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● *Original Contribution*

IMPACT OF FILLING GAS ON SUBHARMONIC EMISSIONS OF PHOSPHOLIPID ULTRASOUND CONTRAST AGENTS

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Abstract—Subharmonic signals backscattered from gas-filled lipid-shelled microbubbles have generated significant research interest because they can improve the detection and sensitivity of contrast-enhanced ultrasound imaging. However, the emission of subharmonic signals is strongly characterized by a temporal dependence, the origins of which have not been sufficiently elucidated. The features that influence subharmonic emissions need to be identified not only to better develop next-generation microbubble contrast agents, but also to develop more efficient subharmonic imaging (SHI) modes and therapeutic strategies. We examined the effect of microbubble filling gas on subharmonic emissions. Phospholipid shelled-microbubbles with different gaseous compositions such as sulfur hexafluoride (SF_6), octafluoropropane (C_3F_8) or decafluorobutane (C_4F_{10}), nitrogen (N_2)/C_4F_{10} or air were insonated using a driving frequency of 10 MHz and peak negative pressure of 450 kPa, and their acoustic responses were tracked by monitoring both second harmonic and subharmonic emissions. Microbubbles were first acoustically characterized with their original gas and then re-characterized after substitution of the original gas with air, SF_6 or C_4F_{10}. A measureable change in intensity of the subharmonic emissions with a 20- to 40-min delayed onset and increasing subharmonic emissions of the order 12–18 dB was recorded for microbubbles filled with C_4F_{10}. Substitution of C_4F_{10} with air eliminated the earlier observed delay in subharmonic emissions. Significantly, substitution of SF_6 for C_4F_{10} successfully triggered a delay in the subharmonic emissions of the resultant agents, whereas substitution of C_4F_{10} for SF_6 eliminated the earlier observed suppression of subharmonic emissions, clearly suggesting that the type of filling gas contained in the microbubble agent influences subharmonic emissions in a time-dependent manner. Because our agents were dispersed in air-stabilized phosphate-buffered saline, these results suggest that the diffusivity of the gas from the agent to the surrounding medium is correlated with the time-dependent evolution of subharmonic emissions. (E-mail: Ayache.bouakaz@univ-tours.fr) © 2016 World Federation for Ultrasound in Medicine & Biology.

Key Words: **Ultrasound contrast agents, Microbubbles, Subharmonic, Non-linear imaging, Gas diffusion.**

INTRODUCTION

Microbubbles, comprising gaseous cores encapsulated with a protein, polymer or lipid shell, are widely used in diagnostic contrast imaging (Faez et al. 2013; Ferrara et al. 2007; Forsberg et al. 1998; Ophir and Parker 1989). To ensure their longevity within the bloodstream, current-generation microbubbles are typically filled with highly insoluble fluorinated carbon or sulfur gases such as sulfur hexafluoride (SF_6), octafluoropropane (C_3F_8) and decafluorobutane (C_4F_{10}), which greatly promote microbubble stability. These gas-encapsulated microbubbles are efficient reflectors of ultrasound waves (Calliada et al. 1998) through two mechanisms: First, microbubbles act passively because of the large mismatch in acoustic impedance between their compressible gaseous interiors and the bloodstream into which they are injected. The second mechanism is active as microbubbles act like a resonator by undergoing resonance phenomena including non-linear behavior. During use, microbubbles oscillate non-linearly in an ultrasound field and *may* generate harmonic components that appear at integer, for example, second-harmonic (*i.e.,* $2f_0$), and fractional, for example, super-harmonic (*i.e.,* $1.5f_0$) and subharmonic (*i.e.,* $f_0/2$), multiples of the fundamental driving frequency, f_0. This has given rise to a number of ultrasound contrast-specific imaging modes such as pulse inversion (Wilson and Burns 2001) and amplitude modulation (Brock-Fisher et al. 1996;

