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The Effects of Hydrostatic Pressure on the Subharmonic Response of SonoVue and Sonazoid

Ipshita Gupta[1]*, Amanda Q.X. Nio[2]*, Alessandro Faraci[1], Mehnoosh Torkzaban[1], Kirsten Christensen-Jeffries[2], Kibo Nam[1], Jason L. Raymond[3], Kirk Wallace[4], Mark J. Monaghan[5], Daniel Fuster[6], Robert J. Eckersley[2], Pablo Lamata[2], Flemming Forsberg[1]
(*Equal contributions in alphabetical order)

[1] Thomas Jefferson University, Philadelphia, PA 19107, USA, [2] King's College London, London, United Kingdom, [3] University of Oxford, Oxford, United Kingdom, [4] GE Global Research, Niskayuna, NY 12309 ,USA, [5] King's College Hospital, London, United Kingdom, [6] Sorbonne Université, Paris, France
Email: ipshitagupta89@gmail.com / amanda.nio@kcl.ac.uk

***Abstract*—It has been previously established that the subharmonic signal of microbubble ultrasound contrast agents follows an inverse linear relationship with the ambient hydrostatic pressure. However, contradictory results have been reported for SonoVue (Bracco Spa, Milan, Italy). Therefore, the aim of this study was to investigate the subharmonic response of SonoVue across a range of physiologically-relevant hydrostatic pressures (10–220 mmHg), and to compare it with Sonazoid (GE, Oslo, Norway). A modified GE Logiq 9 scanner was used to acquire the subharmonic signals from Sonazoid and SonoVue in a sealed water tank. In addition, the Ultrasound Advanced Open Platform (ULA-OP) was used to acquire the subharmonic signal of SonoVue at 0–200 mmHg in a cell culture cassette over approximately 3 min. Sonazoid showed an inverse linear relationship between subharmonic amplitude and hydrostatic pressure (slope -0.11 dB/mmHg; r = -0.81), as expected. SonoVue exhibited an increase in subharmonic amplitude from 0–100 mmHg hydrostatic pressure (slope 0.06 dB/mmHg, r = 0.81), a plateau between 100–140 mmHg, and a decrease from 140–220 mmHg (slope -0.26 dB/mmHg, r = -0.98). The subharmonic amplitude of SonoVue increased over 3 min at 0 and 25 mmHg hydrostatic pressure, did not change with time at 50–100 mmHg, and decreased over time at 125–200 mmHg. However, the effects of time on the subharmonic amplitude of SonoVue were smaller than the effects of hydrostatic pressure. The subharmonic response of SonoVue to hydrostatic pressure differs from Sonazoid and other microbubble ultrasound contrast agents.**

Keywords: subharmonic, ultrasound contrast agents, pressure estimation

I. Introduction

Ultrasound contrast agents (UCAs) are gas-filled microbubbles designed to act as echo-enhancers. They are currently used throughout the world in both clinical and research settings [1-4]. The gas within these microbubbles has high compressibility and thus, a much higher echogenicity than the surrounding tissues in the body [5]. At acoustic pressures typically above 200 kPa, the UCAs start to oscillate nonlinearly [6]. This behavior has enabled the development of subharmonic contrast imaging — an imaging mode that transmits at double the resonance frequency (f_o) and receives at half the transmit frequency ($f_o/2$).

Using subharmonic imaging, our group has proposed and validated the use of UCAs as pressure sensors (i.e., SHAPE) for noninvasive, quantitative pressure estimation (e.g., in cardiovascular and portal hypertension) [1-3]. This novel application of UCAs is based on a decreasing subharmonic amplitude with increasing hydrostatic pressure. Although our in-human studies have focused on the UCAs Sonazoid (GE Healthcare, Oslo, Norway) and Definity (Lantheus Imaging, N. Billerica, MA, USA),[1-3] *in vitro* studies from our group and others suggest that this technique may be extended to most UCAs [7-9]. However, two studies have found a contradictory *increase* in subharmonic amplitude with hydrostatic pressure with SonoVue and SonoVue-like microbubbles [10, 11]. Specifically, Nio et al. observed a 10 dB increase in the subharmonic amplitude of SonoVue as hydrostatic pressure increased from 0 to 75 mmHg, followed by a plateau and a decreasing phase from 75 mmHg to 200 mmHg [11, 12]. Further work is therefore required to verify and understand the subharmonic response of SonoVue to changes in hydrostatic pressure.

