

# Characterizing the subharmonic response of four new microbubble formulations compared with three commercially-available ultrasound contrast agents

Ji-Bin Liu\*, Amanda Q.X. Nio\*†, Cara Esposito\*‡, Jie Chen§, Jie Zhang¶, Xing Zhong||, Renfa Liu\*\*, Jinrui Wang††, Zhifei Dai\*\*, Flemming Forsberg\* and Jaydev K. Dave\*

\*Department of Radiology, Thomas Jefferson University, Philadelphia, PA, USA,

†Department of Biomedical Engineering, King's College London, United Kingdom,

‡School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA, USA,

§Shanghai Jiao Tong University School of Medicine, Shanghai Sixth Peoples Hospital, Shanghai, People's Republic of China,

¶Tianjin Medical University General Hospital, Tianjin, People's Republic of China

||The First Affiliated Hospital of Jinan University, Guangzhou, People's Republic of China

\*\*Department of Biomedical Engineering, College of Engineering, Peking University, Beijing, People's Republic of China

††Beijing University Third Hospital, Beijing, People's Republic of China

Email: jaydev.dave@jefferson.edu

**Abstract**—Subharmonic imaging (i.e., receiving signal at half the transmitting frequency) is a promising technique to detect ultrasound contrast agents in the circulation. The aim of this study was to characterize the subharmonic enhancement of four new microbubble formulations (MBF-1 to 4), compared with three commercially-available contrast agents (Definity, Sonazoid and SonoVue), and to compare this subharmonic enhancement with the fundamental response. The new microbubble formulations consisted of either C<sub>3</sub>F<sub>8</sub> or 2% C<sub>3</sub>F<sub>8</sub> and 98% N<sub>2</sub> as the inner gas, and had different shell configurations containing polyethylene glycol 4000. Equal concentrations of each reconstituted contrast agent were investigated *in vitro* using a flow phantom. Radiofrequency data were acquired at four transmitting frequencies (2.5, 3.0, 3.5 and 4.0 MHz), four pulse-inversion configurations (1–4 cycles/pulse) and four scanner acoustic output levels (25, 52, 77 and 100%). Enhancement was derived as the relative increase in signal amplitude after contrast was added. Subharmonic enhancement of the new microbubble formulations were generally less affected by transmitting frequency, pulse length and acoustic output level than the commercially-available contrast agents ( $p < 0.05$  was considered statistically significant for main and interaction effects). Mean subharmonic enhancement over all transmitting conditions for the new microbubble formulations (MBF-1 8.4 ± 4.0; MBF-2 8.5 ± 3.8; MBF-3 6.5 ± 3.1; MBF-4 7.5 ± 3.2 dB) were within the range exhibited by the commercially-available contrast agents (Definity 6.1 ± 3.4; Sonazoid 7.0 ± 3.4; SonoVue 10.7 ± 3.2 dB). The subharmonic enhancement was similar to or higher than the fundamental response across all contrast agents investigated. Subharmonic imaging may be applied to detect the new microbubble formulations in future work.

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**Keywords:** *ultrasound contrast agents, subharmonic imaging, pulse-inversion, transmitting frequency, acoustic output*

## I. INTRODUCTION

Microbubble ultrasound contrast agents are currently used clinically to improve tissue-to-blood delineation in the heart [1] and to assess vascularity in liver lesions [2], amongst other applications. The enhanced signal generated by ultrasound contrast agents is due to differences in acoustic impedance and compressibility between microbubbles and their surrounding media [3]. In particular, subharmonic imaging (i.e., focusing on the acoustic signal at half the insonating frequency) has the potential to improve visualization of the vasculature due to the lack of subharmonic generation from tissues [3].

Building upon the non-invasive nature and low cost of using ultrasound contrast compared with traditional imaging methods, such as magnetic resonance imaging and computerised tomography scans, there is a broad array of pre-clinical and clinical research to further expand the applications of ultrasound contrast agents. Some examples are super-resolution imaging [4], targeted molecular imaging and drug delivery [5] and non-invasive pressure estimation [6]. Amongst the viable inner gas and shell composition combinations that make up a microbubble, polyethylene glycol (PEG) has emerged as a molecule that may be incorporated into the shell to reduce immunogenicity and enhance longevity of the microbubble in circulation [5].

