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Development of an ultrasound sensitive oxygen carrier for oxygen delivery to hypoxic tissue

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Abstract

Radiation therapy is frequently used in the treatment of malignancies, but tumors are often more resistant than the surrounding normal tissue to radiation effects, because the tumor microenvironment is hypoxic. This manuscript details the fabrication and characterization of an ultrasound-sensitive, injectable oxygen microbubble platform (SE61O₂) for overcoming tumor hypoxia. SE61O₂ was fabricated by first sonicating a mixture of Span 60 and water-soluble vitamin E purged with perfluorocarbon gas. SE61O₂ microbubbles were separated from the foam by flotation, then freeze dried under vacuum to remove all perfluorocarbon, and reconstituted with oxygen. Visually, SE61O₂ microbubbles were smooth, spherical, with an average diameter of 3.1 μ m and were reconstituted to a concentration of 6.5 E7 microbubbles/ml. Oxygen-filled SE61O₂ provides 16.9 $\pm$ 1.0 dB of enhancement at a dose of 880 μ l/l (5.7 E7 microbubbles/l) with a half-life under insonation of approximately 15min. In *in vitro* release experiments, 2 ml of SE61O₂ (1.3 E8 microbubbles) triggered with ultrasound was found to elevate oxygen partial pressures of 100 ml of degassed saline 13.8 mmHg more than untriggered bubbles and 20.6 mmHg more than ultrasound triggered nitrogen-filled bubbles. In preliminary *in vivo* delivery experiments, triggered SE61O₂ resulted in a 30.4 mmHg and 27.4 mmHg increase in oxygen partial pressures in two breast tumor mouse xenografts.

Keywords

Tumor hypoxia; Ultrasound contrast agent; Oxygen delivery; Surfactant; Microbubble

1. Introduction

The highly chaotic process of tumor angiogenesis (whereby cancerous tumors recruit and develop new blood vessels) results in hypoxic conditions on the cellular level, due to oxygen

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transport within the tumor that is inadequate to compensate for the high metabolic rate (Richard et al., 1999). Whereas healthy subcutaneous tissue generally exhibits oxygen partial pressures from 40–60 mmHg, many cancers exhibit partial pressures between 2 and 18 mmHg (Brown and Wilson, 2004). Hypoxic cells have been shown to be more resistant to death from radiation exposure than aerobic cells (Rockwell et al., 2009). This phenomenon provides an innate level of tumor resistance to radiotherapy, a resistance that results in decreased tumoral response and increased odds of recurrence. Cellular resistance to radiation is most pronounced at oxygen levels of less than 20 mmHg (Brown, 2007). Interestingly, a relatively small increase in oxygen partial pressure (pO_2) in hypoxic cells can result in significant sensitization to radiation therapy and this can occur almost instantaneously (Brown 2007; Hodgkiss et al., 1987). Hence, a platform that could increase pO_2 by as little as 10 mmHg immediately prior to radiation could provide significant sensitization to radiation therapy and ultimately improve patient outcomes.

Ultrasound contrast agents (UCA) are intravenously injected gas microbubbles stabilized by an outer shell, generally consisting of a protein or phospholipid monolayer (Goldberg et al., 2001). These UCA function as vascular contrast agents, which can enhance echogenicity by up to 27 dB (Davidson and Lindner, 2012; Goldberg et al., 2001; Miller and Nanda, 2004). Additionally these agents permeate into the tumor microvasculature and are useful for imaging angiogenesis (Eisenbrey and Forsberg, 2010). While first generation UCA initially relied on an air core to provide ultrasound enhancement, newer generations use lower solubility, higher molecular weight gases such as perfluorocarbons or sulfur-hexafluoride to slow diffusion into the media for prolonged enhancement times (Goldberg et al., 2001).

At sufficient acoustic pressures (although still within FDA guidelines of ultrasound outputs), UCA undergo microbubble rupture (Goldberg et al., 2001). Although this rupture, termed inertial cavitation, causes the microbubbles to “disappear” on imaging, destruction by the ultrasound beam is useful for drug delivery. A great deal of literature exists on efforts to encapsulate therapeutics (primarily anticancer drugs) within UCA. Cavitation of these UCA within the tumor vasculature using noninvasive focused ultrasound has been reported as a means to provide localized drug delivery (Eisenbrey et al., 2009, 2010; Bekeredjian et al., 2006; Wang et al., 2010; Escoffre et al., 2011; Ferrara et al., 2007). The use of UCA for delivering gases is less well explored, with most studies focusing on the delivery of nitric oxide for controlling arterial thrombosis or cardiovascular disease (Cavalieri et al., 2008; Postema et al., 2006; Klegerman et al., 2010; Tong et al., 2013). Nanobubble UCA have been proposed as a mechanism for O_2 delivery using nanobubbles stabilized with either chitosan (Cavalli et al., 2009a) or dextran (Cavalli et al., 2009b). Micron-sized agents have also been developed using protein-shelled UCA (Swanson and Borden, 2010), and lipid-shelled UCA (Swanson et al., 2010; Kwan et al., 2012; Polizzotti et al., 2014). Despite this, development of surfactant-shelled, O_2 microbubbles, which could potentially provide a better delivery platform, has yet to be investigated.