The aim of this study was to investigate the subharmonic response of SonoVue to physiologically-relevant hydrostatic pressures using two different *in vitro* phantoms and ultrasound systems. In addition, a direct comparison between SonoVue and Sonazoid was conducted with a commercial scanner to verify the contradictory response of SonoVue.

II. Methods

A. SonoVue vs. Sonazoid at 10–220 mmHg

1) Static pressure chamber

Contrast signals at hydrostatic pressures from 10 to 220 mmHg were measured using a 2.25 L water tank. The water tank was equipped with an acoustic window made of thin plastic (thickness 1.5 mm). The pressure inside was varied by injecting air through an inlet on the back wall of the tank and was monitored by a pressure gauge (OMEGA Engineering Inc, Stamford, CT, USA; model DPG1000B-05G). An inlet on the top of the tank was constructed for injecting microbubbles and placing the pressure gauge.

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2) GE Logiq 9

A modified Logiq 9 scanner (GE, Waukesha, WI, USA) with a 4C curvilinear array was used to transmit a 2.5 MHz Gaussian windowed binomial filtered square wave and to acquire the subharmonic response (at 1.25 MHz). A power optimization algorithm implemented on the Logiq 9 scanner was used for selecting optimum power for maximum SHAPE sensitivity [13, 14]. The scanner was used to acquire radiofrequency data at the optimized acoustic power associated with each individual waveform (in triplicate) for each hydrostatic pressure value following injection of SonoVue and Sonazoid separately (0.2 mL/L) into saline (Isoton II; Coulter, Miami, FL, USA). The mixture was kept homogenous by the use of a magnetic stirrer. Data were collected at Thomas Jefferson University (Philadelphia, PA, USA).

3) Data processing and analysis

Radiofrequency data from each acquisition was extracted using proprietary software (GE Global Research) as described above. Regions of interest on maximum intensity projection of B-mode images (compiled from reconstructed images from the radiofrequency data) were fixed throughout the 6 s acquisition (27–30 frames). The subharmonic amplitude was calculated in a 0.5 MHz bandwidth around 1.25 MHz. Correlation coefficients and regression line slopes were calculated to check for the waveform with the best sensitivity and correlation with pressure.

B. SonoVue at 0–200 mmHg over 3 min

1) Static pressure chamber

As part of a multi-center collaborative effort to investigate the subharmonic response of SonoVue at physiologically-relevant hydrostatic pressures, experiments with SonoVue were also performed in the ultrasound laboratory at King's College London (London, UK). Instead of a 2.25 L water tank, the static pressure chamber at King's College London consisted of a 10 mL cell culture cassette with Luer connections (CLINIcell 25, 175 µm membrane, 6.8 cm × 3.9 cm × 3.7 mm, Mabio International, Tourcoing, France). This static pressure phantom has been used previously in microbubble experiments, and is described in more detail in the report by Nio et al.[11, 12]. Briefly, the cell culture cassette was submerged in a water bath, and high pressure PVC tubings (900 PSI, Cole-Parmer, St Neots, Cambridgeshire, UK) connected the cassette to entry and exit Luer stopcocks for administering microbubble solution. Prior to the exit stopcock, a pressure sensor (PRESS-S-000 sensor, PendoTech, Princeton, NJ, USA) connected to a digital pressure meter (INFCS-112B meter, Newport Electronics, Inc., Santa Ana, CA, USA) enabled real-time monitoring of pressure within the cell culture cassette. A 12 mm layer of open-cell melamine foam (Basotect, BASF, Ludwigshafen, Germany) was positioned in front of the cassette as an attenuating layer between the ultrasound transducer and the microbubbles. The total acoustic signal loss through the foam and cassette window has been previously estimated as 6.19 dB at transmit frequency 5 MHz, and was used to estimate the acoustic pressure within the cassette chamber (acoustic pressure in a water bath × $10^{-6.19/20}$) [12].

