HIFU AND OXYGEN LOAD NANOBUBBLES: TWO DIFFERENT APPROACHES FOR CANCER TREATMENT

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ABSTRACT

Use of high intensity focused ultrasound (HIFU) beam has gained rapid agreement in clinical environment as a tool for non-invasive surgical ablation of tumor cells. This technology, applied simultaneously to nano-bubbles filled with oxygen (OLN), realized and characterized at INRiM with the purpose of treating diseases associated to hypoxia (such as tumors), constitute an innovative therapeutic tool for cancer treatment proposed in this article.

Parole chiave: HIFU; Ultrasuoni; Nanobolle; Terapia del cancro.
Keywords: HIFU; Ultrasound; Nanobubbles; Cancer therapy.
1. Introduction

Cancer is the general name for a group of more than 100 diseases where cells in a part of the body begin to grow abnormally and uncontrollably. A century ago the chances of someone surviving cancer was negligible. Today, two out of every three people diagnosed will still be alive at the end of five years. It is evident that cancer diagnosis and treatment continues to improve, although the ‘cure’ for cancer has been once hoped [1]. When the disease is local, surgery and radiation therapy are commonly used to treat cancer with a radical intent. Technological advancements in imaging and non-invasive treatments bring new therapeutic possibilities to the field [2]. Among the minimally invasive methods that have been developed, High Intensity Focused Ultrasound (HIFU) appears the most investigated technology in the new panorama of solid tumor treatment based on ultrasound [3]. In HIFU medical treatments, ultrasound (US) energy emitted by a transducer is focused into a small volume to heat and destroy the targeted tissue while ideally not damaging tissue outside the focal region. In spite of the multitude of medical applications, the effects of ultrasonic waves interactions on living tissues are not completely described. For this reason, a complete characterization of the transducers, concerning acoustic pressure, ultrasound power measurement and monitoring of the temperature rise induced by ultrasound beam in tissues is essential.

Power measurement has been conducted in this work with a radiation force balance, while acoustic pressure and temperature increase measurement has been conducted using an experimental apparatus containing a fiber optic probe hydrophone. Heating effects induced by HIFU transducers were observed in an agar based tissue mimicking material (TMM) realized at INRiM. Among HIFU, other innovative applications that use ultrasound to treat cancer are under development. In this regard, micro- and nano-bubbles represent a new class of agents with both diagnostic and therapeutic applications. Encapsulated gas nano-bubbles are well known as contrast agents for medical ultrasound imaging [4], but they can also be used as drug/gene carriers [5]. Nano-bubbles are capable of penetrating even into the small blood capillaries. Under exposure of sufficiently high-amplitude ultrasound these targeted nanobubbles would rupture, spewing drugs or genes, which are contained in its encapsulating layer, to targeted cells (the phenomena is called sonoporation) or tissues (sonophoresis). At INRiM, a particular type of nano-bubbles have been developed: oxygen-filled nano-bubbles (OLNs), which are gaseous cavity confined by a coating suitably functionalized. OLN are an innovative oxygenating drugs aimed at treating hypoxia-associated pathologies, in particular for re-oxygenation of cancerous tissue. It has been known for nearly a century that hypoxic cells are more resistant to radiotherapy than aerobic cells, and tumor hypoxia is a major factor leading to the resistance of tumors to radiation treatment as well as several cytotoxic agents [6]. In this work, various formulations of OLN have been synthesized and characterized: OLN can be shelled with chitosan or dextran and cored with perfluoropentane (PFP) or decafluoropentane (DFP). Extensive research has been done on OLN both in vitro, in this case nano-bubbles are effective to release oxygen in absence and presence of ultrasound (sonophoresis) both in vivo, where nano-bubbles increased levels of oxygenated hemoglobin (photoacoustic studies) and transcutaneous oxygen pressure (TcPO$_2$) after topical treatment of hypoxic mice both in the absence and in the presence of ultrasound. After a complete characterization of an HIFU field and the development of oxygen nano-carriers, for the first time these two approach have been used simultaneously. The results can be considered preliminary data of a new and non invasive therapeutic modality to treat cancer.
2. High Intensity Focused Ultrasound (HIFU)

In these paragraphs HIFU transducers and their characterization regarding acoustic power, acoustic pressure and temperature rise in the focus of the transducers will be described.

