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RESEARCH ARTICLE

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Orally administered oxygen nanobubbles enhance tumor response to sonodynamic therapy

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Abstract

Suspensions of oxygen-filled bubbles are under active investigation as potential means of relieving tissue hypoxia. Intravenous administration of large quantities of bubbles is, however, undesirable. Previous work by the authors has demonstrated that tumor oxygen levels can be increased following oral administration of phospholipid stabilized oxygen nanobubbles. The aim of this study was to determine whether this would enhance the efficacy of sonodynamic therapy (SDT), which is known to be inhibited in hypoxic tissue. Experiments were conducted in a murine model of pancreatic cancer. Animals were treated with SDT (intratumoural injection of 1 mM Rose Bengal followed by exposure to 1 MHz ultrasound, 0.1 kHz pulse repetition frequency, 30% duty cycle, 3.5 W cm^{-2} for 3.5 minutes) either with or without a prior gavage of oxygen bubbles. A statistically significant reduction in the rate of tumor growth was observed in the groups receiving oxygen nanobubbles either 5 or 20 minutes before SDT. Separate measurements of tumor oxygen using a fiber optic probe and expression of hypoxia inducible factor (HIF)1α following tumor excision, confirmed the change in tumor oxygen levels. These findings offer a potentially promising new approach to relieving tissue hypoxia in order to facilitate cancer therapy.

KEYWORDS
hypoxia, nanobubbles, oxygen delivery, sonodynamic therapy, ultrasound

1 | INTRODUCTION

Hypoxia, that is, an imbalance between oxygen supply and consumption, is a common feature of solid tumors. Its primary cause is the disorganized microarchitecture of vascular networks in cancerous tissues which results in impaired blood flow and inadequate oxygen delivery to cells.[1] Hypoxia can be further exacerbated by disease- or treatment-associated anemia.[2] Tumor hypoxia is associated with impaired therapeutic response, malignant progression and poor clinical outcomes.[3,4] In particular it has been shown to lower the efficacy of treatments dependent on reactive oxygen species (ROS) generation, such as chemo-, radio- and photodynamic therapy.[5,6] This is

likely due in part to the fact that a low oxygen concentration inhibits efficient ROS generation. Hypoxia is also believed to play a role in the development of aggressive cell phenotypes which have suppressed apoptosis pathways, greater metastatic potential and are more resistant to therapeutic interventions.[7] In addition, impaired vascularization, which is the major cause of low oxygen content in tumors, also prevents therapeutic agents, such as drugs or sensitizers, from efficiently diffusing into cancerous cells that are too remote from blood vessels.[8]

It is important to state that the role of ROS in cancer progression, although undoubtedly significant, is complex and not yet fully understood.[9] ROS have been associated with increased cell proliferation and survival, while at the same time they are also capable of inducing cell death pathways.[10] This notwithstanding, multiple clinical and pre-clinical studies have shown that alleviating hypoxia can improve the efficacy of ROS-dependent therapies.[11] Multiple methods have been explored and can be roughly classified as: (i) manipulating breathing gas composition; (ii) manipulating circulatory system structure and components; (iii) changing oxygen consumption of cells; and (iv) introducing exogenous oxygen carriers. It should be noted that there has also been extensive research into developing bioreductive drugs and sensitizers for hypoxic tissue, but these are not directly relevant to the present study.

Arguably the most obvious solution for alleviating hypoxia is increasing oxygen concentration in the breathing gas of the patient. Hyperbaric oxygen (HBO) therapy is a well-established procedure, involving breathing 100% oxygen under increased pressure, and has been widely used for treatment of conditions such as decompression sickness and carbon monoxide poisoning. It has also shown promise in cancer treatment when used in combination with radio-, chemo- and photodynamic therapy.[12] A number of studies, however, have reported no benefit;[13] and the risk of systemic oxygen toxicity, as well as technical difficulties and safety concerns associated with combining hyperbaric oxygen chambers with other therapies, have prevented its widespread use in oncology.

