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Ultrasonic Characteristics and Cellular Properties of Anabaena Gas Vesicles

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ABSTRACT

Ultrasound imaging is a common modality in clinical examination and biomedical research, but has not played a significant role in molecular imaging for the lack of an appropriate contrast agent. Recently, biogenic gas vesicles (GVs), naturally formed by cyanobacteria and halobacterium, have demonstrated great potential as an ultrasound molecular imaging probe with much smaller size (~100 nm) and improved imaging contrast. But the basic acoustic and biological properties of GVs remain unclear, which hinder future application. In the present paper, we studied the fundamental acoustic properties of rod shape gas vesicle from Anabaena, a kind of cyanobacteria, including attenuation, oscillation resonance, scattering as well as biological behaviors (cellular internalization and cytotoxicity). We found GVs have two resonance peaks (85 MHz and 120 MHz, respectively). We also demonstrate a significant non-linear effect and its pressure dependent ~~(threshold of 0.36 MPa)~~ property as well. Ultrasound B-mode imaging demonstrates sufficient echogenicity of GVs for ultrasound imaging enhancement in high frequency. Biological characterization also reveals endocytosis and non-toxicity.

KEYWORDS: Gas vesicle, Nanobubble, Ultrasound, Acoustic characterization, Resonance frequency, Scattering property, Contrast agent, Molecular imaging

INTRODUCTION

Ultrasound imaging is one of the most common imaging modalities both in clinical diagnosis and biological research due to its high spatiotemporal resolution, deep penetration and low cost (Shung 2015). With the adoption of gas-cored microbubbles, contrast enhanced ultrasound (CEUS) imaging can significantly increase the contrast at the region-of-interest and provide differentiable ultrasound signal, due to harmonic signal from oscillating bubble under sonication (Hoff 2001, Leighton 2012). CEUS imaging is widely used in diagnosing cardiovascular disease and vasculature related dysfunction with microbubbles administered within vessels (Bhatia and Senior 2008, Klibanov 2013, Partovi, et al. 2012). A recent study using microbubbles to map detailed brain vessels demonstrated superior quality with a super resolution strategy (Errico, et al. 2015). Linked with a targeting ligand, CEUS can detect dysfunctional endothelial cells and tumor angiogenesis with high sensitivity and specificity (Anderson, et al. 2011, Zhang, et al. 2015). In addition to diagnosis, microbubbles are also used for drug/gene delivery and blood brain barrier opening due to bubble oscillation-induced permeability enhancement of cell membrane and vessel wall (Geis, et al. 2012, Ting, et al. 2012, Yan, et al. 2013).

Microbubbles are small bubbles filled with gas and encapsulated by a shell of lipid, polymer or albumin with a diameter of several micrometers (De Jong, et al. 2009). They are mainly restricted within blood vessels due to the relatively large size, and are unable to penetrate endothelial cells, where only particles smaller than 400 nm can pass owning to the enhanced permeability and retention effect (EFR) (Anderson, et al. 2011, Maeda 2001, Yuan, et al. 1995). Previous studies have attempted to reduce bubble size to hundreds of nanometers, but the synthesized microbubbles seldom can survive for more than 30 minutes, which is not long enough for microbubbles to reach and accumulate in the target (Jokerst, et al. 2011, Tong, et al. 2013, Yang, et al. 2015, Yin, et al.

2012). ~~The stability of microbubble is determined by the pressure difference between inner and outside, which is theoretically inversely proportional to the bubble radius assuming the mechanical property of shell constant (De Jong, et al. 2009).~~

Therefore, to explore the extravascular cellular and molecular activity for diagnosis or therapeutic purpose, bubbles with nanometer size and stable structure are needed as the ultrasound contrast and therapeutic agent. Recently, gas vesicles (GVs), formed by cyanobacteria and halobaterium ~~as a mean to control buoyancy for optimal access to light and nutrients,~~ was reported as a nanometer size ultrasonic contrast agent and potential molecular imaging probe with advantage of good stability and high contrast (Shapiro, et al. 2014). The porous shell of the GV protein shell excludes water but permits gas to maintain a dynamic balance between internal and external pressure, which ensures that GV can last a very long time even to years (Buckland and Walsby 1971). Besides, it can be genetically engineered for better imaging quality and multiple imaging function (Lakshmanan, et al. 2016).

