Published in final edited form as:

Ultrasound Med Biol. 2018 December ; 44(12): 2728–2738. doi:10.1016/j.ultrasmedbio.2018.08.003.

“Optimization of Phase-Change Contrast Agents for Targeting MDA-MB-231 Breast Cancer Cells”

Kyle P. Hadinger^a, Joseph P. Marshalek^a, Paul S. Sheeran^{b,c}, Paul A. Dayton^d, and Terry O. Matsunaga^{a,*}

^aDepartment of Medical Imaging, University of Arizona, Tucson, AZ, USA

^bPhysical Sciences Department, Sunnybrook Research Institute, Toronto, ON, Canada

^cDepartment of Medical Biophysics, University of Toronto, Toronto, ON, Canada

^dJoint Department of Biomedical Engineering, The University of North Carolina and North Carolina State University, Chapel Hill, NC, USA

Abstract

Breast cancer remains a leading cause of death for women throughout the world. Recent advances in medical imaging technologies and tumor targeting agents signify vast potential for progress toward improved management of this global problem. Phase-change contrast agents (PCCAs) are a dynamic imaging agent with practical applications in both the research and clinical settings. PCCAs possess characteristics that allow for cellular uptake where they can be converted from liquid phase PCCAs to gaseous microbubbles via ultrasound energy. Previously, we reported successful internalization of folate-targeted PCCAs in MDA-MB-231 breast cancer cells followed by ultrasound-mediated activation to produce internalized microbubbles. This study examines the binding, internalization, and activation of folate-receptor targeted PCCAs within MDA-MB-231 breast cancer cells as a function of gaseous core compositions, incubation time, and ultrasound exposure period. *In vitro* results indicate that internalization and ultrasound-mediated activation of PCCAs was significantly greater using a 50:50 mixture of decafluorobutane: dodecafluoropentane compared to other core compositions- 50:50 octafluoropropane: decafluorobutane ($p < 0.0001$), decafluorobutane ($p < 0.04$) and dodecafluoropentane ($p < 0.0001$). Furthermore, it was shown that PCCAs composed of perfluorocarbons with higher boiling points responded with greater activation efficiency when exposed to 12 seconds of ultrasound exposure as opposed to 4 seconds of ultrasound exposure. When evaluating different incubation times, it was found that incubating the PCCAs with breast cancer cells for 60 minutes did not produce significantly greater internalization and activation compared to 10 minutes; this was concluded from comparing the number of microbubbles present per cell prior to ultrasound versus post-ultrasound, and finding a ratio of intracellular MB Post-US/Pre-US, 3.46 vs. 3.14, respectively. The data collected in this

*Corresponding author. Tmatsunaga@radiology.arizona.edu, 520-626-6689.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

study helps demonstrate further optimization of folate-receptor targeted PCCAs for breast cancer targeting and imaging.

Keywords

Phase-Change Contrast Agents; Nanodroplets; Microbubbles; Targeting Agent; Vaporization; Internalization; Ultrasound-Mediated Activation

Introduction

Breast cancer is the second most common type of cancer for women in the United States both in terms of incidence and cause of death. Furthermore, it is the leading cause of premature mortality from cancer in women as measured by total years of life lost (Smith et al. 2015). It is evident that advancements in breast cancer diagnosis and treatment are needed.

Several approaches to drug delivery and/or molecular imaging in certain modalities have utilized the enhanced permeability and retention (EPR) effect of “leaky” solid tumor vasculature (Mulder et al. 2006). Utilization of the EPR effect results in gradual accumulation of particles on the order of several hundred nanometers into the extravascular space by passive diffusion (Mulder et al. 2006). PCCAs, which are liquid targeting agents ranging from 100 – 750nm in size (Sheeran et al. 2012), offer stability and the ability to conjugate with different biomarkers for cell specific delivery. The current target for this study, the folate receptor, has been shown to be overexpressed in ovarian, colorectal, and breast cancers (Wang et al. 2008). Furthermore, data suggests that the folate receptor is upregulated in approximately 40% of all cancers (Low and Kularatne 2009) and this overexpression can be seen in malignant tumor cells (Luo et al. 2017). Consequently, it may be a useful biomarker for targeting imaging agents and/or therapeutics as they have the potential to bind and enter these cells via receptor-mediated endocytosis (Luo et al. 2017). Therefore, strategies to take advantage of extravasation and cell-specific targeting may be advantageous.

Ultrasound is an inexpensive, portable, noninvasive and nonionizing modality that is used clinically to produce anatomic images from backscattered acoustic waves (Wilson et al. 2013), and has emerging clinical utility in molecular imaging and drug delivery. The water content of tissue is relatively uniform across a wide range of tissue types resulting in little variation in their acoustic impedance, oftentimes resulting in poor image contrast (Wilson et al. 2013). Microbubbles consist of a gas core encapsulated by a lipid coating. They are highly echogenic and scatter ultrasound at the frequencies used in medical diagnostic imaging with great efficiency. This is due to the differences in acoustic impedance, a function of density and compressibility, of the gas core and the surrounding blood pool or tissues. When they encounter a propagating ultrasound wave, microbubbles are forced to undergo radial oscillation, such oscillations significantly increase the efficiency with which they scatter ultrasound and is the basis for their use as contrast agents. Addition of a ligand to the microbubble formulation that is selective for an up-regulated receptor provides the basis for targeting. However, microbubbles are generally restricted to the intravascular space

due to size (typically 1 – 5 microns) (Sheeran 2011). The size of a microbubble must allow them to travel freely through capillary beds, but their size range restricts them from extravasating through vessels into tissue, and so they serve primarily as intravascular imaging agents. Given their size restrictions, microbubbles targeting to regions inside of cells, tissues, and tumors becomes problematic. Additionally, microbubble circulation *in vivo* is short, with most formulations measured to be 10 minutes or less due to gas exchange in the lungs (Sirsi, Borden 2009). Sonazoid formulations have demonstrated half-lives as long as 30 to 45 minutes (Landmark 2008), however the microbubbles formulated in this study were similar to DEFINITY + 2mol% folate.