2.1. HIFU transducers

Two HIFU transducers are measured and compared (Fig. 1):
- Transducer 1 (Sonic Concepts SU-102), \( f = 3.5 \text{ MHz} \) (Fundamental);
- Transducer 2 (Sonic Concepts H-106-MR.), \( f = 2.0 \text{ MHz} \) (Fundamental) and \( F = 6.38 \text{ MHz} \) (3rd Harmonic).

![Fig. 1 - Transducer 1(left), transducer 2 (right).](image)

2.2. HIFU transducer characterization: ultrasonic power

The time-average ultrasonic power emitted by a transducer is one of the key values to be taken into account for characterizing an ultrasonic source. At INRiM, the measuring system for the determination of US power emitted by an HIFU transducer has been developed. The system is based on radiation force balance (RFB) method using a submergible load cell (SLC system) that allows measurement for ultrasonic power from 15 W to 200 W. The ultrasonic power is determined from the measurement of the force exerted on a target by the field generated by the ultrasonic source. The absorbing target (diameter 150 mm) is connected to a submersible load cell (Honeywell model 31) which measures mass variation due to the ultrasonic field when the source is alternatively switched on and off. The signal produced by the load cell is conditioned by a strain gauge amplifier (Sensotec model UV-10) and then measured by a nanovoltmeter (Agilent 34420A). Ultrasonic power is calculated as

\[
P_{\text{out}} = g \cdot u(T) \cdot \Delta M \quad \text{[W]}
\]

where \( g \) is the gravity acceleration evaluated in the INRiM laboratory, \( u(T) \) is the speed of sound in water, as a function of the temperature \( T \), and \( \Delta M \) is the apparent mass variation induced by the ultrasound field. The electroacoustic radiation conductance \( G \) is calculated according to the relation

\[
G = P_{\text{out}} \cdot U_{\text{in}}^{-2} = g \cdot u(T) \cdot \Delta M \cdot U_{\text{in}}^{-2} \quad \text{[S]}
\]

The RMS voltage, \( U_{\text{in}} \), of the transducer driving signal is determined, simultaneously with the mass variation, by means of a Rohde&Schwarz model URE-3.
voltmeter. The input voltage $U_{in}$ refers to the transducer input and is to be measured at a point as near as possible to the transducer input connector. $G$ is expressed in Siemens, S, or decimal submultiples of this unit. In Fig. 2 the measurement apparatus is shown, with a detail of load cell and target in the upper right box.

![Fig. 2 - SLC measurement apparatus.](image)

For each transducer and for each frequency, the final measured value of ultrasonic conductance has been calculated. The $G$ values are evaluated as the mean of the four measures, realized for each measurement condition. The highest uncertainty among the four obtained for each conductance for each fixed power level and frequency is taken as the uncertainty for the mean value (Fig. 3).

![Fig. 3 - Transducer 1 (left) and 2: (right) ultrasonic conductance at four different power level.](image)

### 2.3. HIFU transducers characterization: acoustic pressure

Despite its importance, ultrasonic power is not an exhaustive quantity. It gives a value of the energy output of the source, but doesn’t provide any information about how this energy is distributed in space and in time. Such information can be obtained by local measurements of other acoustic quantities, such as the acoustic pressure. Acoustic pressure can be measured directly with an hydrophone. Hydrophones, which could be considered as the equivalent of microphones for the ultrasonic frequencies in water, are tools able to generate an electrical voltage proportional to the acoustic pressure incident...
on their sensitive elements. Once the hydrophone has been correctly calibrated, it is possible to know the time variation of acoustic pressure in a measurement point from the electrical output of the transducer.

A typical measurement system for pressure field characterization (Fig. 4) is described below. A water tank is where the measurement takes place. The tank should be large enough to allow a good movement range for the hydrophone and the possibility to perform free field measurement. The water tank is plexiglass made with 12 mm thick transparent walls. The dimensions of the base are 1.0 x 0.5 x 0.5 m. It is usually filled with 200 l of distilled and deionised water. The HIFU source and the hydrophone are immersed into the water tank. To allow the movement of the hydrophone within the measurement tank there are three linear motion stages for controlling the hydrophone movement along the three orthogonal axes. The signal from the output of the hydrophone have been acquired by means of a digital oscilloscope (LeCroy Waverunner 6000A). A PC is used for remote controlling of the position, for the acquisition systems and for the data analysis by means of dedicated LabVIEW™ based software.