Our group has studied the fabrication, characterization, and *in vivo* imaging potential of surfactant-stabilized microbubbles extensively (Wheatley and Singhal, 1995; Forsberg et al., 1997, 1999; Basude et al., 2000; Basude and Wheatley, 2001). The low toxicity of microbubbles, combined with the ability to control microbubble size, provides a safe and

easily customizable platform for delivery of bioactive gases (Forsberg et al., 2010; Wheatley et al., 2006). Finally, a recent method of freeze-drying these particles under vacuum (Solis et al., 2010) allows the nascent bubble to be preserved for extended periods of time and resuspended with a desired gas. In this manuscript we characterize the ability of a microbubble agent to noninvasively elevate O₂ concentrations as a mechanism for overcoming tumor hypoxia-associated radiotherapy resistance.

2. Materials and methods

2.1. Microbubble fabrication

The UCA used in this study (termed SE61_{O2}) was fabricated using a well-described method for fabricating surfactant UCA (Wheatley and Singhal, 1995). Span 60 (sorbitan monostearate; 1.5 g; Sigma–Aldrich, St. Louis, MO) and water soluble vitamin E (D-alpha tocopheryl polyethylene glycol 1000 succinate or TPGS; 1.3 g; Eastman Chemical Company, Kingsport, TN) were dissolved in 50 ml of phosphate buffered saline (PBS; Sigma–Aldrich) and autoclaved for 30 min. The mixture was cooled under magnetic stirring and placed in an ice bath and continuously sonicated for 3 min at 110 W using a 0.5 in. probe horn (CL4 tapped horn probe with 0.52" tip, Misonix Inc., Farmingdale, NY). The solution was purged with a steady stream of octafluoropropane before and during the sonication. Microbubbles were extracted from the solution *via* gravity separation in a 250 ml glass separation funnel, washed 3 times with cold (4 °C) PBS every 90–120 min using the same separation funnels. Two milliliters aliquots of native bubble suspension were pipetted with a pipet specifically designed for viscous fluids (Gilson Pipetman, Middleton, WI), into 15 ml lyophilization vials. Finally, 0.5 ml 400 mM glucose (Sigma–Aldrich) was added to each vial as a lyoprotectant.

The 2.5 ml samples in lyophilization vials were freeze-dried as described in the literature (Solis et al., 2010). In brief, Fluortec® lyophilization stoppers were inserted into the vials to the first groove (leaving a gap for air to escape). The samples were flash-frozen in liquid nitrogen for 5 min and subsequently freeze-dried for 24 h on a Virtis Benchtop freeze-dryer (Gardiner, NY) fitted with a two shelf assembly that had been previously cooled to –80° C. The conditions during this process were –76.5 °C (in the vacuum drier chamber) and 17–20 µBar which removes all octafluoropropane. Before removing the samples, the piston was lowered to depress the stoppers, effectively vacuum sealing the vials. The gas of choice (nitrogen or oxygen, Airgas LLC, Radnor, PA) was introduced through the Fluortec® stoppers using a sterile syringe needle, after passage through a sterile 0.22 µm Nalgene filter (Nalge Nunc International, Rochester, NY) at an initial flow rate of 50ml/min for 5–10 s then 20ml/min for 1 min to insure the vials were filled. The procedure was performed under an aseptic laminar flow hood and vials sealed with parafilm until use. Immediately prior to use, the vials were charged with 2 ml of deionized water. As a control group (to ensure all ultrasound enhancement is attributed to the addition of O₂), samples were maintained under vacuum and reconstituted in the usual way with deionized water prior to use (thereby creating particles without additional gas).