GVs produced by cyanobacteria, are normally rod shape (Figure 1); the narrowest diameter is about 100 nm, unlike conventional microbubbles which are spherical (Walsby 1994). The shell structure and material of GVs are naturally formed, composed of ~~a 2 nm bilayer protein molecule named~~ Gas Vesicle Protein A (GvpA) and Gas Vesicle Protein C (GvpC) (Walsby 1994). This combination forms uniform small pores which enable gas permeability, different from most of the microbubbles (EzzEldin and Solares 2009, Walsby 1994). Hence, the acoustic behavior and biological properties of GVs, especially from cyanobacteria, are expected to deviate substantially from those of microbubbles. In this regard, we designed unique experiments to investigate the interaction between GVs and ultrasound, and to understand the contrast origin and oscillation resonance. The results are expected to reveal the GVs acoustic properties and unique features,

which may help to identify potential utilities for ultrasound imaging and therapy. In addition, we explored the interaction between tumor cell and GVs, particularly the internalization and toxicity, which are important issues affecting the potential performance as a molecular imaging probe (Rampazzo, et al. 2013).

MATERIALS AND METHODS

GV isolation and characterization. Anabaena flos-aquae (FACHB-1255, Freshwater Algae Culture Collection, Wuhan, China) was cultured in sterile BG-11 medium at 25°C under fluorescence lighting with 14 hours/10 hours light/dark cycle. GVs were isolated according to Walsby's method (Buckland and Walsby 1971). Briefly, hypertonic lysis was used to release GVs by quickly adding sucrose solution to a final concentration of 25%. GVs were isolated by centrifugation at 400g for 3 hours after hypertonic lysis. The isolated GVs formed a white creamy layer on top of the solution was collected by syringe To purify the GVs solution, it was washed by the same centrifugation process 3 times and stored in PBS at 4 °C. GVs concentration was estimated by literature based formula (450 nM per OD_{500}) (Walsby 1994), where the value of OD_{500} means optical density at 500 nm wavelength measured by UV-Visible spectrophotometer (2100 pro, GE Healthcare Ltd, Piscataway, NJ, USA). Volume fraction was estimated using approximated gas volumes of 8.4 μl per mg and molar weight of 107 MDa based on literature information (Walsby and Armstrong 1979). GVs morphology was imaged using transmission electron microscopy (TEM) (JEOL 2100F, JEOL, Tokyo, Japan) operating at 200 kV. Gas vesicle samples in ID water (0.5 nM) were deposited on a carbon-coated grid and dried in room temperature overnight. Hydrodynamic size was obtained by dynamic light scattering method with zetasizer (ZetaPlus, Brookhaven Instruments Corp., NY, USA).

B-mode ultrasound imaging. Ultrasound B-mode image of GVs solution was acquired using a high frequency ultrasound system with a transducer of MS550D (Vevo2100, FUJIFILM VisualSonics, Toronto, ON, Canada). The center frequency and output energy level were set to 40 MHz and 1%, respectively. GVs were diluted in PBS and imaged in a container made of agar gel.

Attenuation characterization. In the attenuation characterization system, a single pulse with broad bandwidth was transmitted from pulser/receiver (5900 PR, Olympus, NDT, USA) and passed through 7.5 mm length GVs solution (0.25 nM) in Isoton solution (Isoton II; Coulter, Miami, FL, USA). Then the attenuated signal was reflected by quartz plane, passed GVs solution again and received by the same transducer. After that, attenuated signal from pure Isoton solution without GVs was acquired by the same process as reference. Attenuation coefficient of GVs was calculated by normalizing the GVs signal to the reference in power spectrum. To verify and confirm the result, totally 4 transducers (NIH Resource on Medical Ultrasonic Transducer Technology, University of Southern California, Los Angeles, CA, USA) at different center frequencies of 22 MHz, 40 MHz, 48 MHz and 60 MHz were well characterized covering the spectrum from 7 MHz to 150 MHz (-20dB), within which, the attenuation result is valid (De Jong, et al. 2009). During the attenuation test, stimulation acoustic pressure was kept at low pressure level (< 0.08 MPa) to avoid non-linear response. Besides the single pulse test, attenuation coefficient was also measured by tone burst at discrete frequencies. The testing system were modified from *Shi and Forsberg* (Shi and Forsberg 2000). Different from the first test, the transmit signal in this setup was 10 cycles pure tone burst at frequencies from 50 MHz to 120 MHz with 2 nm step. The acoustic pressure is lower than 0.1 MPa to avoid non-linear response.