Another breathing gas that has been used for tumor oxygenation is carbogen—a mixture of carbon dioxide and oxygen. The carbon dioxide content in carbogen is usually 5% but can be as low as 1.5% or as high as 50% in different compositions. Its action is based on increasing blood levels of carbon dioxide, which triggers mechanisms associated with suffocation, such as deeper breathing and increase in heart rate, and lead to better blood oxygenation. However, similar to HBO, its therapeutic efficacy has not been conclusively demonstrated.[14]

A different approach to increasing local oxygen levels is manipulating the circulatory system or its components. Methods of enhancing oxygen transport include stimulation of red blood cell production by erythropoietin treatment[15] and introducing hemoglobin oxygen release co-factors, for example 2,3-diphosphoglycerate.[16] Conversely, some researchers have attempted to mediate tumor oxygen levels by introducing drugs that inhibit oxygen consumption,[17] glucose (making use of the Crabtree effect),[18] or by simple mechanisms such as manipulating local tumor temperature.[19] Unfortunately, while enhancing the existing mechanisms in the body to fight hypoxia is an elegant concept, a major problem of such methods is insufficient selectivity that can lead to serious systemic side effects.

The fourth category is the use of exogenous oxygen sources or carriers. Tumor hypoxia is often partly caused by disease- or treatment-related anemia, which limits the oxygen-carrying capabilities of blood and can thus be directly addressed by additional carriers. The most obvious choice of carrier is a red blood cell. As mentioned above, however, attempts to stimulate red blood cell production in patients often results in unwanted systemic side effects. Similarly, the use of blood transfusions in this context carries several risks, for example, transfer of infection, and has not been shown to give a sufficient advantage in cancer therapy.[20] Alternative strategies have been put forward for in situ oxygen production using nanoparticles,[21,22] or oxygen-generating implants.[23] The major limitation of artificial oxygen generation in the body, however, is the need for extremely careful regulation and localization.

Artificial blood products have been widely explored as a means of exploiting the efficiency of hemoglobin as an oxygen carrier, without the risk of eliciting an immune response. However, clinical trials of these blood substitutes, though promising, have largely failed because their complication rates were still higher than those of traditional blood transfusions. Research in this area continues[24] to date but remains problematic.[25] Another important class of oxygen transporters that has been investigated is perfluorocarbon-based (PFC-based) agents. PFCs have low solubility and diffusivity in water, and therefore form relatively stable particles, and offer the possibility of high oxygen loading. Unlike hemoglobin, they have a linear oxygen release response as a function of surrounding oxygen concentration. They are, however, non-reactive, stable and biologically neutral; and a further advantage for cancer therapy is the possibility to use them in combination with focused ultrasound (US) as cavitation nuclei to enhance extravasation of therapeutics. To this end, low molecular weight PFCs (perfluoropropane and perfluorobutane) have been used to form microbubbles that can be loaded with oxygen for localized delivery under ultrasound exposure. Several groups have shown

that oxygen loaded microbubbles are capable of reducing hypoxia in vitro and in vivo.[26–30] McEwan et al.[27] have shown enhanced response to ultrasound-responsive drugs (sonodynamic therapy) in vivo in pancreatic tumors, and Eisenbrey et al.[29] have successfully increased breast tumor oxygenation levels in vitro by 20 mmHg. Encouragingly, no adverse side effects were observed in these studies. There have also been multiple studies of smaller diameter oxygen loaded bubbles for enhancing various cancer therapies in recent years.[31,32]