The aim of this study was to investigate the impact of transmitting frequency, pulse length and driving acoustic pressure on four new microbubble formulations composed of different combinations of inner gases and phospholipid-PEG shell configurations. We assessed the enhancement of the new microbubble formulations at the subharmonic ( $f_0/2$ ) and

fundamental ( $f_0$ ) frequencies, and compared the enhancement with three commercially-available ultrasound contrast agents.

## II. MATERIALS AND METHODS

### A. Ultrasound contrast agents

Four new microbubble formulations (MBF-1 to 4; Department of Biomedical Engineering, Peking University, Beijing, China) were compared with three commercially-available contrast agents. Both MBF-1 ( $2.57 \times 10^9$  microbubbles/mL) and MBF-2 ( $1.86 \times 10^9$  microbubbles/mL) had a solid weight of 50 mg/vial, with C<sub>3</sub>F<sub>8</sub> as the inner gas and contained PEG 4000 in the bubble shell. However, the phospholipid shell of MBF-1 contained phosphatidylglycerol and phosphatidylcholine, while that of MBF-2 contained only phosphatidylglycerol. In contrast to MBF-1 and MBF-2, the inner gas in MBF-3 and MBF-4 consisted of 2% C<sub>3</sub>F<sub>8</sub> and 98% N<sub>2</sub>. Less PEG 4000 in the bubble shell differentiated MBF-4 (solid weight 25 mg/vial;  $3.41 \times 10^9$  microbubbles/mL) from MBF-3 (solid weight 50 mg/vial;  $1.44 \times 10^9$  microbubbles/mL). The three commercially-available ultrasound contrast agents were Definity (Lantheus Medical Imaging Inc., N. Billerica, MA, USA), Sonazoid (GE Healthcare, Oslo, Norway) and SonoVue (Bracco Spa, Milan, Italy). The new microbubble formulations were reconstituted with 1 mL of saline/vial, and the commercially-available agents according to manufacturers' instructions.

### B. Flow phantom

The seven different microbubble formulations were investigated in a Doppler flow phantom (ATS Laboratories, Inc., Bridgeport, CT, USA; 6 mm vessel diameter; 30 mm depth; Figure 1). Equal concentrations of each reconstituted ultrasound contrast agent (0.12 mL MBF-1, 0.16 mL MBF-2, 0.21 mL MBF-3, 0.09 mL MBF-4, 0.025 mL Definity, 0.3 mL Sonazoid, 0.9 mL SonoVue) were added to 750 mL of isotonic diluent (VT390F, Val Tech Diagnostics, Brackenridge, PA, USA) contained in a reservoir connected to the flow phantom. A magnetic stirrer was used to maintain a homogenous mixture of microbubbles in the reservoir, and a continuous-flow roller pump (S10K II, Sarns Inc., Ann Arbor, MI, USA) was used to circulate this mixture through the flow phantom. A physiologically-relevant range of hydrostatic pressures were thus successfully simulated *in vitro* (pressure range of approximately 120 mmHg), which was continually measured using a solid state pressure catheter (Mikro-Tip Transducer SPR-350S and PCU-2000 Pressure Control Unit, Millar Inc., Houston, TX, USA; LeCroy 9350AM Oscilloscope, NY, USA; LabVIEW, National Instruments, Austin, TX, USA).

### C. Subharmonic and fundamental signals

The acoustic signal of each contrast agent was assessed at four transmitting frequencies (2.5, 3.0, 3.5 and 4.0 MHz), four pulse-inversion configurations (1–4 cycles/pulse) and four acoustic output levels (25, 52, 77 and 100%) on a commercially-available SonixTablet ultrasound scanner (BK Ultrasound, Richmond, BC, Canada). With a 4-cycle pulse,