Scattering characterization.

The system used for characterizing the frequency-dependent backscattering property of GVs was similar to the one for attenuation characterization. First, a single pulse with broad bandwidth was transmitted from pulser/receiver and scattered by GVs solution (0.25 nM) in Isoton solution. The backscattered signal then was amplified by low noise RF amplifier (AU1467, Miteq Inc., Hauppauge, NY, USA) and acquired by a digital oscilloscope (LeCroy 715Zi, LeCroy Corporation, Chestnut Ridge, NY, USA). Samples were filled within agar gel container positioned at the focal depth of the transducer. The peak acoustic pressure at the focal depth was characterized to be 0.08 MPa, which was kept at low level to avoid non-linear response. Backscattered signal from GVs were normalized against reference signal reflected from the agar gel-air interface of the empty container to eliminate the spectral features from the measurement system. 200 traces were recorded for averaging in power spectrum offline and smoothed with step of 3.

Harmonic scattering property was measured by pulse-echo measurement system modified from the method proposed by *Shi and Forsberg* (Shi and Forsberg 2000). A programmable function generator produced pulses (tone burst sinusoidal signal at 20 MHz, 40 MHz and 60 MHz of 30 cycles) at PRF of 100 Hz. The transmitted signals were first amplified in a broadband RF power amplifier (Model 500A250C, AR Inc., Souderton, PA, USA) and then supplied to a high frequency ultrasonic transducer to transmit ultrasonic pulse to GVs solution. Signals scattered from contrast GVs or linear scatterer (20 um tungsten fiber) as control group were sensed by a receiving transducer placed at a 90^{o} angle and then amplified by a low-noise RF amplifier. The amplified signals were acquired at a digital oscilloscope and transferred to a personal computer for frequency analysis. The transmitting and receiving transducer properties are listed in Table 1 by pulse-echo calibration. In both scattering and attenuation measurement, GVs were diluted in Isoton solution. To test the frequency dependence property of harmonic response, stimulation ultrasound signals

at 2 different frequencies (40 MHz and 60 MHz) were maintained at the same acoustic pressure level (0.65 MPa) characterized by hydrophone HGL-0200 (Onda Corporation, Sunnyvale, CA, USA). Acoustic pressure dependence property was tested by changing ultrasonic insonification pressure (0.3 MPa, 0.36 MPa, 0.44 MPa, 0.5 MPa, 0.58 MPa and 0.65 MPa), which had been calibrated using hydrophone before the experiment.

PpIX coating. For visualization of GVs using fluorescence microscopy, the shell of GVs was labeled with fluorescent dye, Protoporphyrin IX (PpIX, $C_{34}H_{34}N_4O_4$) (Sigma-Aldrich, St. Louis, MO, USA). The amine group at the shell protein was conjugated to the carboxyl group at the end of PpIX molecule using EDC/NHS (N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide/N-hydroxysulfosuccinimide).(Hermanson 2013) Briefly, EDC (40 mM) and sulfo-NHS (100 mM) were added to PpIX solution (4 mM) in 0.1 M sodium phosphate with pH 7.4. After 15 minutes at room temperature, the reaction solution was added to the GVs (10 nM) and incubated for 2 hours at room temperature. The excess PpIX and EDC/NHS was washed out by centrifugation and stored in PBS at 4 °C.

Cell culture. HeLa cells were cultured at 37 °C, 5% CO_2 in a humidified atmosphere. The HeLa cells were grown in a low-glucose Dulbecco's modified Eagle's medium (DMEM, 1.0 g/L glucose) containing 10% fetal bovine serum (FBS) and 1% antibiotic (100 μg/mL penicillin and 100 μg/mL streptomycin). All of the above chemicals are from Thermo Scientific Company (Waltham, MA, USA). The cells were maintained in the above medium and subcultured once every 4 days.