PFC based carriers for oxygen delivery have, however, suffered from the same problems as hemoglobin-based agents, that is, unwanted side effects at high intravenous concentrations;[33] and the stability of pure oxygen bubbles is low due to its high diffusivity.[26] Consequently both intraperitoneal and oral administration of oxygen-loaded bubbles have also been investigated.[34,35] Oral administration in particular offers significant advantages, as it reduces the risk of infection, improves patient acceptability and compliance and reduces cost. Importantly, it also reduces the risks of embolism and/or lipid toxicity associated with intravenous injection of bubbles that severely limit the quantities that can be administered. For example, the maximum injectable dose of microbubble contrast agent for a human is < 5 mL. Previous work by the authors demonstrated that an orally delivered suspension of oxygen nanobubbles could produce an increase in tumor oxygen levels comparable to that generated by intravenously injected microbubbles.[35] A subsequent study reported similar findings and a direct anti-tumor effect of the nanobubbles themselves in a breast cancer model.[36] The aim of the present study was to determine whether this approach could be used to improve therapeutic response to sonodynamic therapy, by exploiting the temporary increase in tumor oxygen levels to facilitate production of ROS and hence achieve a localized cytotoxic effect.

2 | RESULTS

2.1 | Bubble size distribution and concentration

The size distribution and concentration of the nanobubbles were measured before and after sparging with either oxygen or nitrogen gas to investigate any changes in the population. The size distribution was not found to be significantly different between the three conditions (Figure 1A). A small increase in concentration was observed following sparging, presumably due to the additional agitation of the liquid during this process.

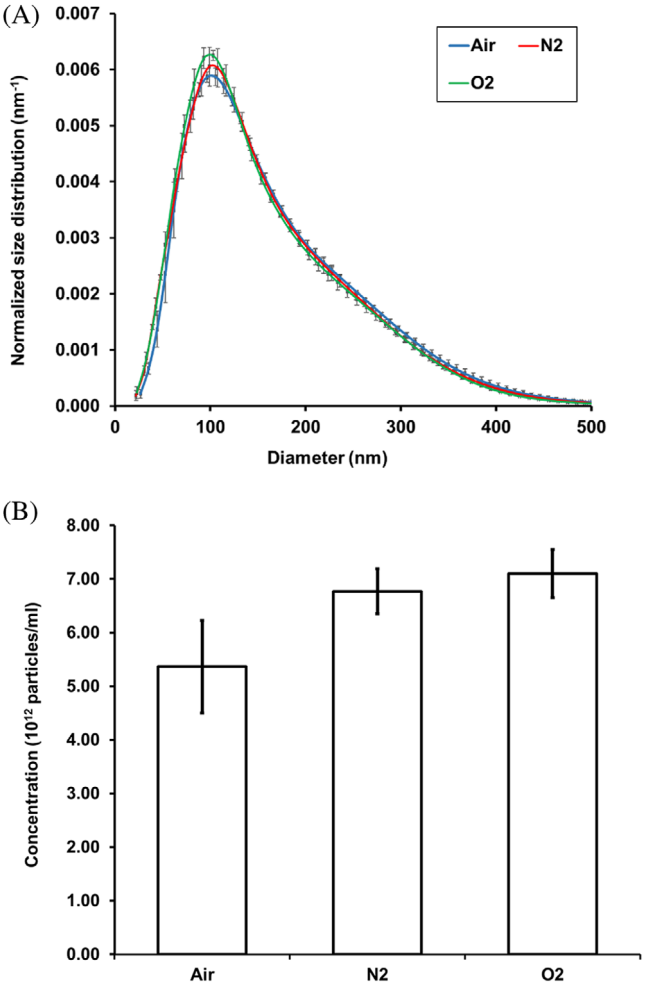

FIGURE 1 A, Size distributions and (B) concentrations of the nanobubble suspensions after production (air) and after sparging with nitrogen or oxygen measured using the nanoparticle tracking analyzer (NanosightNS300). ($n = 3$; error bars indicate standard deviation)

2.2 | Bubble oxygen content

The measurements of oxygen release after introducing oxygenated nanobubbles into deoxygenated (nitrogen-saturated) medium were consistent with those reported by Owen et al.[35] showing a rapid elevation in oxygen concentration upon injection of either oxygenated nanobubbles or oxygenated milliQ water (control), with the bubble suspension producing a more sustained change (Figure 2).

