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Designing Oxygen Microbubbles for Treating Tumor Hypoxia

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Here we report on an oxygen microbubble that has increased circulation and contrast persistence to treat tumor hypoxia during X-ray therapy. In recent work, oxygen microbubbles injected directly into tumors were shown to oxygenate hypoxic tumors and improve radiation therapy [1]. Here, we investigate oxygen microbubbles designed for intravenous, rather than intra-tumoral, injection. Oxygen microbubbles were designed with two phospholipid shell compositions, DSPC (C18:0) and DBPC (C22:0). DBPC microbubbles showed a significant increase in ultrasound contrast persistence *in vivo* in the mouse kidney, and this composition may be ideal for an intravascular microbubble injection to treat tumor hypoxia in radiation oncology.

Keywords—oxygen microbubble, DBPC, tumor hypoxia

I. Introduction

In recent work, oxygen microbubbles (OMB) have been used for oxygenation of hypoxic tumors for radiotherapy treatment [1], [2]. Relieving hypoxia in the tumor with ultrasound-guided OMB destruction during radiotherapy could significantly improve the treatment outcome. Additionally, ultrasound can be used for real-time visualization of the presence of OMB at the tumor location. Ultrasound-induced destruction of OMB has been shown to increase dissolved oxygen content *in vitro* [3] and increase *in vivo* oxygen levels and tumor control in a fibrosarcoma rodent model when injected directly into the tumor [1]. However, an intravenous injection, rather than intra-tumoral injection, of OMB would be highly advantageous for clinical translation. Unfortunately, conventional OMB have short circulation lifetimes and may be ineffective. The goal of this study was to redesign the OMB to improve circulation lifetime.

Oxygen microbubbles made in our lab comprise a phospholipid shell with a polyethylene glycol (PEG) brush that encapsulates a gas core of pure oxygen as shown in Fig. 1. Longer acyl-chain phospholipids have been shown to stiffen the shell [4], [5] and increase circulation persistence of perfluorocarbon gas microbubbles [6]. We therefore designed our OMB with longer acyl-chain phospholipid and tested the *in vivo* stability. We focused on two acyl-chain phospholipids: shorter chain (C18:0) DSPC and longer chain (C22:0) DBPC. Microbubble volume dose (MVD) was kept constant at 300 ± 10.8 µL/kg throughout the experiments [7]. The OMB was tested for *in vivo* stability by imaging the mouse kidney.

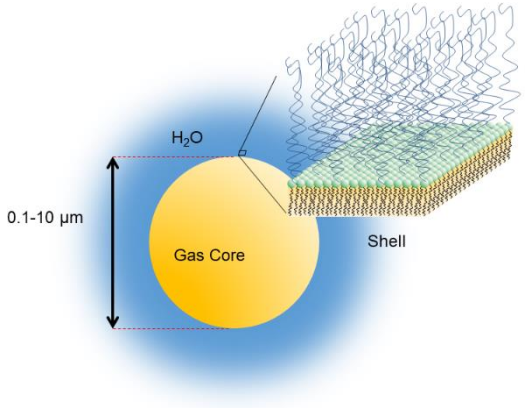

Fig. 1. Cartoon illustration of oxygen microbubble design for *in vivo* studies.

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II. Materials And Methods

A. Materials

Phosphate buffered saline (PBS) solution was prepared by diluting 10x stock solution from Fisher Scientific International, Inc. (Hampton, NH, USA) 9:1 with deionized water (Direct-Q, Millapore, Billerica, MA, USA) and filtered through 0.2 μm diameter nylon filter attached to a vacuum. High purity oxygen was obtained from Airgas (Radnor, PA, USA). The two phospholipids, 1,2-distearoyl-sn-gylcero-3-phosphocholine (DSPC) and 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC) and the emulsifier 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol 2000) (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

B. Microbubble Preparation

Individual lipid solutions were prepared by combining the different acyl-chain phospholipids with DSPE-PEG2000 in a 9:1 molar ratio, respectively, to a final lipid concentration of 12 mg/mL. Lipids were added to PBS and gently stirred using a magnetic stir bar and heated to reach their main phase transition temperature and make a homogenous suspension. The lipid suspension was then sonicated using Branson Digital Sonifier SFX 550 (Danbury, CT, USA) for 10 min at 30% power to disperse lipids into small unilamellar liposomes [8]. The suspension was then cooled to 4°C before OMB generation.

Oxygen microbubbles were synthesized using an ultrasonic horn reactor enclosed in a water-cooled, continuous-flow chamber (Branson, Danbury, CT, USA) [9]. Lipid solutions were flowed through the chamber with room temperature oxygen gas at full sonication power and then collected into a glass collection column with oxygen gas headspace. The final OMB microfoam was collected into 30-mL syringes (BD, Franklin Lakes, NJ, USA) to be further processed.