2.2. Particle morphology, size, and concentration characterization

Particle morphology was assessed by light microscopy using an Olympus IX71 microscope (Tokyo, Japan) at 40× magnification. The size distribution of the microbubbles was analyzed using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) in Z-average mode, using dynamic light scattering techniques. Using this technique a 50 µl sample was dispersed in 950 µl PBS in tapered cuvettes for size-analysis. Particle counting was performed using a flow cytometer, LSR II (BD Biosciences, San Jose, CA). With this technique 10 µl of microbubbles were added to 0.5 ml of deionized water and 10 µl of UV Countbright absolute counting beads (containing 9800 beads used as a counting standard; Life Technologies, Grand Island, NY). Counting beads and SE61_{O2} particles were separated using FSC-A and PE-A filters.

2.3 Particle stability and ultrasound enhancement characterization

A custom-built acrylic plastic sample holder with a clear acoustic window (2.5 cm × 2.5 cm) was placed in a tank filled with 75l of deionized water (temperature-controlled to 37 °C) for *in vitro* acoustic testing of the UCA. A single element 5 MHz transducer (Panametrics, Waltham, MA; 12.7 mm diameter, -6dB bandwidth of 91% and focal length of 50.8 mm) was focused through the acoustic window using an *x-y* positioning system (Edmund Scientific, Barrington, NJ) and a 5072 pulser-receiver (Panametrics) was used to generate acoustic pressures of 0.45 MPa peak negative pressure with a pulse repetition frequency of 100 Hz. This ultrasound signal is expected to be strong enough to detect microbubble response, but not expected to generate inertial cavitation. A magnetic stir bar constantly recirculated the bubbles through the focus of the transducer. Reflected signals from the UCA were detected by the same transducer and amplified 40 dB before being read by a digital oscilloscope (LeCroy 9350A, LeCroy Corporation, Chestnut Ridge, NY). Data acquisition and processing was done on a computer with LabView 7.1 (National Instruments, Austin, TX).

To determine the dose dependence of ultrasound signal enhancement, a cumulative dosage response curve was generated for UCA dosages of 0–1080 µl/l (0–7 E7 microbubbles/l) and ultrasound enhancement expressed in dB relative to the baseline (0 µl/l). To measure microbubble stability, 280 µl/l (1.8 E7 microbubbles/l) of UCA were added to the sample container and enhancement measured once per minute over 15 min. Ultrasound enhancement at each time point was then normalized to the initial signal enhancement. The ability to detect the UCA using a commercial ultrasound scanner was also investigated using a Logiq 9 ultrasound scanner with 9 L probe operating in nonlinear contrast imaging mode (GE Healthcare, Milwaukee, WI). A bolus injection of 1 ml/l of UCA was circulated through a flow phantom (model 524; ATS Laboratories, Bridgeport, CT) with a 6-mm diameter vessel embedded at a depth of 2 cm in urethane rubber using a roller pump set at 250ml/min. Ultrasound images were then obtained every minute for 15 min.

2.4. Determination of release kinetics after ultrasound triggering

Oxygen release kinetics were measured using an Oxy Lite 2000 with bare fiber pO₂ probe (Oxford Optronix, Oxford, United Kingdom). Ultrasound triggering was performed using a SonixRP scanner with a PA4–2 cardiac probe operating at 100% acoustic output

(approximately 3.6 MPa peak–peak pressure) at 4 MHz in power Doppler mode. Two milliliters of reconstituted agent was added to 100 ml of degassed saline. Samples were triggered with ultrasound over 20 min with readings obtained every 30 s. The experimental group consisted of 2 ml of O₂ filled SE61O₂ combined with ultrasound, while control groups consisted of 2 ml of O₂ filled SE61O₂ without ultrasound, 2 ml of nitrogen filled SE61 combined with ultrasound, and 2 ml of deionized water with ultrasound. All pO₂ values were normalized to baseline levels and expressed as change in mmHg.

2.5. In vivo proof of concept studies

In vivo proof of concept experiments were performed in two mice with breast tumor xenografts. All animal work was approved by Thomas Jefferson University's Institutional Animal Use and Care Committee. MDA-MB-231 human breast cancer cells were grown to confluency in a culture media of 94% RPMI culture media, 5% fetal bovine serum, and 1% penicillin/streptomycin (Sigma–Aldrich), before being injected into the mammary pad of two nude mice (Charles River Laboratories, Wilmington, MA; 10⁶ cells/injection). Tumors were then grown 2–3 weeks until reaching diameters of 4 and 7 mm. Mice were anesthetized by an isoflurane and 2–3% oxygen mixture through a nose cone for the duration of the experiments. For intravenous access, a 24 G angiocatheter was placed in a tail vein of each animal. Additionally, the bare fiber pO₂ probe was introduced into the tumor *via* a 21 G percutaneous catheter.

