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The Effect of Freeze/Thawing on the Physical Properties and Acoustic Performance of Perfluoropropane Nanobubble Suspensions

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***Abstract*— Nanobubble (NBs) contrast agents have the potential to augment the molecular imaging capabilities of ultrasound (US), especially in cancer imaging. However, as with most bubble-based formulations, these shell-stabilized perfluoropropane (C_3F_8) bubbles are fragile, last only several hours after self-assembly, and size isolation. Longer storage of the NB preparation leads to bubble dissolution. The short shelf-life after activation has limited broader bubble adoption and can hamper the diagnostic and therapeutic potential. Distribution of the NBs is also limited by the access to the amalgamator and centrifuge used for NB activation and isolation. In this work, we tested two simple freeze/thaw strategies for long term NB storage and examined their effect on NB size and concentration, initial acoustic activity, and US signal decay. Aliquoted NBs were either stored at -80°C for gradual cooling (GC-80), or flash frozen by submerging in liquid nitrogen prior storing at -80°C (FF-80). Our results suggest that NB suspension could be stored at -80°C for at least 28 days with only minor change in physical properties and acoustic performance. FF-80 NBs exhibited preserved NB size distribution and acoustic activity for over 28 days. GC-80 NBs showed a narrower size distribution but decrease in NB concentration at day 28. These differences are likely a result of shell composition changes induced by the freezing process, leading to less stable bubbles. The specific mechanism for the observed changes will be evaluated in future studies.**

Keywords—nanobubble, ultrasound, contrast agent, freezing, freeze-thaw, storage, perfluoropropane

I. Introduction

Shell-stabilized perfluorocarbon microbubbles (MBs) have been widely used as an ultrasound contrast agent (UCA) for molecular imaging and biomedical applications. However, their clinical application is limited by their large size (1-8 μm) which restricts them to the vasculature [1, 2]. Alternatively, submicron echogenic bubbles, or nanobubbles (NBs), have the potential to augment the molecular imaging capabilities of UCAs. This is particularly true in cancer imaging, where NB with size of 100-500 nm, can promote extravasation by taking an advantage of enhanced permeability and retention (EPR) effect, resulting targeting of biomarkers outside of the vasculature[3-5]. Recently, we reported a new generation of NBs which the shell structure was engineered to present improved stability under prolonged insonation [6, 7]. This formulation is composed of a perfluoropropane (C_3F_8) gas core surrounded by a phospholipid layer modified with propylene glycol and glycerol. In order to be clinically viable and augment the theranostics and drug delivery efficiency, the NBs must be stable when stored for an extended period of time, and must maintain consistent physical and acoustic properties after activation. However, the major limitation of most bubble-based formulations is their short shelf life; bubbles last only several hours after self-assembly and size isolation. This is because the acoustic performance of any bubbles, including NBs, is directly related to volume of the entrapped gas. The rate of gas dissolution from the bubble core is relatively rapid once the bubbles are formed and removed from the pressurized environment. This greatly limits the distribution of NBs to different location. The distribution of NBs is also limited by the access to the amalgamator and centrifuge used for NB activation and isolation. To overcome these limitations, in this study, we developed a protocol to preserve NBs longer by freezing in a C_3F_8-saturated environment. The frozen NBs can be stored or shipped, and thawed before use. We tested two simple freeze/thaw strategies for long-term NB storage. Specifically, we examined the effect of gradual cooling or flash frozen by submerging in liquid nitrogen prior storing at -80°C on

physical and acoustic properties of NBs. Size and concentration of thawed NBs, and their acoustic performance were then compared to freshly prepared NBs.

II. Methods

A. *Materials*

Lipids including DBPC (1,2-dibehenoyl-sn-glycero-3-phosphocholine), DPPA (1,2 dipalmitoyl-sn-Glycero-3-phosphate), and DPPE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine) were obtained from Avanti Polar Lipids (Pelham, AL), and mPEG-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)) was obtained from Laysan Lipids (Arab, AL). Propylene glycol (PG) and glycerol were purchased from Sigma Aldrich (Milwaukee, WI) and Acros Organics (Morris, NJ). Phosphate buffered saline solution (PBS, Gibco, Life Technologies) was purchased from Fisher Scientific 1(Pittsburgh, PA). C_3F_8 gas was from AirGas (Cleveland, OH).

B. *NB formulation*

NBs were formulated as previously reported [7, 8] by dissolving lipids including DBPC, DPPA, DPPE, DSPE-mPEG in propylene glycol, and hydrated with glycerol and PBS. Following air exchange with C_3F_8, NBs were activated by mechanical agitation using a ViialMix dental amalgamator for 45 sec. NBs were then isolated from larger bubbles by centrifugation at 50 *g* for 5 min.