![Fig. 4 - Experimental apparatus for the measurement of pressure induced by an HIFU beam.](image)

As described before, the hydrophone is the instrument for acoustic pressure measurement. Due to the wide range of ultrasonic fields to be measured, it is not possible to have a single transducer suitable for each measurement. At INRiM laboratories, hydrophones with different characteristics and different applications fields are available: three needle type with different sizes and sensitivity, one membrane hydrophone, a special HIFU addicted needle type and a Fiber Optic Probe Hydrophone (FOPH). FOPH is the mainly used hydrophone in this work and for this reason in the following section a more detailed description of this instrument is provided.

2.3.1 Fiber Optic Probe Hydrophone (FOPH)

Fiber optic probe hydrophone (FOPH-2000, RP Acoustics) [7] is an advanced transducer for the measurement of the acoustic pressure, $p$, with a spatial and temporal resolution of 100 µm and 3 ns respectively. The sensitive element for measuring pressure is the tip of an optical glass fiber. Its functioning is based on the measurement of the refractive index, $n$, of a medium, which depends on the medium $p$ and $T$ values ($n(p,T)$). A laser ($\lambda = 808$ nm) is coupled to the glass fiber and its light is partly transmitted through the sample and partly reflected at the fiber tip, depending on the sample $n$ value. The reflected light is converted to an electric signal which reproduces,
after being amplified, the value of acoustic pressure incident on the fiber on the oscilloscope screen (Fig. 5). Optical hydrophones have important advantages compared to a conventional hydrophone: the calibration is carried out in a simple and fast way; a damaged glass fiber tip can easily be repaired, in addition to being a tool completely compatible with MRI.

The equation to perform sound pressure measurement with the FOPH hydrophone is:

$$\rho = \left( \frac{1 - \frac{0.0019867 \times \Delta V}{V_0}}{1 + \frac{0.0019867 \times \Delta V}{V_0}} \right)^{-1/0.329} \times 295.6 - 295.5 \quad [\text{MPa}]$$

where $\Delta V$ is the voltage variation in Volt, $V_0$ is the DC- photodetector signal at atmospheric pressure in V and $\alpha$ is the internal light scattering factor of fiber optic system (adimensional).

Due to the fact that the refractive index of a liquid depends on the pressure and temperature, the hydrophone not only can be used as pressure measuring instrument, but it can also be used to measure the temperature:

$$T = \left[ \frac{1.72 \times 10^4 \times \frac{V_0}{\Delta V} - 5}{1 + \alpha} - 0.0146 \right] \times (1 + \alpha) + 0.0146 \times 1.72 \times 10^4 + 5 \quad [\text{°C}]$$

where $T$ is the water temperature in °C, $T_0$ is the water initial temperature in °C.

2.3.2. Pressure calculation

Acoustic pressure measurement has been conducted in the scanning tank described in paragraph 2.3. To obtain an acoustic pressure field characterization, a series of planar scans are performed around the focus and the pre-focus regions (10 mm from the focus) for each transducer.

The aim of these scans has been to define the beam dimension and shape. Ultrasound sources are set for operating at 50 W. In Figg. 6 and 7, a planar scan for transducers 1 and 2, in the focus region and in pre-focus region are shown. These are representative images from several independent experiments. The pressure 3-d diagram of an HIFU fields was performed using the optical hydrophone. The surfaces scanned have a dimension of 30 x 30 points with 0.20 mm resolution, so that each planar scan requires 900 points. The planar scanning is always performed in x-z plane which is
perpendicular to the propagation direction of the ultrasound beam. For each measurement point, the oscilloscope has acquired waveforms with a sampling frequency of 5 GS/s for a time period of 1000 ns. In order to reduce the random noise, each acquired electrical waveform has been the result of 10 averages.

Fig. 6 - Three-dimensional representation of acoustic pressure measured in the focus (left) and pre-focus region (right) for transducer 1.

Fig. 7 - Three-dimensional representation of acoustic pressure measured in the focus (left) and pre-focus region (right) for transducer 2.

From the graphs obtained, it is possible to evaluate the pressure reached in the focus (1.6x10^8 Pa for transducer 1 and 2.3x10^8 Pa for transducer 2). The pressure level reached in the pre-focused region is still high and, especially for the smaller transducer (Transducer 1), it is evident the presence of external “lobes” with high acoustic pressure level due to the more tapered shape of this transducer.