2.3 | Response to sonodynamic therapy

Mice bearing human pancreatic BxPC-3 xenograft tumors were dosed with oxygen bubbles via gavage followed by an

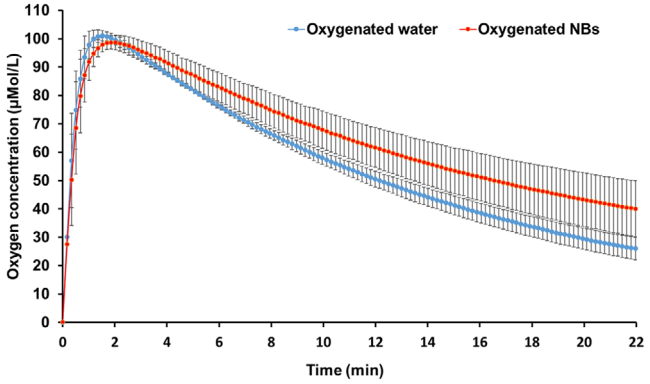

FIGURE 2 Measurements of oxygen concentration after addition of 1 mL of oxygenated sample (nanobubble solution or Milli-Q® water) to 10 mL of deoxygenated (nitrogen-saturated) water under constant mixing of solution and nitrogen flow in vial headspace ($n = 3$; measurement every 10 seconds; error bars indicate standard deviation)

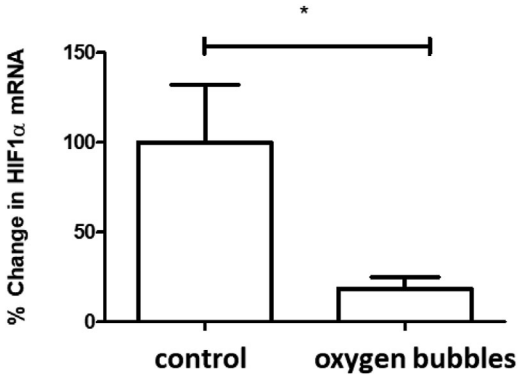

FIGURE 4 Expression of HIF-1α at a transcriptional level in tumours extracted from mice 30 minutes after administration of oxygen bubbles via gavage ($n = 5$; $^*P < 0.05$ determined via one tailed t-test; error bars indicate standard error)

intratumoural injection of a sonosensitizer (Rose Bengal) and exposure to ultrasound either 5 or 20 minutes post gavage. Control groups receiving no treatment, oxygen bubbles only or Rose Bengal and ultrasound only were also included. A statistically significant difference in the rate of change tumor volume was observed between the groups receiving SDT only and those receiving both SDT and oxygen bubbles 19 days after treatment (Figure 3). There was no statistically significant difference between the results for those animals receiving the bubbles 5 minutes before treatment and those receiving them 20 minutes before. The bubbles alone had no significant effect upon tumor growth (please see the supporting information for individual tumor volume curves and final weight).

2.4 | HIF-1α expression

HIF1-α was selected as a physiological probe for oxygen delivery as its rapid degradation in the presence of oxygen provides a direct and immediate indicator of increased tumor oxygen levels. Consistent with previous experiments,[35] a statistically significant reduction in the expression of HIF-1α was measured in excised tumors from mice receiving oxygen bubbles as compared to those receiving the control (unsparged) gavage (Figure 4).