Cell endocytosis test. HeLa cells were first seeded in confocal dishes (200350, SPL Life Science company, Hong Kong, China) at a concentration of $3x10^4$ cells/dish. The PpIX-GVs were diluted in DMEM to a concentration of 0.5 nM. GVs solution of 200 μl was added to and incubated with

cells in confocal dish for overnight. Cells were washed with PBS 3 times and were observed under confocal microscopy (TCS SP8, Leica Corporation, Wetzlar, Germany) with 405 nm excitation light.

Cell viability. Cytotoxicity of GVs was evaluated in HeLa cell by an MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay. Cells were cultured in 96-well plates at a density of 5000 cells/well, and GVs were added to the wells at concentrations from 0 to 2.5 nM for overnight incubation. MTT assay was performed and measured at the wavelength of 570 nm using an Infinite 200 microplate reader (Tecan Inc., Maennedorf, Switzerland). Cytotoxicity test was conducted on both intact GVs and broken GVs. During the incubation, cell-GVs solution was kept shaking by thermomixer (Eppendorf Inc., Hamburg, Germany) to allow fully contact between GVs and cell. To test the toxicity of GVs' shell, the GVs were broken by ultrasound using ultrasonic cleaner (WUC-D06H, Labortechnik & Laborgeräte Inc., Germany) to release the inner gas before incubation. Incubation processes were all conducted in 37 ℃, 5% CO_2 condition.

RESULTS AND DISCUSSIONS

Gas Vesicle as ultrasound contrast agent

Gas vesicles (GVs) were isolated and purified from cyanobacteria (*Anabaena flos-aquae*). TEM image of a GV in Figure 1 shows a cylindrical structure with average length of 395 ± 85 nm and diameter of 98 ± 17 nm (n = 5). The size of the GVs was also measured by dynamic light scattering (DLS). Result from dynamic light scattering test (DLS) shows that the polydispersity index (PDI) of GVs is 0.09 and that almost no aggregation happens. Stability of GVs are confirmed in the

condition with and without ultrasound pulse, not showing obvious decrease for longer than 6 hours. Both the size, structure and stability of the gas vesicle is similar to the result from Shapiro's group, indicating biological consistence within the same cell line (Shapiro, et al. 2014).

A series of ultrasound images of GVs ~~solution~~ (Figure 2) were obtained by a high frequency (40 MHz) ultrasound imaging system by changing GVs concentrations from 50 pM to 800 pM. An increased ultrasound signal is observed at the lowest GVs concentration of 50 pM compared to pure PBS solution, showing a good echogenicity. GVs concentrations in this experiment are approximated to the gas volume fraction of 0.005% to 0.073%, representing the volume of gas in the solution. Compared to commercial microbubbles, these gas volume fractions are rather small ~~because the same volume can contain much more GVs than microbubbles due to the small GV size~~ (Kang, et al. 2015, Zhao, et al. 2014). In this regard, the echogenicity of GVs are very good considering the small volume fraction. Figure 2 shows a consistent increase of signal intensity with increased GVs concentration, which sets the ground for quantitative evaluation of GVs concentration and possible GVs targeted biomarker assessment by an ultrasound molecular imaging strategy. In contrast to Shapiro's result, here we demonstrated the imaging performance in higher frequency range around 40 MHz (Shapiro, et al. 2014).

When the GVs are irradiated with a burst of higher intensity ultrasound (~1 MPa), the signal suddenly drops to the baseline (pure PBS signal) suggesting that the GVs are broken at such ultrasound intensity, which confirms the results from *Shapiro et al* (Shapiro, et al. 2014). This experiment demonstrates a controllable and instantaneous elimination of gas from GVs, important for enhancing imaging contrast by signal difference before and after GVs rupture as well as for reperfusion measurement.