2.3.3. HIFU transducers characterization: TMM

The characterization of ultrasonic fields generated in water by transducers is surely a fundamental tool for the control of device performance and for patient safety protection, but the information they give is not complete. As a matter of fact, they describe the morphology and intensity of the field, but not the effect induced in real tissue. Therefore, the issue is to realize new materials for simulation of acoustic properties of real tissue, new parameters which really refer to biophysical effects, and a robust methodology for measuring them. In this work many phantoms have been realized: Agar-based TMM, Poliacrylamide TMM and a thermocromic silica gel.

By means of these materials it has been possible to analyze the different behaviours and the heating effects caused by focused ultrasonic transducers in function of the
power, frequency and irradiation time. Agar-based TMM are the most used and for this reason, a detailed description is provided. Agarose is derived from agar, a hydrophilic colloid that is extracted by boiling algae. To produce the TMM based on Agarose polymer, the liquid component (distilled water) is mixed to the dry components (Agar, 3% in weight). The solution is hated at 90 °C and then cast into a cylindrical mould. Benzalkonium chloride (0.9% in weight) or Silicon Carbide (SiC, 1% in weight) can be added to the solution while it is left to cooling, respectively to control microbial invasion, and to vary the backscatter coefficient.

2.3.4. HIFU transducers characterization: TMM and temperature

Beside acoustic power and pressure, another important parameter, in particular for the clinical use, to provide a complete characterization of an ultrasonic field is the temperature increase in the focus region of the transducer in the specified medium.

In the previous paragraph the fiber optic hydrophone and an agar based TMM have been described. Now, using theses instrument, temperature increase induced by a focused ultrasound field (Transducer 2) in an agar-based gel is evaluated. The experimental set up is the same used for pressure measurement. By means of a micro-positioning system, TMM under study, including the optical fiber, is moved to reach the point of maximum revealed temperature, which is supposed to be the focus region of the ultrasound beam.

Firstly, the temperature elevation generated by an HIFU source (set to work at $P = 100 \text{ W}$) in the TMM phantom is spatially mapped in the focal plane. An $x-z$ axes planar scan perpendicular to the propagation direction of the ultrasound beam is performed. In Fig. 8, a three-dimensional representation of the temperature distribution is shown. The colour map represents temperature variation from 20 to 70 °C. Subsequently to a spatial scan of the temperature reached around the focus, measurements on temperature rise induced by HIFU transducer using a FOPH have been conducted, in particular, modifying the chemical composition of the gel in order to tuning its acoustic properties. Four different Agar-based TMMs have been prepared, in order to study the changing in temperature increase when the percentage in volume of scattering agents (kieselguhr) is varied. Agar TMM have no scattering agents, while in Agar 2, 3 and 4 there is an increasing percentage in volume of them, respectively 2%, 3% and 4%. Data are acquired by means of a fiber optic hydrophone when the gel is subjected to a focused ultrasound field produced at four different values of power (20, 50, 75, 100W). The exposure time is 5 s every measurement. Temperature values are calculated as the mean of five measures realized for each condition.

![Fig. 8 - Three-dimensional representation of the temperature spatial distribution in the focus (left). Temperature increase at different power values, in four Agar-based TMM (right).](image)
From the right graph, it has been found that increasing the percentage of scattering and absorbing agents (kieselguhr) the temperature reached in the focus is lower, revealing that part of the energy of the ultrasonic beam has been absorbed by wave interactions with inhomogeneities in the medium. Error bar associated to temperature values are calculated as a quadrature combination of two components: the uncertainty relative to the fiber optic system and the uncertainty associated to the repeatability of the measuring data. The total uncertainty \( u(T) \) related to temperature measurement is 3.30%. The principal contribution to the uncertainty is due to \( \alpha \), which is 2.44%.