The collected OMB microfoam was further processed by centrifugation using a bucket-rotor Centrifuge 5804 (Eppendorf, Hauppauge, NY, USA). Microbubble cake was collected by centrifuging the initial MB suspension at 130 relative centrifugal force (RCF) for 1 min. Excess lipid solution from the infranatant was collected and reused to generate more OMB. The final cake was collected into a 30-mL syringe to be size-selected. Differential centrifugation was performed to size-select OMB [8]. The cake was diluted to 30 mL with oxygen saturated PBS and centrifuged at 130 RCF for 1 min to wash out OMB that were smaller than 2-μm diameter. The remaining OMB was transferred to a 12-mL syringe (Covidien Monoject, Mansfield, MA, USA) and washed with PBS once more, with the final product being concentrated OMB.

C. Characterization of Oxygen Microbubbles

Oxygen microbubbles were characterized by particle size, concentration and gas content. Microbubble size and concentration was measured using an electrozone sensing method (Coulter Multisizer III, Beckman Coulter, Opa Locka, Fl). Size and concentration were measured in triplicate. Gas content was measured using an oxygen headspace sensor (MO-200 Oxygen Sensor, Apogee, Logan, UT). Gas volume fraction was measured by weight and volume of the OMB sample [10], [11]. Microbubble volume dose, i.e., oxygen gas volume injected per weight of animal, was calculated by using OMB concentration, size and total injection volume [7].

D. Animal Preparation and In Vivo Injections

All animal experiments were approved by the University of Colorado Denver Institutional Animal Care and Use Committee. Contrast persistence studies were performed in male and female C57Bl/6 mice at 6 weeks of age (The Jackson Laboratory). Mice were anesthetized using 2% isoflurane with oxygen carrier gas and placed supine on a heated platform. Heart rate, respiratory rate and temperature were monitored using Vevo 2100 Physiological Monitoring Unit. Mice were kept under anesthesia via nose cone for the duration of the experiment. Hair was removed from the lower left kidney region using Nair Hair Removal Lotion. A modified 27-gauge, one half-inch winged infusion catheter (Terumo, Tokyo, Japan) with tubing removed and replaced with polyethylene tubing (Warner Instruments, Hamden, CT, USA), was placed in the mouse tail vein.

A VisualSonics Vevo 2100 small-animal ultrasound imaging scanner (Toronto, ON, Canada) with an MS250, 18-MHz transducer at 10% power was placed on the shaved kidney region using Medline Aquasonic acoustic coupling gel. Mice were injected with OMB, MVD = 300 μL/kg, while continuously imaging (n=6-8 per group). Each mouse was imaged once per imaging session and was then removed from anesthesia and placed in a recovery cage on top of a heating pad. Once regaining consciousness, the mouse was returned to its cage.

E. Data Analysis

The Vevo 2100 small-animal ultrasound provided Digital Imaging and Communications in Medicine (DICOM) files that were used to extract individual video frames and measurements. These files were post-processed using MatLab R2018b (MathWorks, Inc., Natick, MA, USA) DICOM reader and were analyzed for gray scale intensity versus time. For *in vivo* mouse studies, the kidney was located using B-Mode, and Contrast Mode was used to measure the change in contrast intensity over time. The region of interest (ROI) was chosen as the entire kidney rather than just a portion because signal attenuation and shadowing showed minimal effect. The average video intensity was determined by averaging the intensities over the entire ROI for each frame. The data was baseline adjusted and plotted as mean video intensity versus time. Lower envelope detection in MatLab was used to adjust for respiratory motion of the mouse and produce a smoothed time intensity curve (TIC). Data was fit to an exponential decay model using OriginPro 2019 (OriginLab Corp., Northampton, MA) software. The decay rate, was used to determine the half-life of the microbubble. Total integrated signal enhancement (area under the curve, AUC) was measured using the TIC data.

III. Results and Discussion

A. Oxygen Microbubbles

Size-selection was conducted to obtain OMB between 2-10 μm in diameter (Fig. 2). The size distribution was bimodal for both lipid acyl-chain microbubble shell compositions: DSPC (C18:0) and DBPC (C22:0). The first peak had a size ranging

approximately from 0.5-2 µm in diameter and the second peak ranged from 2-10 µm in diameter.

The initial OMB concentration was measured to be 6.0×10^9 MB/mL for DSPC and 8.5×10^9 MB/mL for DBPC. After concentration by centrifugation, the volume fraction was 71% for DSPC and 79% for DBPC. The OMB were diluted using oxygen-saturated PBS to a volume fraction of 50% for *in vivo* animal injections.

