Acoustic Behavior of a Reactivated, Commercially Available Ultrasound Contrast Agent

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Background: Commercially available microbubbles such as Definity contain octafluoropropane encapsulated in a lipid shell. This perfluorocarbon can be compressed into liquid nanodroplets at room temperatures and activated with transthoracic diagnostic ultrasound. The aim of this study was to determine the size range and acoustic characteristics of Definity nanodroplets (DNDs) compared with Definity microbubbles (DMBs).

Methods: An in vitro flow system was used with a diagnostic ultrasound transducer (S5-1, iE33). DMBs were prepared using package insert instructions. DNDs were prepared by cooling DMBs in a −10°C to −15°C isopropyl alcohol bath before hand-pressurizing the solution. The formed DNDs were sized, diluted to 1% solutions, and infused continuously into a phosphate-buffered saline solution running within Silastic tubing. Acoustic intensity (AI) was compared with equivalent dilutions of DMBs at different mechanical indices (MIs) ranging from 0.2 to 1.4 (n = 6 comparisons at each MI) using real-time 56-Hz and triggered 2-Hz frame rates (FRs). A 3-cm-thick tissue-mimicking phantom was used to simulate transthoracic attenuation. In vivo transthoracic studies were performed in four normal pigs infused with 10% intravenous infusions of DMBs or DNDs at real-time and triggered end-systolic FRs to compare differences in myocardial and left ventricular cavity AI.

Results: DNDs were smaller than DMBs and ranged in size from 50 to 1,000 nm. In vitro studies revealed that at an MI of 0.2 and an FR of 56 Hz, DMBs had high AI (37 ± 2 dB), but AI dropped to 25 ± 2 dB at an MI of 1.0 ($P < .001$, analysis of variance). In comparison, DNDs had virtually no AI at MIs of 0.2 to 0.6 at both triggered and 56-Hz FRs (1 ± 0 dB), but AI increased to 34 ± 2 dB at an MI of 1.4 using an FR of 56 Hz ($P < .001$ vs MI of 0.2). AI also persisted longer at 56 Hz with DNDs when using higher MIs. In vivo studies demonstrated higher myocardial AI for DNDs at higher MIs when using real-time FR, most likely from microvascular nanodroplet activation.

Conclusion: These data indicate significant differences in acoustic responses of the commercially available DMBs when administered as an equivalent number of DNDs. The DND formulation may render them more useful for high-MI real-time imaging and other targeted transthoracic diagnostic applications. (J Am Soc Echocardiogr 2016;■:■-■.)

Keywords: Microbubbles, Droplets, Acoustic activation

The use of microbubbles (MB) for contrast enhancement during diagnostic ultrasound imaging has become common over the past few decades.[1] MBs have been used as a contrast agent in a wide variety of medical applications, including solid-organ tumor detection and enhanced cardiac imaging.[1,2] Current US Food and Drug Administration–approved MBs have also been used for off-label applications such as free intravascular tracers for myocardial perfusion analysis.[3] MBs are typically 1 to 5 μm in diameter and are composed of a gaseous center encapsulated by a protein, lipid, or polymer shell. This size prohibits crossing into extravascular spaces.[1,4]

Phase-change ultrasound contrast agents are nanometer-sized droplets, or nanodroplets (ND), that also contain fluorocarbons in their liquid form. These fluorocarbons stay in a liquid form well above their boiling points. Their size (50–1,000 nm) results in increased Laplace pressure that prevents boiling and also increases the acoustic energy necessary to vaporize the NDs.[5] Because of this significant limitation, it has been difficult to formulate a phase-change agent that is stable at physiologic temperature but can be activated into stable MBs after vaporization.[5,6]

Recently, it has been shown that even perfluoropropane (the fluorocarbon gas that is used to formulate commercially available contrast agents such as Definity)[7] can remain in the liquid form at body temperatures, even though its boiling point is −36.7°C.[8] We have been able to condense Definity MBs (DMBs) into Definity NDs (DNDs) using a modification of protocols published by Matsunaga *et al.*[5] and Sheeran *et al.*[6] Some investigators have shown the reactivated MBs

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Abbreviations

AI = Acoustic intensity
CPS = Contrast pulse sequencing
DMB = Definity microbubble
DND = Definity nanodroplet
FR = Frame rate
LV = Left ventricular
MB = Microbubble
MI = Mechanical index
ND = Nanodroplet

formed from lipid-encapsulated NDs may have different acoustic responses that affect their destruction rates and stability.[1,9] A variety of potential explanations exist for the altered acoustic behavior of the reactivated MBs, including altered shell characteristics upon reactivation, larger size distribution, and more resistance to acoustic destruction.[1,10] We hypothesized that DNDs could be reactivated using a technique observed with other perfluoropropane NDs.[10] Furthermore, we postulated that their behavior in the presence of the ultrasound imaging protocols used for contrast imaging (real time and triggered) would be different from DMBs because of the requirement that they first must be activated before being detected with imaging. In this study, we compared the acoustic responses of DNDs with those of standard DMBs and the potential for specifically activating the <220-nm NDs that may cross defective vascular endothelial barriers and be used for extravascular applications.