Contrast agent was reconstituted in sterile water for each animal immediately before use. Each animal received a 0.05 ml intravenous injection of SE61O₂ followed by 0.1 ml saline flush during flash-replenishment imaging at the fiber tip using a Vevo 2100 scanner (Visualsonics, Toronto, Canada) in nonlinear imaging mode at 18 MHz. This setting generates 5 s destructive pulses intermittent with approximately 12 s of nondestructive imaging to allow contrast reperfusion. Partial oxygen pressures were recorded every 5 s until levels returned to baseline. As controls, release profiles were compared to untriggered SE61O₂, and triggered SE61N₂ in both animals. Ultrasounds images and pO₂ levels were recorded and stored for later comparison between groups.

2.6. Statistical analysis

All experiments were repeated in triplicate from 3 independent samples and averaged. Statistical significance between groups was determined using a one-way ANOVA with a Bonferroni multiple comparison post test. All statistics were performed in GraphPad Prism (Version 5.0, GraphPad Software, San Diego, CA) with significance determined by $\alpha = 0.05$.

3. Results

The SE61O₂ microbubbles were successfully charged with O₂ and remained intact when reconstituted with DI water. Reconstituted particles demonstrated a spherical shape and smooth morphology on light microscopy as shown in Fig. 1a. These results demonstrate the presence of microbubbles after reconstitution. Analysis of the particle size distribution by dynamic light scattering (Fig. 1b) showed an average diameter of 3.1 ± 0.1 μ m with a

polydispersity index of 0.89 ± 0.18 indicating a broad size distribution. Particle counting by flow cytometry (example plot shown in Fig. 1c) showed the SE61O₂ microbubbles consisted of approximately $6.5 \pm 0.8 \times 10^7$ microbubbles/ml after suspension in 2 ml of saline.

Results of ultrasound enhancement and stability testing are shown in Fig. 2. *In vitro* enhancement at 5 MHz (Fig. 2 left) increased with SE61O₂ dose, with a peak enhancement of 16.9 ± 1.0 dB at a dose of 880 μ l/l (5.7 E7 microbubbles/l). As a control, freeze-dried SE61 was reconstituted with deionized water alone (no gas), to detect the presence of any remaining octafluoropropane. No detectable enhancement was observed (Fig. 2 left) with enhancement levels remaining at baseline levels (average enhancement 1.4 ± 0.9 dB, $p < 0.0001$ relative to SE61O₂). The SE61O₂ bubbles were insonated at low level ultrasound (0.45 MPa) to determine microbubble stability (Fig. 2 right). These microbubbles demonstrated good overall stability, with a half life over 15 min. Finally, 880 μ l/l of SE61O₂ microbubbles were imaged in a flow phantom using a commercial scanner (Fig. 3). The agent demonstrated substantial enhancement within the 6 mm vessel lumen at a depth of 2 cm after injection (bottom), relative to baseline (top) in both grayscale B-mode ultrasound (left split screen), and nonlinear contrast mode (right split screen, in gold).

Oxygen release experiments were performed to determine the ability of SE61O₂ to locally increase O₂ concentrations when triggered with ultrasound (Fig. 4). All groups showed a gradual rise in partial pressure of O₂ (pO₂) due to gas exchange between the degassed saline and atmospheric air (average of 40 mmHg over 20 min). However, pO₂ levels were found to be significantly elevated over a 20 min period when SE61O₂ bubbles were insonated relative to ultrasound alone, uninsonated SE61O₂, and insonated nitrogen filled SE61 ($p < 0.001$). This difference became apparent after 1 min of insonation (with differences of 12.0, 11.4, and 7.7 mmHg, respectively) and remained consistent throughout the full 20 min (23.0, 13.8, and 20.6 mmHg, respectively at 20 min). Untriggered SE61O₂ showed higher pO₂ levels at 20 min relative to triggered nitrogen filled SE61 (6.8 mmHg), although their release curves were not found to be significantly different ($p = 0.5$).

In vivo proof of concept studies demonstrated an ability to elevate hypoxic tumor oxygenation levels in both animals, albeit with a large degree of variability. Fig. 5 shows example ultrasound images and tumor oxygenation levels over time in both animals. Ultrasound images on the left show the triggering sequence of contrast arrival within the periphery of the tumor (top image, slight peripheral enhancement denoted with green arrows), the flash-destructive ultrasound pulse used to destroy the microbubbles (middle), followed by a slight decrease in peripheral enhancement immediately after microbubble destruction. The pO₂ probe introduced into the tumor through the catheter was observed in all images (white arrow).