3. Oxygen load nano-bubbles

Oxygen nano-carriers have been developed at INRiM to treat a variety of hypoxia-related diseases and in particular hypoxic cancerous tissues. This is an innovative, effective, almost non-invasive and low-cost nano-technological method. Different formulations of oxygen-loaded nano-bubbles (OLNs) were realized to improve the available nanotechnology of gas delivery by studying new core/shell nano-carriers. The main feature of these new nano-carriers consisted of employing 2H,3H-decafluoropentane (DFP) or Perfluoropentane (PFP) for the oxygen-storing core structure. Notably, DFP displays oxygen-solubilizing capabilities as high as OLNB core-associated PFP. However, unlike PFP, which has a boiling point of 32 °C and is gaseous at body temperature, DFP is a clear, colorless fluorocarbon with a boiling point of 51 °C, thus resulting liquid at 37 °C. For that reason, the new nano-carriers were named oxygen-loaded nano-droplets (OLNDs). Dextran was kept as the main constituent of the polysaccharidic shell as for former OLNs, since dextran-based formulations have been extensively tested for biocompatibility, and dextran-based hydrogels are currently used as matrices in tissue engineering, without showing signs of inflammation \textit{in vivo}. On the other hand, chitosan was also chosen to build the polysaccharidic shell of OLNDs. This polysaccharide is a positively charged, partially deacetylated form of chitin, a natural substance found abundantly in the exoskeletons of insects and in the shells of crustaceans. Chitosan also displays high biocompatibility, healing capabilities, anti-cancer activity and anti-microbial properties against some bacteria (e.g. methicillin-resistant \textit{Staphylococcus aureus}) and fungi (e.g. \textit{Candida albicans}). Due to these characteristics, it has been investigated for use in several biomedical applications, including wound dressings and drug carriers. Along with OLNDs, complementary control preparations were also evaluated, including OLNBs, oxygen-free nanodroplets (OFNDs), oxygen-free nanobubbles (OFNBs), and oxygen-saturated solution (OSS). All preparations were made either in liquid (water) or gel (hydroxyethylcellulose, HEC) formulations. As described before, two different type of polysaccharidic have been chosen to build the shell of OLNs: dextran and chitosan. In the following paragraph, a detailed description of dextran OLNs characterization is presented.

3.1 Dextran OLNs: preparation

For oxygen loaded nanodroplet (OLND) liquid formulations, 1.5 ml DFP along with 0.5 ml PVP and 1.8 ml Epikuron® 200 solved in 1% w/v ethanol and 0.3% w/v palmitic acid solution were homogenized in 30 ml water or phosphate buffered saline (PBS) for 2 min at 24000 rpm by using Ultra-Turrax SG215 homogenizer. Thereafter, the solution was saturated with O\(_2\) for 2 min. Finally, 1.5 ml dextran sulfate solution was added drop-wise whilst the mixture was homogenized at 13000 rpm for 2 min. For oxygen-loaded nano-bubble (OLNB) water formulation, the protocol developed by
Cavalli et al. [8] was applied by using PFP as a core fluorocarbon. Oxygen-free nanodroplet (OLND) and nanobubble (OFNB) water formulations were prepared according to OLND and OLNB protocols without adding \( \text{O}_2 \). For oxygen-saturated solution (OSS) water formulation, OLND preparation protocol was applied omitting dextran sulfate and DFP addition. To obtain gel formulations, 0.8 mg hydroxyethylcellulose were solved in 20 ml water, and subsequently mixed 1:1 with OLND, OFND, OLNB, OFNB, or OSS water formulations.

### 3.2 Physical-chemical characterization

Immediately after manufacturing, OLNs and control preparations were characterized for morphology, by optical and transmitting electron microscopy (TEM). TEM analysis was carried out using a Philips CM10 instrument, whereas optical microscopy was carried out using a XDS-3FL microscope.

Dextran OLND and OLNB are prepared in liquid (water) or gel (HEC) formulations and has been checked for morphology by TEM or by optical microscopy. Results are shown in Fig. 9 as representative images from ten different preparations for each formulation. Panel A. TEM image of OLND water formulation. Magnification: 15500X. Panel B. Optical microscopy image of OLND water formulation. Magnification: 60X. Panel C. Optical microscopy image of OLND HEC formulation. Magnification: 60X. Panel D. TEM image of OLNB water formulation. Magnification: 15500X. Panel E. Optical microscopy image of OLNB water formulation. Magnification: 60X. Panel F. Optical microscopy image of OLNB HEC formulation. Magnification: 60X.

