mice were monitored by photoacoustics for oxy-Hb and deoxy-

Hb levels before (0 min, upper row), during (0-10 min, central row) and after (10 min, lower row) topical treatment with OSS (first column), OLND (second column) and OFND (fifth column) gel formulations. White/red pixels: oxy-Hb; blue pixels: deoxy-Hb. Data are shown as representative images from three independent experiments (three mice per experiment) with similar results.

References

1 J. Jose, R.G. Willemink, S. Resink, D. Piras, J.C. van Hespen, C.H. Slump, W. Steenbergen, T.G. van Leeuwen, S. Manohar, *Opt. Express.* 2011, **19**, 2093-2104. [Pubmed: 21369026]

2 S. Resink, J. Jose, R.G. Willemink, C.H. Slump, W. Steenbergen, T.G. van Leeuwen, S. Manohar, *Opt. Lett.* 2011, **36**, 2809-2811. [Pubmed: 21808320]

3 J. Laufer, D. Delpy, C. Elwell, P. Beard *Phys. Med. Biol.* **52**, 141-168. [Pubmed: 17183133]