2) ULtrasound Advanced Open Platform (ULA-OP)

To verify the subharmonic response of SonoVue across ultrasound systems, the ULtrasound Advanced Open Platform (ULA-OP, MSD Lab, University of Florence, Florence, Italy) instead of the Logiq 9 was used in experiments at King's College London. The ULA-OP was used to drive a commercially available linear ultrasound transducer, to transmit pulse-inversion sequences consisting of 16 cycle 5 MHz pulses (LA332E Marzo 2014, bandwidth 3–7 MHz, Esaote, Genoa, Italy). The transducer was positioned 45° to the cell culture cassette to minimize backscatter from the cassette windows, which concomitantly increased the effective depth of the region of interest to 5.2 mm [12].

3) Experimental protocol with SonoVue

SonoVue was reconstituted according to manufacturer's instructions, and diluted in gas-equilibrated water to yield a typical concentration used in the clinic (0.4 µL/mL water). With the exit port open, approximately 25 mL of diluted microbubble solution was added to the static pressure phantom (at a rate of ≈0.5 mL/s). A 1.5 mm magnetic stirrer in the cell culture cassette maintained a homogenous concentration of microbubbles within the pressure chamber. The exit port was then closed and the desired hydrostatic pressure achieved by adding more microbubble solution.

Radiofrequency data were recorded on the ULA-OP at ambient hydrostatic pressure (0 mmHg) from 3.5–100% maximum scanner acoustic output (n = 40, equally spaced on a logarithmic scale) [15]. The mean subharmonic amplitude over a 40% bandwidth (i.e., 2–3 MHz) was extracted using MATLAB to identify the growth phase of the subharmonic signal of SonoVue [12, 16]. Data were then recorded at the acoustic pressure corresponding to the middle of the growth phase (18% maximum scanner acoustic output; 127 kPa peak negative acoustic pressure) at 200 to 0 mmHg hydrostatic pressures in 25 mmHg decrements (n = 20), and repeated to get three sets of data.

To disentangle the effects of time and acoustic pressure, data were recorded intermittently three vs. twenty times over 2.5–3 min at 0, 75 and 200 mmHg hydrostatic pressures (18.7% maximum scanner acoustic output for the acoustic pressure corresponding to the middle of the growth phase on a separate day; 131 kPa peak negative acoustic pressure). This was repeated to obtain five datasets of three insonations, and four datasets of twenty insonations. The microbubble solution in the phantom was replenished after each set of acoustic output levels. All experiments were performed at room temperature (≈21°C), and were completed within 4.5 h of microbubble reconstitution.

4) Data processing and analysis

A zero-phase digital filter was applied with a finite impulse response (FIR) band-pass filter to isolate the signal amplitude over the subharmonic bandwidth. A 15.5 × 3 mm rectangular region of interest was defined from the B-mode image. The average subharmonic amplitude was calculated as the mean amplitude across three frames of the region of interest (i.e., 3 frames × 64 lines/frame). Linear regressions between subharmonic amplitude and time were performed at

each hydrostatic pressure level, to investigate (i) the interaction of time at each hydrostatic pressure level on the subharmonic response of SonoVue, and (ii) the effects of three vs. twenty exposures to acoustic pressure on the relationships in (i). The analysis of covariance (ANCOVA; *aoctool* in MATLAB) was used to compare the subharmonic response of SonoVue between three and twenty acoustic insonations.