Both animals showed an increase in pO₂ during triggering of SE61O₂, although measurements appeared to be highly dependent on probe position (Fig. 5 right). Ultrasound triggering in mouse 1 showed an increase of 27.4 mmHg, with elevated tumor oxygen levels lasting 1.7 min after injection before returning to baseline. SE61O₂ triggering in mouse 2 resulted in a 30.4 mmHg increase, with elevated tumor oxygen levels lasting over 4 min. Ultrasound-triggering of SE61N₂ resulted in no discernible increase in oxygen partial

pressure in either mouse. Untriggered SE61O₂ (*i.e.*, without ultrasound exposure) resulted in no increase in oxygen partial pressure in one animal, and a brief (20 s) 5.6 mmHg increase in the second animal.

4. Discussion

Results of this study demonstrate the feasibility of using SE61O₂ as an oxygen carrier for localized ultrasound-triggered gas delivery to hypoxic tissue. The osmolality and biocompatibility of the particle and all excipients should make the platform safe for clinical translation. The estimated osmolality of the samples are approximately 375 mOsm/kg (from the PBS and glucose prior to freeze drying). This estimate is well below the tolerance of peripheral venous endothelial cells in animal models (reported as 820 mOsm/kg for 8 h (Kuwahara et al., 1998). Additionally, toxicity studies in rats injected with a similar surfactant-shelled microbubble using Span 60 and Tween 80 without the glucose cryoprotectant have shown the microbubbles to be well tolerated (Forsberg et al., 2010), and replacement of Tween with water soluble vitamin E is expected to further improve this tolerability. While removal of Span would further improve tolerability, the stability of the bubble shell is dependent on the presence of at least one non-water soluble surfactant (results unpublished).

Light microscopy and particle sizing demonstrate the particles to be well formed, with diameters of approximately $3.1 \pm 0.1 \mu\text{m}$. These particles showed a relatively wide size distribution (a polydispersity index of 0.89), which could potentially compromise ultrasound sensitivity. Microfluidic and differential centrifugation approaches for creating monodisperse bubble populations have been described (Seo et al., 2010; Feshitan et al., 2009; Oeffinger et al., 2004) and may be useful to adopt in the future for improved gas payload and delivery efficiency. Cavalli et al. developed O₂ filled nanobubbles stabilized with either chitosan (Cavalli et al., 2009a) or dextran (Cavalli et al., 2009b), and showed that the addition of ultrasound (260 W at 45 kHz for chitosan bubbles or 2.4 MPa at 2.5 MHz for dextran bubbles) more than doubled the O₂ release profile relative to the uninsonated controls (Cavalli et al., 2009a,b). The group also demonstrated that these nanobubbles were capable of being internalized in cells, thereby providing intracellular gas delivery (Cavalli et al., 2009b). However, because the volume of O₂ incorporated within the core of the bubble is proportional to the cube of the radius, nanobubbles have limited capacity for *in vivo* drug payloads to solid tumors. Microbubbles can potentially improve gas payload, because even bubbles with diameters up to 8 μm can still traverse the capillary bed (Goldberg et al., 2001). Our group has previously demonstrated the ability to separate surfactant microbubbles based on size using centrifugation and floatation techniques (Wheatley et al., 2006). Similar size optimization and particle concentration techniques are planned in the future to maximize O₂ payloads.

The SE61O₂ microbubbles constituted with O₂ showed significant enhancement with a single element transducer ($16.9 \pm 1.0 \text{ dB}$) and with a commercial scanner (Fig. 3), indicating their responsiveness to ultrasound. Additionally, the particles demonstrated a half-life of over 15min in suspension (long enough for multiple passes through the circulatory system and potentially to the tumor vasculature). Borden and co-workers group first reported on

protein shelled (Swanson and Borden, 2010) and phospholipid shelled (Swanson et al., 2010b) O₂ microbubbles. These agents carried significant quantities of O₂ due to their larger diameters (3–10 μm) and an overall shelf life (up to 3 weeks), but immediately released their oxygen payload once injected in unsaturated solutions (Swanson et al., 2010b). Such diffusion limitations pose a major hurdle to microbubble-based delivery of O₂, as the bubble must remain stable enough to carry the gas to the desired target (roughly 10–30 s for an intravenous injection to reach a tumor site). Kwan et al. (2012) have since modified these phospholipid O₂ bubbles with the incorporation of a small (< 5%) amount of perfluorobutane. This small percentage of gas acts as a ‘trapped species’ and significantly improves the bubble stability due to Raoult’s law effects, in which the tendency of O₂ to diffuse out of the bubble is counterbalanced by the chemical potential tendency of oxygen to diffuse into the bubble to dilute the trapped species. These ultrasound-sensitive O₂ carriers display similar levels of stability to SE61O₂ while in solution. However, SE61O₂ does not appear to need incorporation of a trapped species. Freeze-dried SE61 that was not recharged with any gas (control in Fig. 2 left), showed no ultrasound enhancement, indicating a lack of gas within the vials when capped under vacuum. Thus, we have ruled out the presence of residual octafluoropropane (which shows considerable enhancement (Solis et al., 2010)) acting as a trapped species, and hypothesize that the surfactant-based shell provides a superior resistance to gas diffusion across the microbubble wall.