![Dextran OLN and OLNB morphology](image)

Fig. 9 - Dextran OLN and OLNB morphology

Average diameters \( d \), polydispersity indexes, and zeta potentials of OLNDs and OLNBs were determined by dynamic light scattering, using Delsa Nano C instrument, displaying a 0.6 nm – 7 \( \mu \)m range for measurements of particle size distribution. The polydispersity index indicates the size distribution within a OLND or OLNB population. For zeta potential determination, formulation samples were placed into an electrophoretic cell, where an electric field of approximately 30 V/cm was applied. Note that since the zeta potential measures charge repulsion or attraction between particles, it is a fundamental parameter to determine nanoparticle physical stability, with zeta potentials lower than \(-30 \) mV or larger than \(+30 \) mV being generally required for physical stability of colloid systems [9]. Although nano-droplets and nano-bubbles displayed zeta potentials slightly larger than \(-30 \) mV, our formulations proved to be physically stable over time for the steric repulsion of the polymer chains, as assessed by monitoring their sizes and zeta potential by dynamic light scattering for 72 h after manufacturing. Either OLNDs or OLNBs displayed spherical shapes, nanometric sizes,
with average diameters ranging from $\sim 500$ nm (OLNBs) to $\sim 600$ nm (OLNDs) for oxygen-loaded carriers and from $\sim 210$ nm (OFNBs) to $\sim 240$ nm (OFNDs) for oxygen-free carriers, and negatively charged surfaces as a consequence of the presence of sulfate groups in dextran molecule. OLNDs displayed also a good oxygen capacity, storing $\sim 0.40$ g/ml of oxygen. Current ONDs and ONBs displayed zeta potentials slightly higher than $-30$ mV, this is an evidence that nano-bubbles are surface-charged. This is an exquisitely propriety suitable for topical treatment, as surface charges enhance nanoparticle interaction with skin. Oxygen content of OLNDs, OLNBs and OSS have been evaluated by adding known amounts of sodium sulfite and measuring generated sodium sulfate, according to the reaction: $\text{Na}_2\text{SO}_3 + \frac{1}{2}\text{O}_2 \rightarrow \text{Na}_2\text{SO}_4$

Results are shown as means $\pm SD$ (standard deviation) from ten preparations (average diameters, polydispersivity index, and zeta potential) or three preparations (oxygen content) for each formulation. Results are shown in Tab. 1.

### Tab. 1 - Physical-chemical characterization of OLNDs, OFNDs, OLNBs, OFNBs and OSS.

<table>
<thead>
<tr>
<th>fluorocarbon boiling point</th>
<th>$O_2$ content (g/ml$\pm$SD)</th>
<th>$d$ (nm$\pm$SD)</th>
<th>Polidispersivity index</th>
<th>zeta potential (mV$\pm$SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLND 51 °C</td>
<td>0.42 ± 0.01</td>
<td>596.35 ± 149.09</td>
<td>0.13</td>
<td>25.68 ± 1.00</td>
</tr>
<tr>
<td>OFND 51 °C</td>
<td>/</td>
<td>239.54 ± 96.20</td>
<td>0.10</td>
<td>25.17 ± 1.00</td>
</tr>
<tr>
<td>OLNB 32 °C</td>
<td>0.42 ± 0.01</td>
<td>486.87 ± 147.62</td>
<td>0.11</td>
<td>27.31 ± 1.00</td>
</tr>
<tr>
<td>OFNB 32 °C</td>
<td>/</td>
<td>212.31 ± 94.82</td>
<td>0.95</td>
<td>26.54 ± 1.00</td>
</tr>
<tr>
<td>OSS /</td>
<td>0.40 ± 0.01</td>
<td>/</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

Results related to dextran OLND and OLNB water formulation checked for size distribution by light scattering are shown in Fig. 10, as a representative image from ten different preparations.

![Fig. 10 - Dextran OLN and OLNB size distribution.](image)

### 3.3 In vitro determination of oxygen release from dextran OLNs

After nano-bubbles characterization, their ability to release oxygen *in vitro* has been extensively studied. Firstly, oxygen-loading capacity has been evaluated without ultrasound and then several sonophoresis experiments have been performed. OLNs has been manufactured both in liquid and gel formulations. Gel formulation has been...
prepared in order to simulate a real treatment through the skin. The concentration of oxygen released by diffusion from OLND, OLNB and OSS liquid or gel formulations was monitored up to 6 h through Hach Langhe LDO oxymeter, displaying an accuracy of 0.01 mg/l. Before each measurement, the oxymeter was calibrated in air, waiting for stable temperature and humidity conditions to be reached. Results are shown in Fig. 11, as a representative image from three independent experiments.