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Mor-Avi et al. 2001), which exploit non-linearities in the signals backscattered from microbubbles. Other harmonic imaging components such as the third-, fourth- and fifth-order superharmonics (Bouakaz et al. 2002) and subharmonics (Forsberg et al. 2000; Shi et al. 1999) have been similarly investigated. Although a number of therapeutic and ultrasound diagnostic imaging techniques have sought to exploit microbubble non-linear oscillations, considerable technical challenges remain. For example, whereas the use of second-harmonic emissions for imaging has been found to improve imaging sensitivity, diagnostic applications are limited by the fact that second-harmonic signals are also generated by non-linear tissue propagation (Krishnan et al. 1998), which degrades imaging performance (Goertz et al. 2005). However, investigations into subharmonic emissions have indicated that the ratio of subharmonic signals backscattered from microbubbles compared with that from tissue is stronger than the ratio of backscattered second harmonic signals. This suggests that blood perfused with microbubbles is more easily detectable with respect to tissue if the subharmonic rather than the second harmonic imaging modes are used (Shankar et al. 1998) and implies that subharmonic signals can be frequency discriminated, thus allowing for improvements in the ratio between the signal coming from the microbubbles and the unwanted signals coming from the surrounding tissue.

The potential use of subharmonic emissions has been illustrated in a number of acoustic and imaging studies. Chomas et al. (2002) reported that subharmonic imaging (SHI) with a transmission frequency twice the resonant frequency of the bubble produced subharmonic frequency responses while simultaneously minimizing bubble instability. Forsberg et al. (2006) used SHI to quantify *in vivo* perfusion and reported good agreement with a microvascular staining technique. Goertz et al. (2007) used SHI to identify atherosclerotic plaques in a rabbit aorta. Forsberg et al. (2007) and later Dahibawkar et al. (2015) compared quantifiable measures of tumor vascularity obtained from contrast-enhanced high- and low-frequency SHI and compared their findings against three immunohistochemical markers of angiogenesis in a murine breast cancer model. More recently, SHI was implemented for 3-D and 4-D imaging and exhibited an improvement in contrast compared with 3-D and 4-D harmonic imaging (Eisenbrey et al. 2012a, 2012b, 2015). Shi et al. (1999) found that subharmonic signals were sensitive to ambient pressure changes. This finding was later confirmed by Frinking et al. (2010), who argued that these subharmonic signals may be used for non-invasive *in vivo* detection of pressure changes. Finally, Dave et al. (2012) non-invasively monitored portal vein pressures and hypertensions using subharmonic-aided pressure estimations. Despite a plethora of applications, a detailed acoustic understanding of the mechanism(s) leading to subharmonic generation from contrast microbubbles has not been elucidated.

A number of research groups have examined the parameters leading to the induction and continued generation of subharmonic emissions from microbubbles. Katiyar and Sarkar (2011) reported that both the driving frequency and acoustic pressure are significant contributors to subharmonic scattering. It has been argued that a threshold acoustic pressure has to be reached for subharmonic emissions to emerge, and it has been theoretically and experimentally illustrated that this pressure remains minimal for non-encapsulated microbubbles insonified at twice their resonance frequency (Eller and Flynn 1969; Neppiras 1969; Prosperetti 1974), while the threshold for shell-encapsulated microbubbles remains low (Biagi et al. 2006; Lotsberg 1996). Indeed, theoretical analyses by Sijl et al. (2010) of the changes in microbubble shell elasticity, as proposed by the model of Marmottant et al. (2005), have broadly confirmed the requirement for a low threshold. In a broader context, Sijl et al. (2010) emphasized the importance of the bubble's initial surface tension and its role as a trigger of subharmonic emissions. Specifically, Sijl et al. (2010) observed that a bubble with a small initial surface tension, that is, close to the buckled state, exhibited a large subharmonic response, whereas no subharmonic response was observed for a bubble with an initial surface tension in the elastic regime. Through the use of ultrafast optical analysis, it was observed that microbubbles undergoing subharmonic emissions commonly exhibited "compression-only" behavior. This mechanism was reported to be more favorable for subharmonic generation at acoustic pressures (De Jong et al. 2007; Frinking et al. 2009) <50 kPa. In parallel investigations, Van Rooij et al. (2015) using microbubbles of differing phospholipid shell compositions found that dipalmitoylphosphatidylcholine, one of the most common microbubble shell components, influenced the onset of subharmonic emissions at acoustic pressures <100 kPa.

Although these studies have clearly improved our understanding of some of the factors involved in the triggering and maintenance of subharmonic emissions, very little is known about the effect of the gas inside the contrast microbubble. A recent study by Shekhar et al. (2014) examined the non-linear subharmonic and ultraharmonic emissions of the experimental microbubble agent Targestar-P and reported that both subharmonic and ultraharmonics emission from this agent exhibited a strong temporal dependence. These workers argued that the temporal changes in the non-linear behavior of the agent were mediated primarily by gas exchange through