In vitro oxygen release experiments demonstrate the SE61O₂ bubble’s ability to locally increase oxygen concentration when triggered *via* ultrasound. While all control groups demonstrated a gradual rise in O₂ concentration over time, due to gas exchange between the degassed saline and atmospheric air, O₂ filled bubbles triggered with ultrasound showed significantly greater increases compared to all controls (13.8 mmHg more than untriggered bubbles and 20.6 mmHg more than ultrasound triggered nitrogen- filled bubbles). Additionally the lack of statistically significant change in O₂ concentrations between the untriggered SE61O₂ bubble group and ultrasound groups with and without SE61 nitrogen bubbles demonstrates that relatively little O₂ is released from the particles in the absence of ultrasound triggering. Unfortunately, release experiments must be performed outside of the storage vial to allow ultrasound triggering and insertion of the O₂ probe. Future experiments using a nitrogen blanket to prevent atmospheric gas exchange may be useful in providing a direct quantification of O₂ release from the microbubbles.

Preliminary *in vivo* results demonstrate the ability of the platform to temporarily elevate hypoxic tumor oxygenation levels. This is the first published report on the use of ultrasound-sensitive microbubbles to locally elevate tumor oxygenation levels *in vivo*. While imaging was not optimized for destruction of SE61 and tumor oxygenation appeared to be very dependent on pO₂ probe positioning, triggering of SE61O₂ showed an increase in tumor oxygenation levels in both cases. Mass transport modeling is needed in the future to better understand this variability and predict diffusion of the O₂ into the tissue. However, results of ultrasound triggered oxygen filled SE61O₂ relative to the controls in this pilot study are encouraging and warrant future *in vivo* radiation sensitization studies.

Several potential *in vivo* applications for oxygen delivery using the described platform exist. This platform is primarily envisioned as a method for overcoming tumor hypoxia-associated

radiotherapy immediately prior to therapy. The relationship between pO_2 and radiosensitivity has been well studied *in vitro* (Rockwell et al., 2009). While cells at a pO_2 of <2 mmHg are almost completely radiation-insensitive, cell sensitivity reaches its asymptotic peak at approximately 30mmHg (Rockwell et al., 2009). Thus, while the total volume of oxygen payload may be small within the microbubble, it has been shown that a relatively small increase in oxygen partial pressure can result in significant sensitization. Other applications such as wound or ulcer healing may also benefit from the proposed platform, although the relatively low total gas payload may limit its usefulness in applications where large or continuous long term pO_2 elevations are required for efficacy.

5. Conclusions

Oxygen-filled SE61's size, concentration, morphology, stability, ultrasound enhancement, and ability to elevate oxygen concentrations in solution have been characterized *in vitro*. Preliminary *in vivo* results demonstrate the particle to be a potentially effective platform for noninvasive delivery of O_2 to solid tumors. Future efforts will examine the ability of the particle to effectively elevate intra-tumoral O_2 levels in order to overcome hypoxia-associated radiotherapy resistance *in vivo*.

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Abbreviations:

O_2	oxygen
pO_2	partial pressure of oxygen
SE61 ₀₂	experimental oxygen microbubble consisting of a Span60 and vitamin E shell
UCA	ultrasound contrast agents

References

- Basude R, Duckworth JW, Wheatley MA, 2000 Influence of environmental conditions on a new surfactant-based contrast agent: ST68. *Ultrasound Med. Biol.* 26, 621–628. [PubMed: 10856625]
- Basude R, Wheatley MA, 2001 Generation of ultraharmonics in surfactant based ultrasound contrast agents: use and advantages. *Ultrasonics* 39, 437–444. [PubMed: 11775659]
- Bekeredjian R, Katus HA, Kuecherer HF, 2006 Therapeutic use of ultrasound targeted microbubble destruction: a review of non-cardiac applications. *Ultraschall Med.* 27, 134–140. [PubMed: 16612722]
- Brown JM, Wilson WR, 2004 Exploiting tumor hypoxia in cancer treatment. *Nat. Rev. Cancer* 4, 437–44. [PubMed: 15170446]
- Brown JM, 2007 Tumor hypoxia in cancer therapy. *Methods Enzymol.* 435, 297–321. [PubMed: 17998060]
- Cavalieri F, Finelli I, Tortora M, et al., 2008 Polymer microbubbles as diagnostic and therapeutic gas delivery device. *Chem. Mater.* 20, 3254–3258.