![Fig. 11 - In vitro oxygen release from dextran OLND and OLNB liquid and gel formulation.](image)

In the figure appears that OLNs had a good oxygen-loading capacity. This was due to the presence of fluorocarbons, as DFP and PFP can favor oxygen entrapment. Both OLND and OLNB displayed a very good ability to deliver O\(_2\) at high concentrations and for long times, either in water or gel formulations; nevertheless, DFP-containing OLNs appeared more effective than those with PFP. OSS did deliver high O\(_2\) concentrations, but for shorter times than OLNs, as expected.

It is known that US might elicit sonophoresis, a process that exponentially increases the absorption of topical compounds (transdermal delivery) into the epidermis, dermis and skin appendages by ultrasonic energy.

To verify the ability of ultrasound (US)-activated OLNs to release O\(_2\) through biological membranes, a home-made apparatus consisting of two sealed cylindrical chambers separated by a layer of pig ear skin was employed. One chamber (the oxygen-donor), containing OLNDs OLNB or control solutions (or OFND, OFNB and OSS solutions) was connected to an US-transducer, with the beam directed towards the membrane; the second chamber (the oxygen-recipient) was filled with hypoxic solution and monitored by an oxymeter. The US transducer (\(f = 2.5\) MHz; \(P = 5\) W) was alternatively switched on and off at regular time intervals of 5 min for an overall observational period of 135 min, and oxygen concentration was monitored in the recipient chamber every 45 min. O\(_2\) concentration in the hypoxic chamber was monitored by Hach Langhe LDO oxymeter. Because of the local heating caused by US, the O\(_2\) sensor was positioned laterally in order to prevent possible damage of the oxymeter, whereas the transducer was held in a fixed position, within the donor compartment (see Fig. 12). The acoustic power of the transducer was determined through a balance’s radiation force with a reflecting target, with an uncertainty of 4%.
US improved the ability of both liquid and gel OLND formulations to cross the pig skin membrane and to release oxygen into the hypoxic chamber, being such oxygen release significantly larger than that from OFNDs, OLNBs, OFNBs, and OSS formulations. Even in this case, OLND appears more effective than OLNB. On the contrary, OFNs did not release significant O₂ amounts, whereas OSS did deliver high O₂ concentrations, but for shorter times than OLNs, as expected. The efficacy of OLNs in releasing O₂ on skin models is particularly crucial in order to verify the hypothesis that OLNs might help to restore the physiological level of oxygenation in cancerous tissues in which there is an oxygen deficiency.

3.4 In vivo determination of oxygen release from dextran OLNs

Once proven in vitro that OLNDs were more effective than former OLNBs and OSS in releasing oxygen and that gel formulation appeared suitable for treatment of skin membranes, OLND gel formulation was tested for oxygen delivery in vivo. The skin oxygenation of the shaved hind limbs of nine anaesthetized mice topically treated with OLND, OFND or OSS gel formulations was monitored by visualizing the subcutaneous levels of oxy-Hb and deoxy-Hb through a photoacoustic technique of imaging before, during and after the treatment (10 min). This innovative hybrid imaging technique, based on the light absorption and the acoustic transmission properties of a tissue slice interrogated by a computed tomography photoacoustic imager, 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 [9]. Photoacoustic monitoring revealed that oxy-Hb levels significantly increased for the entire observational period in the animals treated with OLNDs while, as expected. OSS induced a high but only transient peak in oxy-Hb, whereas OFNDs did not affect oxy-/deoxy-Hb balances at all.

Lastly, the OLND ability to improve tissue oxygenation in vivo after US treatment was investigated. The shaved abdomens of five anaesthetized mice were topically treated with OLNDs and sonicated for 30 s (f = 1 MHz). Skin oxygenation was investigated through transcutaneous oxymetry before and after the treatment. This technique measures the oxygen transcutaneous tension (tcpO₂) through a non-invasive method which elicits a heating-related vasodilatation, generating fast diffusion of gases from the vessels to an electrode located on the skin. When capillary oxy-Hb dissociation occurs, the reaction of oxygen reduction generates a current which is directly proportional to capillary oxygen arterial pressure. Monitoring tcpO₂ is a well-
consolidated technique extensively used also in clinical practice. Basal tcpO$_2$ values in mice were inhomogeneous, possibly as a consequence of the different level of peripheral vasoconstriction induced by anesthesia. Nevertheless, after topical administration of US-activated OLNDs, hypoxic mice displayed larger oxygenation levels in a time-sustained manner (up to 1 h). Data are shown in Fig. 13, as representative images from three independent experiments with similar results.