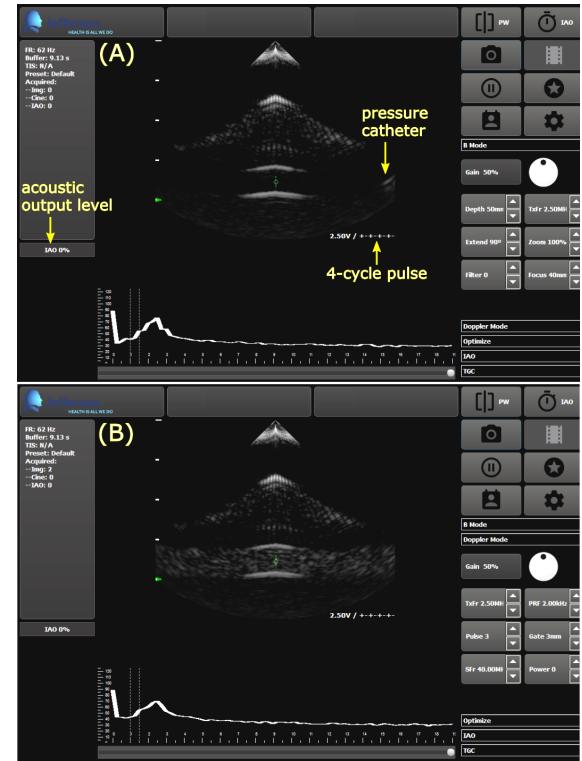


Fig. 1. Customized interactive interface on the SonixTablet ultrasound scanner showing the *in vitro* 6 mm vessel (A) without and (B) with ultrasound contrast (Definity in this example). Transmitting parameters are manipulated on the right panel. The pressure catheter is visible within the vessel, but away from the sample gate such that it does not interfere with the flow of the contrast agent.

100% acoustic output on the scanner corresponded to acoustic pressures of 1.35–2.17 MPa (peak negative) across transmitting frequencies, as measured by a 0.2 mm needle hydrophone (Precision Acoustics, UK) in a water bath. The scanner was modified to run an interactive interface from which transmitting parameters could be manipulated and unprocessed radiofrequency data recorded from a Doppler sample gate (Figure 1). An SA4-2 transducer (bandwidth 2–4 MHz) was clamped above the flow phantom, and a 3 mm sample gate was positioned in the middle of the 6 mm vessel. Radiofrequency data were recorded for 5 s at each transmitting configuration, with a pulse repetition frequency of 2000 Hz ( $n = 3$ ). Signal processing was performed offline (MATLAB R2016a, The MathWorks, Inc., Natick, MA, USA). The mean signal amplitude in a 0.5 MHz bandwidth over each 5-s interval was calculated around the theoretical subharmonic (i.e., 1.25, 1.5, 1.75 and 2.0 MHz), and fundamental frequencies (i.e., 2.5, 3.0, 3.5 and 4.0 MHz). Baseline data without contrast were first acquired at each acoustic output level for each transmitting frequency and pulse configuration (16 permutations in total), and then, enhancement was determined as the increase in signal amplitude after the contrast agent was added to the flow phantom (Figure 2). In between acquisitions with different contrast agents, the flow phantom was rinsed and replenished with new isotonic diluent.

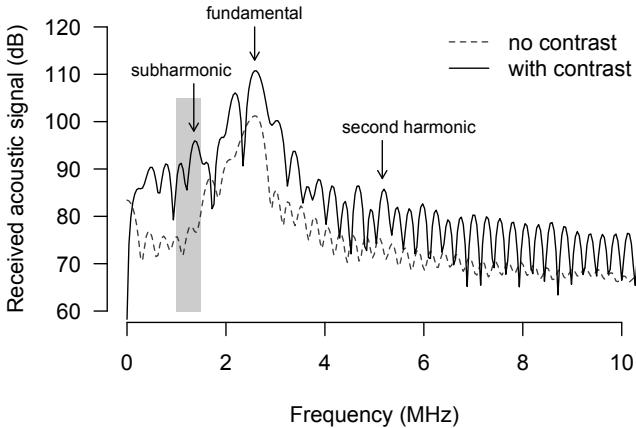


Fig. 2. An example of the frequency spectrum of a single received pulse after ultrasound contrast was added, overlaid on the mean frequency spectrum without contrast. In this example, Sonazoid was added and data recorded at a transmitting frequency of 2.5 MHz, a pulse length of 4 and 25% acoustic output. ■ 0.5 MHz bandwidth around the theoretical subharmonic frequency of 1.25 MHz.

#### D. Statistical analysis

Statistical analyses were performed with R (version 3.3.1). Analysis of variances (ANOVAs) were used to examine the impact of transmitting frequency, pulse length and acoustic output level on the subharmonic enhancement for each contrast agent, and to compare subharmonic and fundamental enhancements. Subharmonic enhancement across contrast agents were compared using another ANOVA. Alpha was set at 0.05 *a priori*, and was not adjusted for multiple comparisons (e.g., Bonferroni correction) to maintain statistical power [7] while comparing seven ultrasound contrast agents. Data are reported as mean and standard deviations.