C. *NB freezing and thawing*

After isolation, 200 µL of NB suspension was gently drawn from the vial using 1mL-syringe with 21G needle and aliquoted into vials with C_3F_8 in the headspace. The aliquoted NBs were stored at either 1) -80°C for gradual cooling (GC-80), or 2) flash frozen by submerging in liquid nitrogen for 1 min prior storing at -80°C (FF-80) for over 28 days. After 2, 14, and 28 days, NBs (n = 3 for each condition) were thawed at room temperature by placing the vial up-side-down and collecting 150 microliters of the milky solution before characterization. The remainder of the thawed solution, including any residual remaining on the vial walls, was discarded.

D. *Size and concentration measurement*

NB size distribution and concentration were measured using resonant mass measurement (RMM) (Archimedes, Malvern Pananalytical Inc., Westborough, MA, USA) using a nanosensor (capable of measuring particles between 100 nm and 2 µm), pre-calibrated using NIST traceable 565 nm polystyrene bead standards (ThermoFisher 4010S, Waltham MA, USA). NBs were diluted at 1:1000 with phosphate buffered saline (PBS, pH 7.4). A total of 1000 particles were measured for each trial (n = 3).

E. *Acoustic characterization*

NBs were diluted in PBS at 1:100 and poured into a tissue mimicking agarose phantom [7] placed directly over an US transducer (PLT-1204BT). Nonlinear contrast images were continuously acquired using a clinical US scanner (AplioXG SSA-790A, Toshiba Medical Imaging Systems, Otawara-Shi, Japan) via contrast harmonic imaging (CHI, 12 MHz, mechanical index 0.1, focus depth of 1.5 cm, 2D gain of 70 dB, dynamic range of 65 dB) at 1 frame per second for 8 min. Raw echo power data was recorded and analyzed using built-in software. Initial signal enhancement, signal decay over time, and percent remaining signal at 8 min were determined.

III. Results and Discussion

Typically, submicron sized colloidal particles, and especially bubble-based formulations, show poor long-term stability due to their inherent instability, physical/chemical instability such as aggregation, dissolution, etc. that can destabilize the system [9]. This is one obstacle that can limit the clinical translation of NBs. To overcome this challenge , in this study, simple freeze/thaw strategies for long term NB storage were investigated. Specifically, we examined the effect of slow cooling at -80°C versus rapid cooling using liquid nitrogen prior storing at -80°C on the properties of NBs over a period of time.

A. *Size and concentration of NBs*

The size of NBs resulting from different storage condition was characterized using RMM, which works by measuring the buoyant mass of particles. The results show consistent size of buoyant and non-buoyant particles in the range of 300-400 nm (Fig 1.c-d) with no particle larger than 1 µm is observed for both GC-80 and FF-80. This is similar to typical size range of NBs (100-600 nm) reported previously [6, 7]. The concentration of freezing NBs measured was typically on the order of 10^{11} particles/mL.

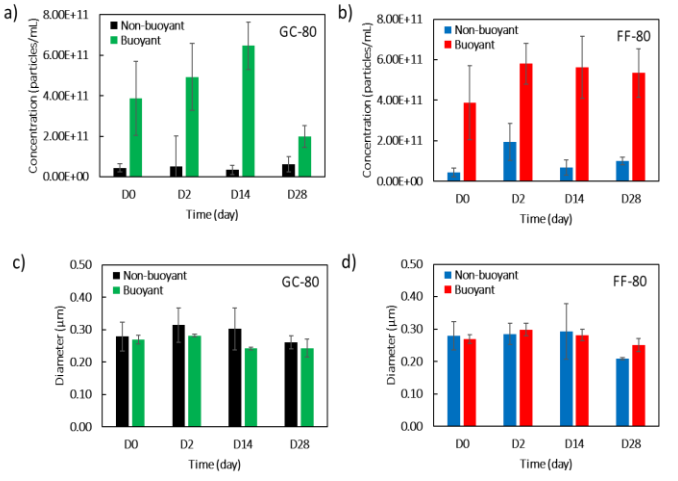

Fig.1 Average size and concentration of buoyant and non-buoyant particles for different storage conditions.

As a result of freezing and thawing process, both GC-80 and FF-80 methods showed a higher NB concentration, with consistency in size, compared to freshly prepared NB at Day 0. Inverting of the NBs in the vial on thawing appeared to concentrate the NBs. In contrast, as previously reported, the concentration of freeze-dried perfluorocarbon microbubbles decreases and their size increases, because bubbles are likely lost and diluted during drying reconstituting and process[10]. Various stresses can be generated during freezing process and often result in significant increase in particle size due to coalescence[11]. With our technique, a higher amount of smaller bubbles with fewer non-buoyant particles was found in GC-80 as shown in the representative size distribution (Fig.3). However, the concentration for GC-80 NBs at day 28 decreased by 48.8% but increased by 37.8% for FF-80 NBs compared to NBs at Day 0 (Fig 1.a-b).