- Cavalli R, Bisazza A, Rolfo A, et al., 2009 Ultrasound-mediated oxygen delivery from chitosan nanobubbles. *Int. J. Pharm.* 378, 215–217. [PubMed: 19501639]
- Cavalli R, Bisazza A, Giustetto P, et al., 2009 Preparation and characterization of dextran nanobubbles for oxygen delivery. *Int. J. Pharm.* 382, 160–165. [PubMed: 19716870]
- Davidson BP, Lindner JR, 2012 Future applications of contrast echocardiography. *Heart* 98, 246–253. [PubMed: 22199019]
- Eisenbrey JR, Huang P, Hsu J, Wheatley MA, 2009 Ultrasound triggered cell death in vitro with doxorubicin loaded poly lactic acid contrast agents. *Ultrasonics* 49, 628–633. [PubMed: 19394992]
- Eisenbrey JR, Forsberg F, 2010 Contrast-enhanced ultrasound for molecular imaging of angiogenesis. *Eur. J. Nucl. Med. Mol. Imaging* 37, S138–S146. [PubMed: 20461376]
- Eisenbrey JR, Mualem-Burstein O, Kambhampati R, et al., 2010 Development and optimization of a doxorubicin loaded poly(lactic acid) contrast agent for ultrasound triggered drug delivery. *J. Control Release* 143, 38–44. [PubMed: 20060024]
- Escoffre JM, Piron J, Novell A, Bouakaz A, 2011 Doxorubicin delivery into tumor cells with ultrasound and microbubbles. *Mol. Pharm.* 8, 799–806. [PubMed: 21495672]
- Ferrara K, Pollard R, Borden M, 2007 Ultrasound microbubble contrast agents: fundamentals and application to gene and drug delivery. *Annu. Rev. Biomed. Eng.* 9, 415–44. [PubMed: 17651012]
- Feshitan JA, Chen CC, Kwan JJ, Borden MA, 2009 Microbubble size isolation by differential centrifugation. *J. Colloid Interface Sci.* 329, 316–324. [PubMed: 18950786]
- Forsberg F, Wu Y, Makin IR, et al., 1997 Quantitative acoustic characterization of a new surfactant-based ultrasound contrast agent. *Ultrasound Med. Biol.* 23, 1201–1208. [PubMed: 9372569]
- Forsberg F, Basude R, Liu JB, et al., 1999 Effect of filling gases on the backscatter from contrast microbubbles: theory and in vivo measurements. *Ultrasound Med. Biol.* 25, 1203–1211. [PubMed: 10576263]
- Forsberg F, Liu JB, Patel M, et al., 2010 Preclinical acute toxicology study of surfactant-stabilized ultrasound contrast agents in adult rats. *Int. J. Toxicol.* 29, 32–39. [PubMed: 20008819]
- Goldberg BB, Raichlen JS, Forsberg F, 2001 *Ultrasound Contrast Agents: Basic Principles and Clinical Applications*, 2nd ed Martin Dunitz Ltd., London.
- Hodgkiss RJ, Roberts IJ, Watts ME, Woodcock M, 1987 Rapid-mixing studies of radiosensitivity with thiol-depleted mammalian cells. *Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med.* 52, 735–744.
- Klegerman ME, Wassler M, Huang SL, et al., 2010 Liposomal modular complexes for simultaneous targeted delivery of bioactive gases and therapeutics. *J. Control Release* 142, 326–331. [PubMed: 19903503]
- Kwan JJ, Kaya M, Borden MA, Dayton PA, 2012 Theranostic oxygen delivery using ultrasound and microbubbles. *Theranostics* 2, 1174–1184. [PubMed: 23382774]
- Kuwahara T, Asanami S, Kubo S, 1998 Experimental infusion phlebitis: tolerance osmolarity of peripheral venous endothelial cells. *Nutrition* 14, 496–50. [PubMed: 9646289]
- Miller AP, Nanda NC, 2004 Contrast echocardiography: new agents. *Ultrasound Med. Biol.* 30, 425–434. [PubMed: 15121243]
- Oeffinger BE, Forsberg F, Merton DA, et al., 2004 Design and acoustic characterization of a nano-scale ultrasound contrast agent. *J. Ultrasound Med.* 23, S77.
- Polizzotti BD, Thomson LM, O’Connell DW, et al., 2014 Optimization and characterization of stable lipid-based, oxygen filled microbubbles by mixture design. *J. Biomed. Mater. Res. B Appl. Biomater.* 102, 1148–1156. [PubMed: 24425069]
- Postema M, Bouakaz A, ten Cate FJ., et al., 2006 Nitric oxide delivery by ultrasonic cracking: some limitations. *Ultrasonics* 44, e109–e113. [PubMed: 16889810]
- Richard DE, Berra E, Pouyssegur J, 1999 Angiogenesis: how a tumor adapts to hypoxia. *Biochem. Biophys. Res. Commun.* 266, 718–722. [PubMed: 10603309]
- Rockwell S, Dobrucki IT, Kim EY, et al., 2009 Hypoxia and radiation therapy: past history, ongoing research, and future promise. *Curr. Col. Med.* 9, 442–458.