### III. RESULTS AND DISCUSSION

#### A. Impact of transmitting frequency, pulse length and acoustic output level on the subharmonic enhancement of each microbubble formulation

*a) MBF-1 and MBF-2:* The subharmonic enhancements of MBF-1 and MBF-2 were similar across the transmitting frequencies, pulse lengths and acoustic output levels investigated ( $p > 0.05$  for all main and interaction effects; Figure 3).

*b) MBF-3 and MBF-4:* The subharmonic enhancement of MBF-3 was highest at 25% acoustic output ( $7.4 \pm 2.5$  dB; main effect  $p = 0.045$ ) and independent of pulse length ( $p > 0.05$ ), while that of MBF-4 was highest at 25% acoustic output and a pulse length of 4 ( $10.3 \pm 4.3$  dB; interaction between pulse length and acoustic output level  $p = 0.029$ ). Transmitting frequency did not affect the subharmonic enhancement of both MBF-3 and MBF-4 ( $p > 0.05$  for the main and interaction effects of transmitting frequency).

*c) Definity:* A transmitting frequency of 2.5 MHz elicited the highest subharmonic enhancement from Definity ( $8.6 \pm 4.1$  dB; main effect  $p = 0.046$ ), independent of pulse length and acoustic output levels considered in this study ( $p > 0.05$  for all other effects).

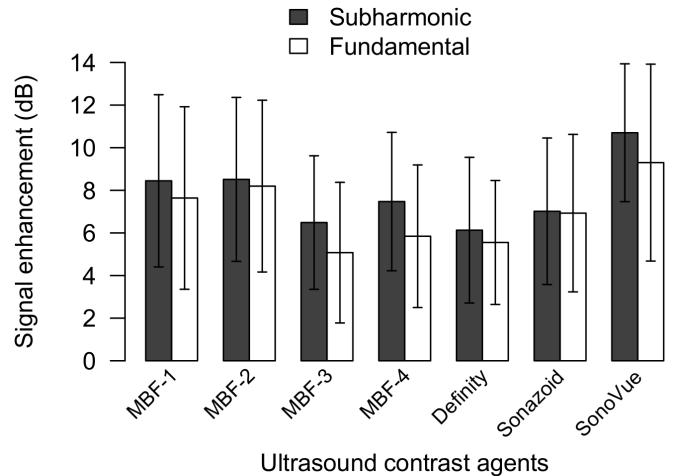


Fig. 3. Subharmonic and fundamental enhancements of ultrasound contrast agents averaged over transmitting frequencies 2.5–4.0 MHz, pulse-inversion configurations of 1–4 cycles/pulse and acoustic output levels 25, 52, 77 and 100%. MBF: new microbubble formulations. Values are mean  $\pm$  one standard deviation.

*d) Sonazoid:* Transmitting frequency, pulse length and acoustic output level all influenced the subharmonic enhancement of Sonazoid ( $p < 0.05$  for interaction effects). The highest subharmonic enhancement for Sonazoid was observed at a transmitting frequency of 2.5 MHz, a pulse length of 4 and 25% acoustic output ( $14.3 \pm 2.0$  dB; Figure 4). In particular, the results of Sonazoid emphasize the importance of selecting a low acoustic output level to minimise microbubble destruction [2], [8], as the lowest enhancement was at 100% acoustic output and was only 15% of the maximum enhancement ( $2.2 \pm 3.4$  dB at transmitting frequency 2.5 MHz and pulse length 3).