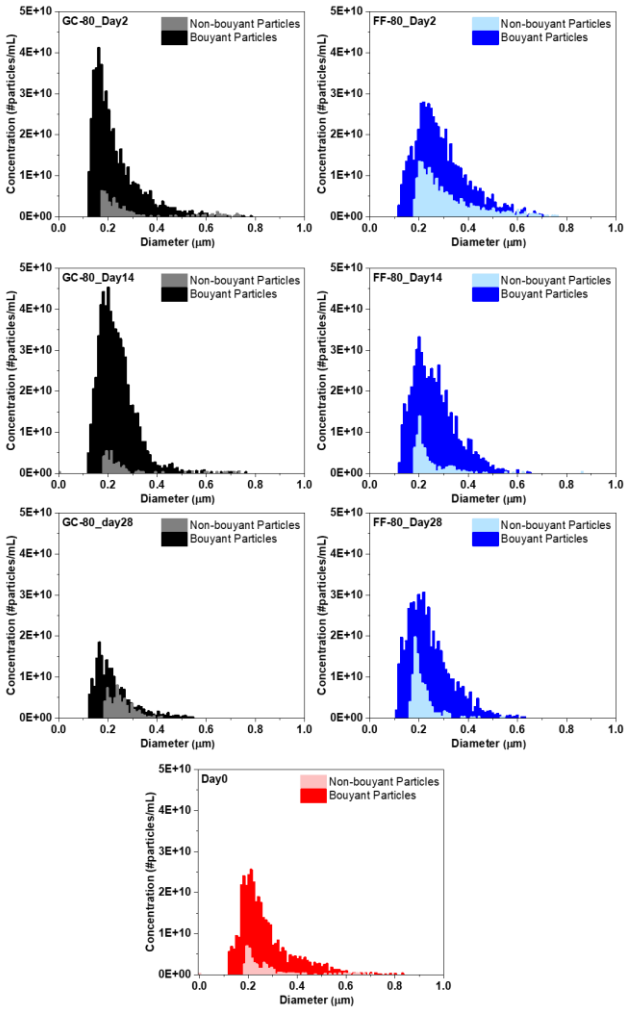

Fig.2 Size distribution of buoyant and non-buoyant particles of NB suspension stored at GC-80 (black and gray) and FF-80 (blue) at different time points as measured by RMM.

It was found that particles larger than 600 nm could be eliminated through the flash freezing process (FF-80) with consistent bubble to solid particle ratios maintained over time. The reason for this is likely to be the effect of faster freezing rate that cause more aggregation of larger particles[12] at the bottom of the vial and could be eliminated by our thawing procedure.

B. *Acoustic activity and stability of NB under ultrasound*

NBs are currently under investigation as an UCAs for molecular imaging applications. The effect of freeze/thaw process on the NB acoustic performance was thus studied by determining their initial signal enhancement and in vitro-stability. Representative US contrast images of GC-80 and FF-80 NBs stored for different times after initiation of US exposure are shown in Fig. 3.

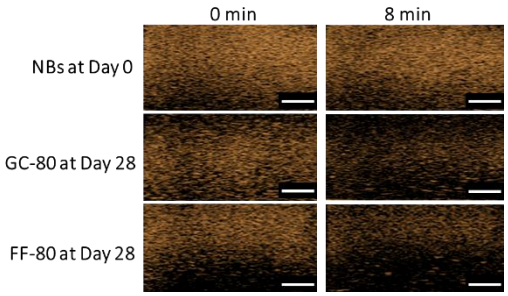

Fig.3 Representative ultrasound images. The scale bar is 0.5 cm.

The corresponding signal enhancement over time is shown in Fig. 4a and 5b. The compressibility of gas core results in bubble signal under ultrasound, thus the effect of freeze/thawing on the integrity of NBs can be evaluated by testing its in vitro acoustic behavior. Here, we examined the initial signal enhancement and signal decay overtime. Both GC-80 and FF-80 NBs show comparable initial signal intensity (IS) and signal decay pattern to freshly prepared NB at Day 0 (Fig.4a, b). After day 28, IS of GC-80 NB and FF-80 NB decreased by 29.4% and 27.7% (Fig.4c) and signal decay is faster as indicated by the lower remaining signal at 8 min (Fig.4d). Although stability is reported to depend on the size and concentration of the particles[13], freezing the NB suspensions at -80°C does not appear to affect the in vitro acoustic behavior of the NBs. Overall, this finding suggests that while gentle cooling results in higher amount of smaller bubble population flash freezing more effectively preserves NBs without significant lost in acoustic performance, which may result from some changes to the bubble morphology. More specifically, these are likely a result of shell composition changes induced by ice crystallization during the freezing process[14].