2.5 | Intratumoural oxygen measurements

Also consistent with previous experiments[35] measurements from an implanted oxygen probe (Figure 5)

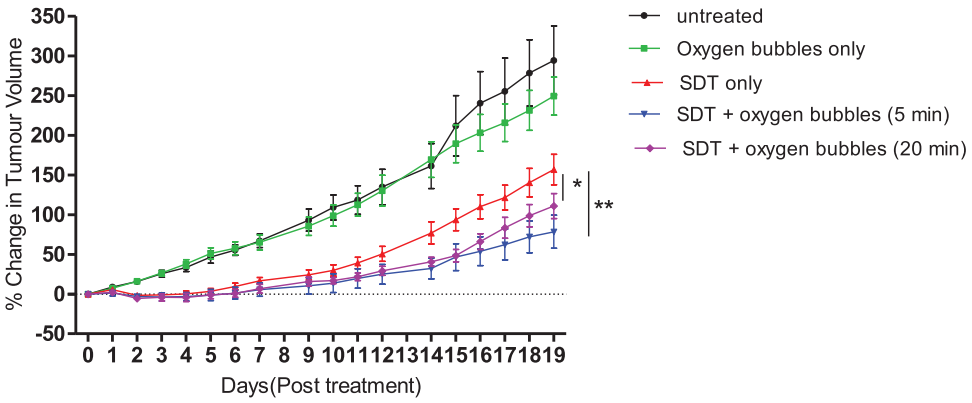

FIGURE 3 Effect of oral administration of oxygen bubbles upon tumour response to sonodynamic therapy. Treated animals received oxygen bubbles by gavage and/or SDT, that is, an intratumoural injection of Rose Bengal followed by exposure to ultrasound for 3.5 minutes. SDT was administered either 5 or 20 minutes after the oxygen bubble gavage. Animals were treated on Days 0, 5, 11 and 15. ($n = 5$, $^*P < 0.05$, $^{**}P < 0.01$; determined via a one-tailed t-test for measurements at day 19; error bars indicate standard error)

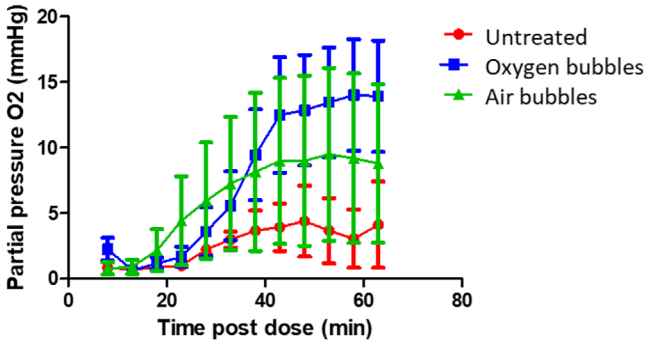

FIGURE 5 Partial pressure of oxygen in a mouse xenograft tumour model for human pancreatic cancer following administration of bubbles sparged with oxygen ($n = 5$) or air ($n = 5$) or no treatment ($n = 3$). No statistically significant differences were observed between oxygen and air bubble groups. Error bars indicate standard deviation

indicated an increase in partial pressure within the tumor following administration of oxygen bubbles (prior to ultrasound exposure). The variance in the measurements was however very large, such that there was no statistically significant difference between the effect of the oxygenated and air sparged bubbles. This was attributed to the fact that, under the terms of the animal license, measurements had to be conducted with a single probe, the position of which could not be varied.

3 | DISCUSSION

3.1 | Orally administered nanobubbles promote SDT

The data shown in Figure 3 support the hypothesis that the increase in tumor oxygenation produced by oral administration of oxygen bubbles has the potential to enhance tumor response to SDT. This finding is also consistent with the results of previous studies using intravenously delivered oxygen microbubbles to promote SDT, in which similar changes in tumor oxygen levels and in hypoxia inducible factor expression to those in Figures 4 and 5 were measured and found to correlate with therapeutic effect;[27,37] and of intravenous nanobubbles to promote photodynamic therapy.[31] No indicators of acute toxicity were observed and there were no statistically significant changes in animal body weight over the course of the study (Figure S7). This is very encouraging for the potential use of oxygen bubbles as a therapeutic adjuvant as it indicates that the current limitations imposed on maximum injected volume may be overcome by changing the administration route. The simplicity of the approach moreover makes it suitable for use with other therapies for which relieving hypoxia is known to be beneficial such as radiotherapy or immunotherapy.