- Seo M, Gorelikov I, Williams R, Matsuura N, 2010 Microfluidic assembly of monodisperse, nanoparticle-incorporated perfluorocarbon microbubbles for medical imaging and therapy. *Langmuir* 26, 13855–13860. [PubMed: 20666507]
- Solis C, Forsberg F, Wheatley MA, 2010 Preserving enhancement in freeze-dried contrast agent ST68: examination of excipients. *Int. J. Pharm.* 396, 30–38. [PubMed: 20540998]
- Swanson EJ, Borden MA, 2010 Injectable oxygen delivery based on protein-shelled microbubbles. *Nano Life* 1, 215–218.
- Swanson EJ, Mohan V, Kheir J, Borden MA, 2010 Phospholipid-stabilized microbubble foam for injectable oxygen delivery. *Langmuir* 26, 15726–15729. [PubMed: 20873807]
- Tong J, Ding J, Shen X, et al., 2013 Mesenchymal stem cell transplantation enhancement in myocardial infarction rat model under ultrasound combined with nitric oxide microbubbles. *PLoS One* 8, e80186. [PubMed: 24244646]
- Wang Y, Li X, Zhou Y, et al., 2010 Preparation of nanobubbles for ultrasound imaging and intracellular drug delivery. *Int. J. Pharm* 384, 148–153. [PubMed: 19781609]
- Wheatley MA, Singhal S, 1995 Structural studies on stabilized microbubbles: development of a novel contrast agent for diagnostic ultrasound. *React. Polym.* 25, 157–166.
- Wheatley MA, Forsberg F, Dube N, et al., 2006 Surfactant-stabilized contrast agent on the nanoscale for diagnostic ultrasound imaging. *Ultrasound Med. Biol.* 32, 83–93. [PubMed: 16364800]

Fig. 1.

A: light microscopy image of SE61O₂ at 40 × magnification (size bar = 20 μm). B: example size distribution measurement of SE61O₂ by dynamic light scattering, showing a mean particle diameter of approximately 3 μm. C: example flow cytometry result showing forward scattered light (FSC-A) on the x-axis and particle counts on the y-axis. Distinct, separate bubble (black; left plot) and counting bead (red; right plot) populations were observed with a total bubble population of approximately 6.5×10^7 microbubbles/ml. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.
Left: *in vitro* ultrasound enhancement of SE61O₂ and the water-filled control microbubble.
Right: oxygen-filled microbubbles also showed good overall stability with a half-life of over 15 min (half-life threshold displayed by dashed line).

Fig. 3.

In vitro ultrasound enhancement of SE61O₂ in a flow phantom using a commercial ultrasound scanner at baseline (top) and 30 s post injection (bottom).

Fig. 4. Relative change in pO₂ levels in 100 ml of degassed saline over 20 min with the addition of SE61O₂ and ultrasound, ultrasound alone, SE61O₂ alone, and nitrogen filled SE61 with ultrasound (* $p < 0.0001$).

Fig. 5.

Results from *in vivo* proof of concept delivery experiments. Left: ultrasound images of the tumor and pO₂ probe (white arrow) showing contrast arrival within the periphery of the tumor (top image, slight peripheral enhancement denoted with green arrows), the flash-destructive ultrasound pulse used to destroy the microbubbles (middle), followed by a slight decrease in peripheral enhancement immediately after microbubble destruction (bottom). Right: intra-tumoral pO₂ levels over time for both mice after injection of SE61O₂ with ultrasound, SE61O₂ alone, and SE61N₂ with ultrasound.