Attenuation and Resonance

Acoustic attenuation characterization is a method to investigate resonance which happens commonly in compressible material like bubble under sonication (De Jong, et al. 2009, Gao, et al. 2003). ~~Attenuation properties of GVs were measured by an experimental setup illustrated in Materials and Methods part~~. Figure 3A plots the signal waveforms acquired from GVs and Isoton solution, respectively. Attenuation coefficient was calculated by normalizing the spectrum of GVs attenuation signal to the Isoton reference signal and the result is shown in Figure 3B. Such a characteristic figure shows an intrinsic property of GVs independent of the transducers and testing systems.

From Figure 3B, the resonance frequency of GVs are found to be around 100 MHz, much higher than that of microbubbles (4-20 MHz) (De Jong, et al. 2009, Shi and Forsberg 2000). Nanoscale size and harder shell material are the major factors causing the significantly higher resonance frequency, because smaller size bubbles response to higher frequency (De Jong, et al. 2009). It is also noted from Figure 3B that two resonance peaks are found: the major one with higher amplitude at around 120 MHz and the minor one at 85 MHz. The appearance of two separated resonant peaks is very different from that of conventional microbubbles.

To further verify the attenuation result, we used pure tone burst at discrete frequencies to measure attenuation coefficient, the result of which is plotted in Figure 3C. A weak peak can be observed at the frequency of 90 MHz, that agrees with the result by single pulse test. Another noticeable peak appears at 120 MHz, but it is not complete because the transmitter frequency covers up to 120 MHz. The amplitude of first peak (90 MHz) is at the same level with the single pulse test, while the second peak is around 2 dB lower than the previous result. This difference may be caused by the biological variance of GVs from different generations. Generally, the results from both measurements agree upon each other, which reveals that the attenuation coefficient

measured by our setup is closed to the intrinsic acoustic behavior of a group of GVs, independent from the testing system.

Two peaks in attenuation coefficient may indicate two different oscillation modes in GVs. One possible reason which may account for the phenomenon is its two oscillation directions (transverse and longitudinal) caused by the rod shape structure. ~~One obvious benefit of this feature is the broadened ultrasound active frequency. Besides, this new property may bring new biomedical imaging and therapeutic application~~. Instantaneous resonance of contrast agent measured by attenuation is determined by both the individual resonance property of gas vesicle and their overall size distribution (De Jong, et al. 2009, Shi and Forsberg 2000). Therefore another possible explanation to this phenomenon is the group effect of GVs, instead of the physical property of single GV. ~~whether the two resonance peak is caused by intrinsic GV property or group effect of GVs cannot be answered from the result~~. ~~To get conclusive illustration to this interesting and novel finding needs more optical imaging results and theoretical simulation researches to individual gas vesicle.~~

Scattering Property ~~and non-linear~~

In addition to attenuation, scattering property of GVs is another important feature that affects ultrasonic imaging quality and reveals resonance frequency as well. Frequency-dependent backscattering of GVs, which is measured by low amplitude (<0.08 MPa) is plotted in Figure 4 as Apparent Backscatter Transfer Function. A dominant peak is observed at frequency around 125MHz, which falls within the major peak of attenuation coefficient. Another minor peak is also observed at around 85 MHz with lower amplitude. While, this minor peak is not as obvious as the one in attenuation result, which may be caused by low signal to noise ratio of backscattering test. Apparent backscatter transfer function confirms our findings from attenuation characterization that

GVs have two resonance frequency both in high frequency region: one around 85MHz, one around 120MHz.

Harmonic response is a scattering property of contrast agent that commonly happens when transmitter acoustic pressure is at high level. Figure 5 plots the spectra of scattered signal from GVs insonified by 0.65 MPa acoustic pressure at 40 MHz and 60 MHz frequency. The second harmonic components are observed at 80 MHz and 120 MHz from GVs insonified by 40MHz and 60 MHz respectively. Meanwhile, second harmonic amplitude from linear scatterer (tungsten fiber) is almost at the level of background noise. The amplitude of the second harmonic component under 40 MHz insonification is 10 dB higher than background noise~~, comparable to that of commercial microbubbles with protein shell such as Quantison® or Optison® (De Jong, et al. 1994, Frinking, et al. 1999, Goertz, et al. 2005, Shi and Forsberg 2000)~~. While, under 60 MHz insonification, the (2^{nd}) harmonic amplitude is 5 dB (compared than control group), relatively lower than 40 MHz insonification. The difference of second harmonic amplitude may be caused by the frequency response of the receiver transducer. Though 60 MHz is closer to the resonance, its second harmonic component (120 MHz) is out of the (-6 dB) bandwidth of receiver transducer (No 4 of Table 1), therefore the detected signal in this frequency region is underestimated. 3^{rd} harmonic component is observed in the spectrum under 40 MHz insonification, however the amplitude is relatively low.