3.2 | The timing of treatment administration did not affect the therapeutic outcome

There was no statistically significant difference between the treatment groups receiving SDT 5 or 20 minutes after oxygen bubble administration. The results shown in Figure 5 suggest that this may be because the treatment should have been delivered at a later point once the oxygen level in the tumor had risen to its maximum level. Unfortunately due to the restrictions of the license under which the experiments were performed, it was not possible to simultaneously measure tumor oxygen and deliver SDT. Further work is needed to make a detailed study of the circulation kinetics of the bubbles, to optimize the treatment protocol and also to assess the effect of multiple treatments which have been shown to enhance the therapeutic effect in the case of SDT with microbubbles.[38]

3.3 | Further work is needed to elucidate the mechanism of oxygen delivery

An interesting question for future investigation is the mechanism of oxygen delivery. The quantity of encapsulated oxygen can be estimated from both the oxygen concentration and bubble size and concentration measurements. The former indicate that the quantity of oxygen in the undiluted suspension is of the order of 10 mg L^{-1} of oxygen. From Figure 1, the total volume of oxygen that could be encapsulated within bubbles of the size indicated in Figure 1 is approximately 10^{-8} m^3 mL^{-1} of suspension. Assuming that there is a sufficiently tightly packed coating on the bubble surface to balance surface tension, this also corresponds to ~10 mg L^{-1} of oxygen. A 0.2 mL dose of oxygen bubble suspension thus contains ~0.002 mg oxygen. Even assuming 100% of the ingested bubbles were transferred to the bloodstream, this quantity of oxygen would be unlikely to produce either a large or sustained effect, being approximately equivalent to the quantity inhaled in 1 breath.[39] This quantity is also inconsistent with the measured changes in tumor oxygen in Figure 5 or reported in[35] that persist over tens of minutes.

A possible explanation for this discrepancy, is that the lipid coated bubbles, being of comparable size to chylomicrons,[40] are passing into the bloodstream via the digestive tract and acting as oxygen carriers. The duration of the change in tumor oxygen levels suggests the bubbles are circulating and thus can potentially absorb oxygen

during their passage through the lung capillary bed and subsequently release it in areas of hypoxia. This is one of the mechanisms by which suspensions of perfluorocarbon nanodroplets are assumed to function as blood substitutes;[41,42] and indeed lecithin micelles have also been investigated in this context on account of the high solubility of oxygen in lecithin and their negligible toxicity. The timescale over which the increase in tumor partial pressure was observed would be consistent with upper gastrointestinal tract absorption.[43] If this hypothesis is correct then the bubble suspension would be increasing the oxygen carrying capacity of the blood by 0.05-0.5 mg L^{-1} depending on the proportion of bubbles passing into the blood stream (for comparison a 5% increase in hematocrit would provide 1.4 mg L^{-1}). Over tens of minutes the corresponding increase in the oxygen concentration gradient in the tumor could deliver much more significant quantities of oxygen than that initially encapsulated in the administered dose. This could potentially explain why the air bubbles in this study and the argon sparged bubbles in the previous study (Figure 4B in reference[35]) were also found to increase tumor oxygen levels and influence HIF-1α when they were not expected to. Further investigation is however needed both to confirm this hypothesis and then understand the rates of gas transfer and why the oxygenated bubbles still produced a more significant effect.