III. Results

A. *SonoVue has a different subharmonic response from Sonazoid*

Sonazoid showed an inverse linear relationship between subharmonic amplitude and ambient pressure, with a slope of -0.11 dB/mmHg and a correlation of -0.81, as expected (Figure 1) [8, 13]. However, SonoVue exhibited an increase in subharmonic amplitude from 0–100 mmHg hydrostatic pressure (slope = 0.06 dB/mmHg, r = 0.81), a plateau between 100–140 mmHg, and a decrease from 140–220 mmHg (slope = -0.26 dB/mmHg, r = -0.98).

B. *Hydrostatic pressure influences the subharmonic response of SonoVue over time*

The subharmonic amplitude of SonoVue increased slightly over 3 min at 0 and 25 mmHg hydrostatic pressures, did not change with time at 50–100 mmHg, and decreased over time at 125–200 mmHg (Figure 2). Duration of acoustic insonation (i.e., three vs. twenty insonations) at 131 kPa peak negative acoustic pressure did not affect the subharmonic response of SonoVue over time (Figure 3; $p > 0.05$).

IV. Discussion

A. *Verifying the subharmonic response of SonoVue to hydrostatic pressure*

Our results with the Logiq 9 clinical scanner are consistent with what has been observed previously with single element transducers and the ULA-OP [10, 12]. Simulations using the Rayleigh-Plesset equation suggest that an increase in the subharmonic response may be driven by a decreasing bubble surface tension [11]. In addition, the change in bubble size

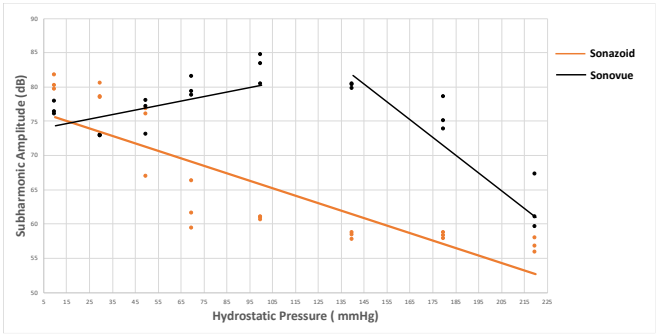

Figure 1: In vitro changes in subharmonic signal amplitude as a function of hydrostatic pressure for Sonazoid and SonoVue along with the best linear fit of the data.

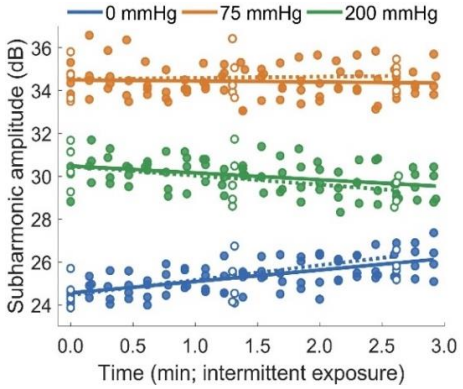

Figure 3. Subharmonic response of SonoVue with 3 (n=5; data as open circles; linear regression as dotted line) and 20 (n=4; data as closed circles; linear regression as solid line) intermittent acoustic insonations at 0, 75 and 200 mmHg hydrostatic pressure.

with hydrostatic pressure and accordingly, the change in its resonance frequency may also explain the presence of ascending and descending phases of SonoVue. Crucially, Katiyar et al. have shown with simulations of a single microbubble that there is an upper critical ratio of excitation frequency (f) to bubble resonance frequency (f_o) above which the subharmonic response increases with increasing hydrostatic pressure [17]. Below a lower critical value of the same ratio, increasing hydrostatic pressure decreases the