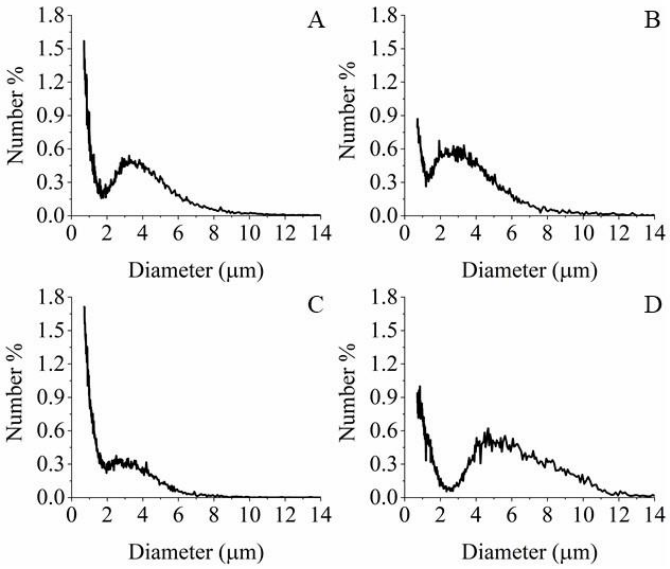

Fig. 2. Multisizer III number weighted size distributions for size-selected: A and C. DSPC (C18:0) and B and D. DBPC (C22:0) oxygen microbubbles.

B. In Vivo Ultrasound Contrast Persistence

In vivo contrast persistence was measured using Vevo 2100 small-animal ultrasound with an 18-MHz transducer placed on the left mouse kidney. All mice were given bolus OMB injections. The MVD was kept constant at 300 µL/kg. OMB have a rapid elimination *in vivo*, so the bolus injection was not followed with a saline flush. However, the catheter tubing length was taken into account and an additional volume of OMB was injected that filled the catheter tubing length, so the mouse received the OMB full dose.

Figure 3 shows a typical grayscale image of a mouse kidney before and after injection of OMB comprised of DBPC OMB with the same volume fraction. The post image was taken at peak amplitude, a few seconds after injection. Mean video contrast enhancement and persistence in the blood stream was analyzed from the TICs for each OMB (Fig. 4). Injection of DBPC OMB showed a significant increase in contrast and persistence *in vivo*. OMB comprised of DSPC did not show a measurable contrast increase above noise. Some sparse contrast was observed, but it could not be accurately quantified due to the low signal-to-noise ratio. However, the minimal signal enhancement over baseline shows that OMB were present.

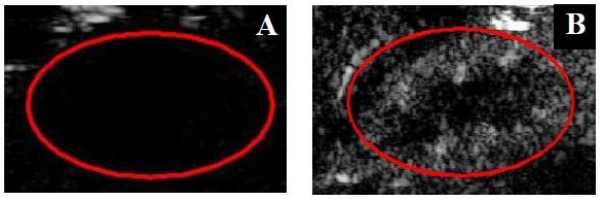

Fig. 3. Contrast enhancement in the kidney: A. before and B. after OMB administration.

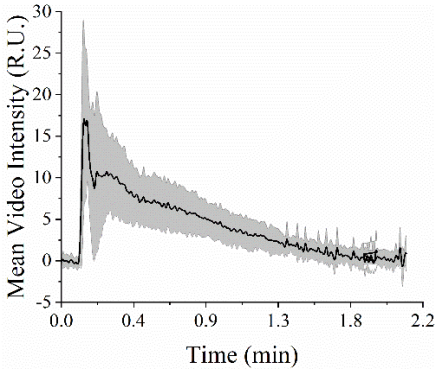

Fig. 4. Time intensity curve for DBPC OMB volume fraction study.

C. Conclusions

In this study, we demonstrate that OMB comprising the long acyl-chain phospholipid, DBPC (C22:0), show an increase contrast enhancement and persistence *in vivo*. The shorter acyl-chain phospholipid, DSPC (C18:0), showed minimal contrast enhancement and short persistence *in vivo*. This result is consistent with contrast agent studies that compared differing acyl-chain phospholipids for increase contrast persistence and circulation *in vitro* and *in vivo* [1], [6], [12]. Future work will examine the capability of this long-circulating OMB for improving radiation therapy of hypoxic tumors.

IV. Conclusion

In this study, we used novel oxygen microbubbles to show an increase in contrast persistence and circulation *in vivo*. Oxygen microbubbles were designed with a long acyl-chain phospholipid to improve microbubble stability for oxygen delivery to hypoxic tumors. We showed that, when compared to shorter acyl-chain phospholipid shells, OMB produced with DBPC (C22:0) shells were visible by ultrasound and had a measurable increase in contrast intensity. Future work will examine the capability of these OMB for radiotherapy treatment of hypoxic tumors.

Acknowledgment

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