4 | CONCLUSIONS

The results of this study showed that an improved response to SDT was observed following oral administration of oxygen nanobubbles in a mouse model of pancreatic cancer. This finding is consistent with previous observations of increased tumor oxygenation produced by orally delivered oxygen bubbles. The duration over which tumor oxygen levels were elevated and calculation of the quantities of encapsulated oxygen suggest that the primary effect of the bubbles may be to increase the oxygen carrying capacity of the blood. A more detailed investigation of the effect of oxygen bubbles upon the spatial distribution of tumor hypoxia and the relationship to perfusion and other tissue characteristics is needed to fully assess their potential as a therapeutic adjuvant.

5 | EXPERIMENTAL SECTION

5.1 | Materials and suppliers

Lecithin and citric acid were purchased from Special Ingredients (Chesterfield, Derbyshire, UK). Rose Bengal (RB), phosphate buffered saline (PBS), cell culture media, glycyrrhizic acid and glycerol were purchased from Sigma-Aldrich Ltd (Gillingham, Dorset, UK). Oxygen and nitrogen cylinders were purchased from BOC gases (Guildford, Surrey, UK). Hypnorm and Hypnovel were purchased from VetaPharma Ltd. (Leeds, West Yorkshire, UK) and Roche (Welwyn Garden City, Hertfordshire, UK) respectively. Matrigel was purchased from BD Biosciences (Erembodegem, Belgium). Anagel ultrasound couplant was purchased from Ana Wiz Ltd. (Surrey, UK). Singlet Oxygen Sensor Green was purchased from Thermo Fisher Scientific (Loughborough, UK). Cells were purchased from ATTC, LGC Standards (Teddington, Surrey, UK). Independent identification was not carried out. Animals were purchased from Envigo (Huntingdon, Cambridgeshire, UK) for the sonodynamic therapy experiments and Charles River (Portishead, Avon, UK) for the oxygen probe experiments described below.

5.2 | Bubble fabrication

The bubble solutions were prepared according to Owen et al.[35] Briefly, filtered, de-ionized water was mixed with glycyrrhizic acid (3 mg mL^{-1}), lecithin (3 mg mL^{-1}), citric acid (5 mg mL^{-1}) and glycerol (0.0125 mL mL^{-1}). The solution was then stirred for 30 mins on a hot plate at 50°C. For individual experiments, 5 mL of the solution was then transferred to a glass vial and sparged with oxygen, nitrogen, or air as required for 3 minutes. The vial was then immediately sealed and mechanically agitated for 30 seconds.

5.3 | Bubble characterization

The size distributions and concentrations of the bubble suspensions were measured at different stages of preparation using a nanoparticle tracking analyzer (NTA) (Nanosight NS300, Malvern, PA). The solution was diluted 1:10,000 with filtered deionized water. Measurements were made before sparging, then after sparging with N_2 or O_2 to examine any changes in the size distribution.

The oxygen content of the bubble suspensions was measured using an optical oxygen sensor (OXY MINI Minisensor Oxygen Meter, PreSens Precision Sensing GmbH, Regensburg, Germany) with temperature compensation. An SP-PSt3-NAU oxygen sensor was attached to the inner surface of a glass vial on the level approximately corresponding to the middle of the solution. The oxygen sensor temperature probe and a magnetic bar were placed inside; the vial was placed on a mixing plate (500 rpm), put into a fume hood and covered to protect it from strong air flow. 10 mL of milliQ water were added into the vial and

nitrogen gas at 1 bar was bubbled through the liquid until the oxygen sensor showed a negligible oxygen concentration. 5 mL of sample (bubbles or Milli-Q® water for control) were sparged with oxygen for 3 mins, and then 1 mL of oxygenated solution was added into the measuring vial. The nitrogen feed was taken out of the solution but kept near the surface of the liquid to maintain the gas concentration gradient. All oxygen measurements were calibrated and carried out at room temperature

5.4 | In vivo experiments

All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with local ethical committee approval. Animals were housed in individually ventilated cages with a maximum of 6 animals per cage, a 12 hour light/dark cycle, unrestricted access to food and water, and environmental enrichment.