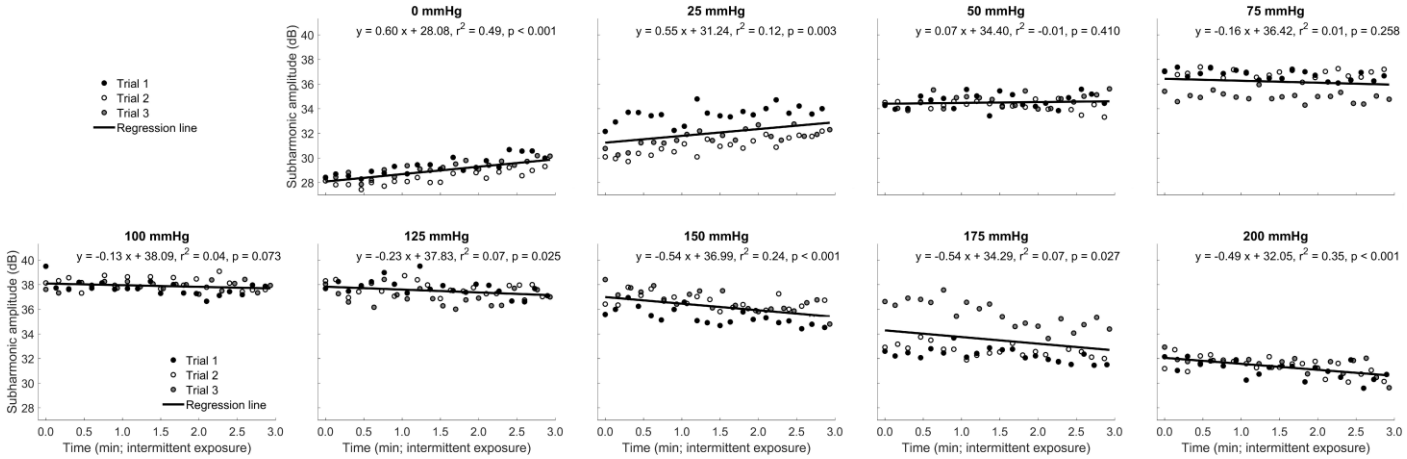

Figure 2. Subharmonic amplitude of SonoVue at 0–200 mmHg hydrostatic pressure over 3 min of intermittent 5-s exposure to 16-cycle 5 MHz ultrasound pulses of 127 kPa peak-negative acoustic pressure.

subharmonic response. However, we do not know why an ascending phase has only been observed with SonoVue or SonoVue-like microbubbles. One possible explanation may be the higher vapor pressure of the gas used inside these bubbles i.e., sulphur hexafluoride (1062 kPa vs. 333 kPa for perfluorobutane gas used in Sonazoid). The difference in subharmonic behavior of SonoVue compared with Sonazoid (and other commercially-available microbubbles) is important for interpreting SHAPE results using SonoVue.

B. *Experiment time influences the sensitivity of the subharmonic-pressure relationship of SonoVue*

As the subharmonic amplitude of SonoVue increased slightly over 3 min at 0 and 25 mmHg hydrostatic pressure, this indicates that a small delay in data collection is likely to reduce the sensitivity (dB/mmHg) of the ascending subharmonic-pressure relationship from 0 mmHg to the plateau phase. Conversely, the decrease in the subharmonic amplitude of SonoVue over time at 125–200 mmHg would increase the sensitivity of the descending phase at higher hydrostatic pressures. The time-dependent evolution of subharmonic emissions may be related to the diffusivity of the sulphur hexafluoride gas from SonoVue microbubbles to the surrounding medium [18]. Our study extends upon previous work by Kanbar et al. [18] by demonstrating that the time-dependent evolution of subharmonic emissions is additionally influenced by the surrounding hydrostatic pressure. Nonetheless, whilst a small delay in data collection will affect the sensitivity of the subharmonic-pressure response of SonoVue, the pattern of an increase-plateau-decrease remains.

V. Conclusion

This study verifies that the subharmonic response of SonoVue to 0–220 mmHg hydrostatic pressure consists of ascending, plateau and descending phases, in contrast to a single descending phase for Sonazoid and most other microbubble ultrasound contrast agents. Whilst time from administration of a bolus of SonoVue influences the sensitivity of the subharmonic-pressure relationship, this effect is smaller than the change in subharmonic amplitude due to hydrostatic pressure. The ascending and descending phases of the subharmonic-pressure relationship of SonoVue may potentially be used to estimate pressures in the body.

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