Pressure dependence of harmonic response was characterized by shifting the insonification acoustic pressure from 0.3 MPa to 0.65 MPa (Figure 6). During experiment within this pressure range, almost no GVs ruptured. ~~As shown in Figure 5, a threshold of 0.36 MPa is observed for the onset of the second harmonic component, indicating the requirement of a minimum ultrasound intensity to generate the non-linear effect.~~ In our experiment setup, the minimum acoustic pressure

to induce second harmonic signal is 0.36 MPa corresponded to a mechanical index of 0.057, far below the FDA required safety threshold of 1.9. A consistent increase of second harmonic intensity plotted in Figure 6D demonstrates a pressure dependent effect~~, which may also facilitate the improvement of imaging resolution, since only the focal area with acoustic pressure beyond the threshold can induce a second harmonic signal.~~ The acoustic pressure dependent non-linear property well explains the higher second harmonic intensity from our experiment than that of Shapiro's result, as the acoustic pressure used in their setup is 98 kPa lower than the threshold (Shapiro, et al. 2014). Finally, this non-linear effect lasts for over 15 minutes under continuous ultrasound irradiation, suggesting long-time imaging ability of GVs.

Biological Properties

To know the cellular bioeffect of contrast agent is important for optimizing imaging strategy and therapeutic efficiency. A lot of research has been done regarding the interaction of microbubble and endothelial cells or microphages, because the conventional application of microbubble are restricted within blood vessels (Bioley, et al. 2012, Yanagisawa, et al. 2007). Because of the nanometer size and superior stability, GVs are potentially suitable for molecular imaging and treatment to tumor cells outside vessels. However our knowledge about the interaction between bubble and tumor cells lacks. Therefore, we conducted research on the interaction between GVs and tumor cells, particularly the internalization and cytotoxicity.

Because of their small size and transparent air core, GVs are scarcely visible under fluorescence microscopy. A fluorescence dye (PpIX) was coated on the shell of GVs illustrated in Figure 7A&B (PpIX-GV) showing a distinct red color under fluorescence microscopy. To test the cellular uptake of PpIX-GV, they were added to Hela cells and incubated for 12 hours. To overcome the buoyance

effect of GVs, we released the inner gas before incubation with cell culture. Figure 7C shows a picture of such HeLa cells acquired by confocal microscopy. The red dots within cell bodies indicated by white arrows demonstrate that GVs are internalized and stay within the cell cytoplasm. The internalization of GVs may be attributed to its nanoscale size, since the same property has been widely reported for gold nanorods and carbon nanotubes with similar diameters (Salem, et al. 2003). In addition to the size, the cylindrical shape and protein shell may also contribute to cellular internalization (Chithrani and Chan 2007, Chithrani, et al. 2006). This study demonstrats that GV is able to be internalized by mammalian tumor cells without any active targeting. This property may benefit ultrasound molecular imaging by increasing the functional resolution from the tissue to the cellular/subcellular level. Therapeutic applications may also improve due to the consequentially increased accuracy.

In addition, MTT based cytotoxicity tests were conducted on both intact GVs and broken GVs. The former one is to test the overall bioeffect (toxicity) to the cells, while the later one aims to test the toxicity of shell material. ~~GVs were added to and incubated with Hela cells for 12 hours at a concentration up to 2.5 nM, 50 times that used for contrast enhanced imaging~~. From Figure 7D cell viability is not influenced by broken GVs even in the highest concentration (2.5 nM). While there is slight decrease of the viability caused by intact GVs at the concentration higher than 0.313nM, though it is not obvious. The results indicate that the protein shell is relative biocompatible to cell not causing any toxicity. The toxicity induced by intact GVs may be caused by the bioeffect after internalization, possibly by mechanical effect of buoyancy force of GVs. ~~plots The MTT results as a function of GVs concentration, and shows a steady response of cell viability without obvious toxicity.~~ Overall, the cellular biocompatibility is superior to the

conventional lipid bubbles (Yang, et al. 2015). Such low toxicity is important for future *in vivo* application and translation to clinical usage.