5.5 | Sonodynamic therapy

BxPc-3 human pancreatic tumor cells (1×10^6, 1:1 media:Matrigel) were subcutaneously implanted into the dorsum of C.B-17/IcrHan Hsd-*Prkdc scid* mice using isoflurane anesthesia. Mice were both male and female and a minimum of 6 weeks old when implanted. They were randomly allocated to groups 4–5 weeks after cell implantation when the tumors had reached an average volume of 150 mm^3 (Day 0). A minimum of 5 mice was included in each group. Mice were anaesthetized with a mixture of Hypnorm and Hypnovel, diluted in an equal volume of water for injection [1:1:2 ratio]. This was administered as a single 100 μL intraperitoneal injection. For those mice receiving Rose Bengal, 100 μL of a 1 mM solution was injected intratumorally. Mice receiving the oxygen nanobubble suspension were given 100 μL using a flexible gavage. Immediately prior to dosing with the oxygenated bubbles, the vial was shaken vigorously for approximately 1 minute. Those mice receiving both Rose Bengal and oxygen nanobubbles, were given the latter 30 minutes after the RB injection while they were still anaesthetized. Ultrasound was applied to the tumor using a Sonidel SP100 probe (Sonidel Ltd., Republic of Ireland) for 3.5 minutes at an intensity of 3.5 W cm^{-2}, center frequency 1 MHz, pulse repetition frequency 0.1 kHz and 30% duty cycle). Ultrasound gel was applied to the probe head to ensure good coupling and the probe was slowly moved over the entire tumor during the treatment. US treatment was given either 5 minutes or 20 minutes after the oxygen nanobubble gavage. Animals were treated on Day 0, 5, 11 and 15 according to a previously optimized protocol. Tumors were measured a minimum of 5 times per week using calipers, tumor volume = (width x length x height)/2.

5.6 | Tumor oxygen measurements

In a separate experiment, BxPc-3 xenografts were again established by subcutaneous injection of tumor cells (1×10^7, 1:1 media:Matrigel) in the right flank of athymic nude female mice (8 weeks old) under isoflurane anesthesia. When the tumors reached an average volume of 150 mm^3, animals were randomly assigned to one of three groups, receiving no treatment (n = 3), air sparged bubbles (n = 5) or oxygen sparged bubbles (n = 5). In these experiments animals were gavaged with 0.4 mL suspension, anaesthetized using isoflurane in air and placed on a heat mat to maintain core body temperature. An oxygen/temperature bare-fiber sensor of tip diameter 350 μm (Oxylite by Oxford Optronix) was inserted into the center of the tumor, and tumor oxygen partial pressure (in mmHg) was recorded every 5 minutes for 1 hour.

5.7 | Hypoxia Inducible Factor (HIF)-1α expression

Following the oxygen measurements described above, the animals were euthanized. The tumors were harvested by surgical excision and RNA extracted using Trizol (Invitrogen, Paisley, UK) and reverse transcribed using a first strand cDNA synthesis kit according to the manufacturer's instructions (Roche, Welwyn Garden City, UK). Real Time Quantitative PCR (RT-Q-PCR) was undertaken using SYBR green (Fermentas, Cambridge, UK) and gene-specific primers in a Lightcycler 480 (Roche, Welwyn Garden City, UK). Using Hypoxanthine-guanine phosphoribosyltransferase (HPRT) as a reference, expression of HIF-1α was calculated using the comparative CT (ΔΔCT) method.

Supporting Information is available from the Wiley Online Library or from the author.

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CONFLICTS OF INTEREST

Joshua Owen and Eleanor Stride are co-inventors on a patent (Stride, E., Averre, R., Owen, J. (2015)

Nanoencapsulated Oxygen. UK Patent Application No. 1512728.5), which has been filed in relation to one of the formulations described in the paper. Avrox Technologies Ltd. has licensed this patent but did not fund this work or play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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