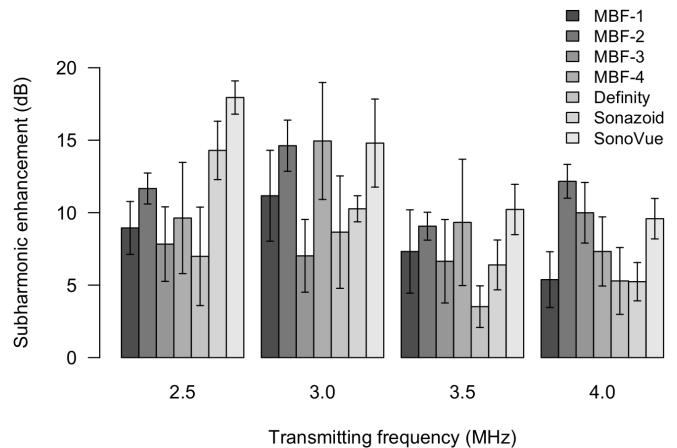


Fig. 4. Subharmonic enhancement of ultrasound contrast agents at four transmitting frequencies (2.5, 3.0, 3.5 and 4.0 MHz), at a pulse length of 4 and 25% acoustic output. Values are mean  $\pm$  one standard deviation.

*e) SonoVue:* Similar to Sonazoid, the subharmonic enhancement of SonoVue was influenced by all three transmitting parameters manipulated in this study (three-factor interaction  $p = 0.028$ ). The highest subharmonic enhancement

for SonoVue was observed at a transmitting frequency of 2.5 MHz, a pulse length of 4, and 25% acoustic output ( $17.9 \pm 1.1$  dB; Figure 4); the lowest subharmonic enhancement was observed at a transmitting frequency of 4.0 MHz, a pulse length of 3 and 77% acoustic output ( $4.8 \pm 1.2$  dB).

Our results suggest that the subharmonic signals from the new microbubble formulations vary less with transmitting frequency, pulse length and driving acoustic pressure, compared with existing commercially-available agents. Notwithstanding, the highest subharmonic enhancement was consistently observed at 25% acoustic output across contrast agents (i.e., MBF-3, MBF-4, Sonazoid and SonoVue), and indicates that applying a low acoustic output level should be prioritised. A longer pulse length also appears to increase the likelihood of a high subharmonic enhancement (e.g. MBF-4, Sonazoid and SonoVue [9]). Whilst not apparent with 4-cycle pulses in this study, it is nonetheless important to recognize that longer pulse lengths destroy microbubbles and thus pulse length should be increased cautiously to maximize enhancement [10]. Interestingly, all three commercially-available contrast agents exhibited their highest subharmonic enhancement at a transmitting frequency of 2.5 MHz, which could be an ideal default setting to ensure enhancement across multiple agents. Taken together, our findings across different contrast agents illustrate the importance of optimizing transmitting parameters over a multi-parameter space to maximize enhancement [11].

#### B. Enhancement at the subharmonic frequency compared with the fundamental frequency

A greater subharmonic than fundamental enhancement was observed for MBF-3 and MBF-4 (main effect of signal  $p < 0.05$ ; Figure 3), but not for any of the other contrast agents investigated in this study ( $p > 0.05$ ). It is likely that our choice of pulse-inversion configurations contributed to a stronger subharmonic signal, despite the subharmonic frequency falling outside the bandwidth of the commercially-available SA4-2 transducer. Accordingly, the potential of subharmonic imaging may be better realised with a transducer of broader bandwidth. In contrast, enhancement at the second harmonic—above the bandwidth of the transducer—was minimal (Figure 2) and thus, could not be accurately assessed in this study. Overall, the subharmonic enhancement generated by all the contrast agents investigated in this study highlight the potential of subharmonic imaging to visualize and track microbubbles when injected into the circulation.

#### C. Subharmonic enhancement of new microbubble formulations in comparison with Definity, Sonazoid and SonoVue

Following on from our earlier findings (above) indicating a high subharmonic enhancement with 4-cycle pulses, the seven contrast agents were compared using data at this specific pulse length. The subharmonic enhancement of the seven contrast agents differed, and was affected by transmitting frequency (interaction between microbubble and transmitting frequency  $p = 0.043$ ). However, the subharmonic enhancement of the new microbubble formulations were generally within the range

exhibited by the commercially-available agents (Figure 3–4), and thus, we conclude that the subharmonic enhancement of the new formulations are comparable to that of existing commercially-available agents.

#### IV. CONCLUSION

Signal enhancement at the subharmonic frequency generated by four new microbubble formulations containing PEG 4000 were comparable to commercially-available Definity, Sonazoid and SonoVue. The subharmonic enhancement was similar to or higher than the fundamental response across all contrast agents investigated. The new microbubble formulations may be useful for subharmonic imaging in the future.

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