CONCLUSIONS

In the present study, acoustic and biological characterizations of GVs with the interaction of ultrasound and cells respectively were carried out, and ~~unique~~ properties distinct from microbubbles, were identified. GVs have adequate echogenicity as a suitable contrast agent for ultrasound imaging enhancement, and the echogenicity is correlated to GVs concentration for potential quantitative assessment of GVs targeted biomarker. GVs also demonstrate a significant non-linear harmonic effect, important for background reduction and image quality improvement. The harmonic property is pressure dependent~~ and the onset threshold is observed at 0.36 MPa acoustic pressure~~. Attenuation measurement reveals two separated resonance peaks (120 MHz and 85 MHz) indicating oscillation behavior different from conventional microbubbles. ~~This unique feature may facilitate future development of new imaging and therapeutic strategies~~. In terms of cellular behavior, GVs without targeting ligand can be internalized by tumor cells, demonstrating that GVs could be a potential intracellular molecular reporter or therapeutic agent, without obvious cytotoxicity.

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LIST OF CAPTIONS

Fig. 1. TEM image of a single GV isolated from Anabeana.

Fig. 2. B-mode image and echo intensity of GVs solution at concentration of 50-800 pM. (Center frequency: 40 MHz)

Fig. 3. Attenuation characterization result of GVs. (A) Transmitted echo signals from GVs and Isoton solution without GV as reference, respectively. (B) Attenuation coefficient in power spectrum resulted from single pulse test (tested by four different transducers marked by color), the transmitting signal iof which is single pulse broad bandwidth signal, 100 Hz PRF, acoustic pressure < 0.08 MPa. (C) Attenuation coefficient in power spectrum resulted from tone burst test at discrete frequencies from 50MHz to 120 MHz with 2 MHz step size (10 cycle, acoustic pressure < 0.1 MPa, 100 Hz PRF). (GVs concentration is 0.25 nM).

Fig. 4. Apparent backscatter transfer function plotted versus frequency for GVs solution. (Concentration is 2.5 nM in scattering characterization, PRF of 100 Hz, acoustic pressure < 0.08 MPa). N=3

Fig. 5. Spectra of the scattered signals of GVs and 20 μm tungsten fiber insonified at frequency of 40 MHz (A) and 60 MHz (B). These spectra were not corrected for the frequency response of the receiving transducer. The experiment for each spectrum was carried out using GVs in Isoton

solution with a concentration of 0.5 nM. Acoustic pulses with an amplitude of 0.65 MPa and a length of 30 cycles were transmitted at a PRF of 100 Hz.

Fig. 6. Spectra of the scattered signals of GVs insonified at acoustic pressure of 0.36 MPa (A), 0.5 MPa (B) and 0.58 MPa (C) for 40 MHz insonification. D, Second harmonic amplitude as the function of acoustic pressure for 40 MHz insonification. The experiment for each spectrum was carried out using GVs in Isoton solution with a concentration of 0.5 nM. Acoustic pulses with a center frequency of 40 MHz and a length of 30 cycles were transmitted at a PRF of 100 Hz.

Fig. 7. Biological characterization in cell culture. (A) Schematic illustration of fluorescence dye coated GV. (B) Superimposed fluorescence and bright field images of PpIX-GVs using confocal microscopy. (C) Superimposed fluorescence and bright field image of HeLa cells incubated with PpIX-GVs (red dot) using confocal microscopy, arrows representing internalized PpIX-GVs. (D) Cell viability as the function of concentration of intact GVs and broken GVs after overnight (12 hours) incubation (n = 3). Scale bar =10 μm.

TABLE

Table 1 Information of transducers in attenuation and scattering characterization.

NO.	Center frequency (MHz)	Bandwidth (-6dB) %	-20 dB region (MHz)
1	22	81.82	7-32
2	40	39.02	21-59
3	48	45.83	30-103
4	60	83	25-150