METHODS

MBs

To create MBs for both imaging and formation of NDs, a vial of Definity was mechanically agitated (Vialmix shaker; Bristol-Myers Squibb, New York, NY) for 45 sec, creating a maximum MB concentration (within the vial) of 1.2×10^{10} microspheres/mL, with diameters ranging from 1.1 to 3.3 μm according to the package insert. Because the measurement techniques described in the package insert may have failed to measure the number of submicrometer MBs produced, these formulated MBs were sized in our laboratory with analysis systems capable of determining the quantity of submicrometer MBs (see below).

ND Formulation

DNDs were formulated according to a protocol adapted from Matsunaga *et al.*[5] and Sheeran *et al.*[6]: 2-mL vials of DMBs were agitated using a Vialmix for 45 sec; a 10% solution of DMBs was created with 0.9% saline as the diluent; the solution was submerged in a 70% isopropanol bath at a temperature between −10°C and −15°C for 3 min; and manual pressure was applied until the solution cleared, indicating condensation. The solution was then warmed to room temperature before administration. A second filtered condensed suspension containing NDs <220 nm was prepared by passing a 10% DND formulation through a 220-nm filter (Merck Millipore, Billerica, MA).

ND and MB Sizing

Because of the polydisperse distribution of the NDs formed following condensation,[9] the DNDs were sized using two different machines: a NanoSight NS300 (Malvern Instruments, Malvern, United Kingdom), which is capable of sizing particles as small as 10 nm in diameter, and a Nano Zetasizer (Malvern Instruments), which can measure particles up to 10 μm in diameter. Initial sizing of the filtered and unfiltered DNDs ($n = 3$ each) was done using the Nano Zetasizer to ensure that the larger NDs were being removed from the samples. The filtered DNDs ($n = 6$) were also sized using the NanoSight NS300 to improve resolution of NDs with diameters <1 μm and to determine the change in diameter of the filtered DNDs over a 24-hour period at 25°C. DMBs ($n = 3$) were analyzed with the Nano Zetasizer to determine the entire range of sizes produced in the Vialmix sample. The sizes produced with manual pressure were compared with those produced with known applied pressures of 6, 9, and 12 atm using a high-pressure inflation device for angioplasty balloon catheters (Merit Medical Systems, Jordan, UT).

In Vitro Experimental Setup

An in vitro flow system was created that has been described previously.[11] This system consists of a pulsatile flow pump (Masterflex; Cole Parmer, Vernon Hills, IL) that propels fluid through a 2-mm-diameter Silastic tube flow system. The solution used for all in vitro studies was phosphate-buffered saline solution running at 20 mL/min at 37°C. An imaging probe (S5-1; Philips Medical Systems, Andover, MA) was placed over the flow system; the probe had a 3-cm tissue-mimicking phantom (Computerized Imaging Reference Systems, Norfolk, VA) placed between it and the tubing to mimic transthoracic attenuation. A port proximal to the imaging chamber allowed either DMBs or DNDs to be infused into the Silastic tubing at dilutions that can be adjusted.

Three different states of Definity ($n = 6$ for each state) were acoustically compared: the MBs, the NDs, and the filtered NDs. DMBs and DNDs were both infused at 5.8×10^8 MBs or NDs/min, while filtered NDs containing just the <220-nm NDs were infused at 3.9×10^{10} NDsmin. A higher concentration of filtered NDs was infused to produce equivalent acoustic intensity (AI) as that achieved with DMBs and unfiltered DNDs. Each formulation was continuously infused at a rate of 1.0 mL/min into the proximal port connected to the 2-mm Silastic tubing containing PBS flowing at 20 mL/min. Using real-time imaging (56-Hz frame rate [FR]) and a triggered FR (2.22 Hz), the diagnostic transthoracic ultrasound transducer (1.3-MHz center frequency, 3.4-MHz received frequency, 3-μs pulse duration) was used in ultraharmonic mode to insonate the infusion at incremental mechanical indices (MIs) (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.45) to (1) determine the threshold required to activate the NDs and (2) determine the AI of the solutions at different regions along the scan plane within the field of insonation (Figure 1). The elevation plane of the transducer was placed in parallel with the Silastic tubing, and AI was measured as the formulations crossed this imaging field. Exposure time to the insonation field at the 20 mL/min flow rate was 5 sec, resulting in cumulative FR exposures of 280 frames at the 56-Hz FR and 11 frames at the 2-Hz FR by the time the MBs or NBs reached region 4 of the imaging plane (Figure 1). Because flow in the tubing was 0.33 mL/sec, and the ultrasound-exposed tubing volume was 1.75 mL, the 500-msec time interval between frames at 2 Hz allowed only partial replenishment of NDs and MBs between frames. AI in the different horizontally placed regions 1 to 4 demonstrated in Figure 1 was analyzed with QLAB software (Philips Medical Systems).

In Vivo Experimental Setup

All studies performed were approved by the institutional animal care and use committee at the University of Nebraska Medical