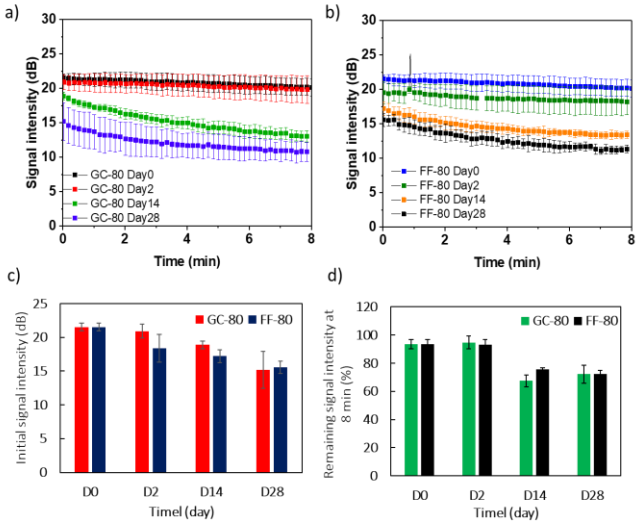

Fig.4 Acoustic characterization. a, b) US signal decay, c) enhancement of initial signal intensity (dB), d) remaining signal (%) at 8 mins

IV. Conclusions

This study demonstrates that freezing isolated NBs is a feasible process for long term storage of this contrast agent. The freeze-thawed NBs exhibited effective acoustic performance after a 28-day storage period, which was similar but not identical to freshly-prepared NBs. The shelf-life of NB is nonetheless prolonged with freezing, which may be an ideal means of improving the scale-up, dissemination and convenience of use of the agents and may open new opportunities for clinical translation

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References

1. Klibanov, A.L., *Microbubble contrast agents: targeted ultrasound imaging and ultrasound-assisted drug-delivery applications.* Invest Radiol, 2006. **41**(3): p. 354-62.
2. Dewitte, H., et al., *Enhancing Nucleic Acid Delivery with Ultrasound and Microbubbles.* Methods Mol Biol, 2019. **1943**: p. 241-251.
3. Greish, K., *Enhanced permeability and retention (EPR) effect for anticancer nanomedicine drug targeting.* Methods Mol Biol, 2010. **624**: p. 25-37.
4. Nittayacharn, P., et al., *Enhancing Tumor Drug Distribution With Ultrasound-Triggered Nanobubbles.* J Pharm Sci, 2019. **108**(9): p. 3091-3098.
5. Perera, R., et al., *Nanobubble Extravasation in Prostate Tumors Imaged with Ultrasound: Role of Active versus Passive Targeting.* 2018 Ieee International Ultrasonics Symposium (Ius), 2018.
6. Abenojar, E.C., et al., *Effect of Bubble Concentration on the in Vitro and in Vivo Performance of Highly Stable Lipid Shell-Stabilized Micro- and Nanoscale Ultrasound Contrast Agents.* Langmuir, 2019. **35**(31): p. 10192-10202.
7. de Leon, A., et al., *Contrast enhanced ultrasound imaging by nature-inspired ultrastable echogenic nanobubbles.* Nanoscale, 2019. **11**(33): p. 15647-15658.
8. Hernandez, C., et al., *Sink or float? Characterization of shell-stabilized bulk nanobubbles using a resonant mass measurement technique.* Nanoscale, 2019. **11**(3): p. 851-855.
9. Abdelwahed, W., et al., *Freeze-drying of nanoparticles: formulation, process and storage considerations.* Adv Drug Deliv Rev, 2006. **58**(15): p. 1688-713.
10. Oeffinger, B.E., et al., *Preserving the Integrity of Surfactant-Stabilized Microbubble Membranes for Localized Oxygen Delivery.* Langmuir, 2019. **35**(31): p. 10068-10078.
11. Date, P.V., A. Samad, and P.V. Devarajan, *Freeze Thaw: A Simple Approach for Prediction of Optimal Cryoprotectant for Freeze Drying.* Aaps Pharmscitech, 2010. **11**(1): p. 304-313.
12. Choi, M.J., et al., *Effect of freeze-drying process conditions on the stability of nanoparticles.* Drying Technology, 2004. **22**(1-2): p. 335-346.
13. Peppin, S.S., J.S. Wettlaufer, and M.G. Worster, *Experimental verification of morphological instability in freezing aqueous colloidal suspensions.* Phys Rev Lett, 2008. **100**(23): p. 238301.
14. Chung, N.O., M.K. Lee, and J. Lee, *Mechanism of freeze-drying drug nanosuspensions.* Int J Pharm, 2012. **437**(1-2): p. 42-50.