**Fig. 13** - Topical treatment with dextran OLND gel formulation effectively enhances oxy-Hb levels (left) and tcpO$_2$ (right) in vivo.

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 (third column) gel formulations. White/red pixels: oxy-Hb; blue pixels: deoxy-Hb. Photoacoustic monitoring revealed that oxy-Hb levels significantly increased for the entire observational period in the animals treated with OLNDs while, as expected. OSS induced a high but only transient peak in oxy-Hb, whereas OFNDs did not affect oxy-/deoxy-Hb balances at all. After topical administration of US-activated OLNDs, hypoxic mice displayed larger oxygenation levels in a time-sustained manner, OLNs significantly increased transcutaneous capillary oxygenation in topically treated mice.

**4. A combined approach: HIFU and OLNs**

In this paragraph, a combined approach, regarding HIFU and OLN is provided. Temperature increase induced by a focused ultrasound field (Transducer 2) in two different TMMs is studied: one of them is prepared according to the receipt reported in paragraph 2.3.4, while, for the second TMM, 20 ml of dextran OLNDs are added before the gelling process is completed. By means of a micro-positioning system, the TMM under study, including the optical fiber, are moved to reach the point of maximum revealed temperature, which is supposed to be the focus region of the ultrasound beam. Temperature data are acquired by means of the fiber optic hydrophone when the gel is subjected to a focused ultrasound field produced at three different power values (50, 75, 100W). The exposure time is 5 s every measurement. Temperature values are calculated as mean of five measures for each measurement condition. In Fig. 14 data obtained are shown.
Fig. 14 - Temperature increment in an agar-based TMM (black bars) and in a TMM in which 20 ml of OLND are added to the solution (red bars).

As it is possible to see from the figure above, temperature rise induced by an HIFU source in the TMM phantom without OLN (black bars) are lower than the temperature reached in a TMM in which 20 ml of OLN are added (red bars) in all the considered cases. The percentage of temperature increment using a TMM containing OLN is about 26% at 50W, 22% at 75W and 34% at 100W. These experimental evidence are probably due to the fact that OLN may act as nuclei for acoustic cavitation inducing formation of shock waves and local temperature increase.

Conclusion

This article summarizes the results of three years activity devoted to the study of two different approaches for cancer treatment based on the use of ultrasound for therapeutic purpose. These two techniques regard the characterization of high intensity focused ultrasound (HIFU) and the synthesis, characterization and applications (both in vitro and in vivo) of oxygen loaded nano-bubbles. Finally, the two approaches have been used simultaneously, revealing the basis of a new therapeutic modality.

The characterization of an ultrasound source has been provided measuring the total emitted power and a spatial description of the pressure field. Beside them, an accurate study of the temperature rise in the focus of an HIFU transducer has been performed using a fiber optic probe hydrophone in different TMM. These measurement are necessary to understand the interaction between ultrasound beam and tissues. Simultaneously, the other research activity has concerned the realization and characterization of oxygen load nanobubbles. OLN developed are constituted by a shell of biocompatible material (chitosan or dextran) and an oxygen-storing core, made of decafluoropentane (DFP) or perfluoropentane (PFP). The behaviors of the different nano-bubbles preparation have been fully studied and compared looking for a further applications to treat hypoxia-associated pathologies of superficial tissues or to treat cancer along other techniques such as HIFU. The OLN formulations has been characterized from a physical-chemical point of view, after that, OLN's ability to across a skin membrane have been evaluated in vitro and in vivo. All experiments have confirmed that topical administration of exogenous $O_2$, properly encapsulated in nano-bubble formulations, might be a new suitable and efficient approach to treat hypoxic tissues, cancerous or affected by other disease. In the end, the two techniques, concerning HIFU field characterization and the development of oxygen nano-carriers, have been used for experiments that merge them in a new and non invasive therapeutic...
modality. Indeed, focused ultrasound destroy cancerous tissues while, in the same time, nano-bubbles are able to counteract hypoxia effects (by improving oxygen delivery to hypoxic tissues) and to increase temperature reached in the focus on an HIFU field. This new therapeutic modality could be useful to improve the effects of HIFU therapy and decrease the time required for the treatment.

References