Phase-change contrast agents (PCCAs) have been developed to extend the utility of the microbubble by avoiding the size limitations and offering increased stability. Recent work has expanded the range of PCCA formulations by utilizing more volatile compounds. Sheeran and coworkers demonstrated fabrication of stable micron and sub-micron PCCAs, and modulated the vaporization pressures by altering the ratios of the perfluorocarbon gases used (2011). By exposing pre-formed perfluorocarbon microbubbles to decreased ambient temperature and increased ambient pressure, a condensation of the perfluorocarbon core occurs and the microbubble changes phase to a liquid nanodroplet (Sheeran et al. 2012). The increase in PCCA stability likely arises from not surpassing a homogenous nucleation energy barrier (Mountford and Borden 2016) as well as the condensation of gas resulting in an increased Laplace pressure, thereby stabilizing the nanodroplets in the liquid state with effective boiling points significantly higher than the bulk perfluorocarbon alone (Sheeran 2012; Rapoport 2012). PCCAs have a longer half-life than microbubbles due to the fact that perfluorocarbon gases in water have a low solubility and their small size assists in their avoidance of elimination via respiration, resulting in increased circulation time compared to gas bubbles (Kripfgans et al. 2000).

PCCAs can be produced by a variety of methods, including the previously mentioned condensation of microbubbles containing highly volatile gases into their liquid phase (Sheeran et al. 2017). Furthermore, PCCAs are sufficiently small to theoretically facilitate extravasation into tumor tissue where they can be vaporized back into microbubbles for ultrasound imaging. This is desirable because liquid nanoscale agents do not provide as sufficient an ultrasound contrast as compared to their gaseous counterparts (Couture et al. 2006). The conversion from liquid-phase nanodroplets to gaseous microbubbles provides a gas-liquid interface which offers a much larger impedance mismatch providing more contrast for imaging. When vaporizing nanodroplets to microbubbles, increased ultrasound reflectively comes from the greater impedance mismatch being provided by the gas, as well as the particle's 5–6 fold diameter increase (consistent with the ideal gas law prediction of liquid-to-gas volume expansion). Given the prospect of circulating initially as a nanoscale droplet and targeting agent prior to vaporization, PCCAs may provide an ideal solution to detect breast cancer noninvasively.

PCCAs decorated with folate ligands could be useful for targeting the cell surface receptor as well as promoting intracellular uptake (Marshalek et al. 2016). MDA-MB-231 breast cancer cells are an example of cells capable of folate-receptor mediated endocytosis (Cao et al. 2015). In these studies, PCCAs were designed to undergo ultrasound-mediated phase

change back into gaseous microbubbles after being taken up by MDA-MB-231 cells, hence making them effective for contrast-enhanced ultrasound. To visualize both the phase transition of the PCCAs and the microbubble contrast, confocal microscopy was used in these experiments to provide optical contrast to the stained MDA-MB-231 cells and contrast agents both before and after application of ultrasound. Assessment of PCCA activation prior to ultrasound was important for noting spontaneous PCCA activation, which may be caused by several factors including thermal effects (determined by the boiling point of the gas within the particle).

Marshalek et al. have studied PCCA accumulation in MDA-MB-231 and MCF-7 cells *in vitro*. In these experiments, folate-targeted PCCAs were incubated for one hour followed by an ultrasound activation pulse to vaporize the droplets that had bound and endocytosed within the breast cancer cells. The vaporization was successfully conducted using clinically utilized ultrasound levels. These studies demonstrated that folate-targeted PCCAs internalized within MDA-MB-231 cells 45x more frequently than untargeted PCCAs (2016).

Although the Marshalek study provided proof-of-principle, clearly a greater understanding of the dependence of internalization on droplet composition, ultrasound energy and exposure, and time, are critical for advancing this technology towards preclinical and clinical evaluation and translation. The work presented here examines the optimization of PCCAs for uptake and activation within MDA-MB-231 cells. Efficiency is measured as a function of 1) varying perfluorocarbon cores to determine the role of core boiling point, 2) number of ultrasound scans used to activate PCCAs, and 3) length of time incubating PCCAs in the presence of cells prior to activation.

Materials and Methods

Microbubble Preparation

Microbubbles were prepared using 1,2-dipalmitoyl-sn-glycerophosphatidylcholine (DPPC), 1,2-dipalmitoyl-sn-glycerophosphatidic acid (DPPA), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-((polyethylene glycol-2000)folate) (ammonium salt), a folate-lipid bioconjugate (Avanti Polar Lipids Inc, Alabaster, AL). The folate conjugate was added at a concentration of 2 mol % relative to the total lipid emulsion. The lipids and bioconjugate ligand were first dissolved in propylene glycol followed by dispersion with phosphate-buffered saline (PBS) and glycerol at a ratio of 16:3:1(v:v:v). The total lipid concentration of the formulation was 1 mg/mL. Following formulation of the folate-decorated lipid membrane, 1.5 mL of the colloid dispersion was added to a 2 mL vial (Wheaton Industries, Millville, NJ) sealed and crimped. The headspace of the vial was then purged with 50 mL of the designated perfluorocarbon gas or gas mixture by inserting the gauge of a needle through the vial septum and a second needle through the vial septum to serve as an outflow, and gas was purged at a constant rate. The lipid membrane of the PCCAs was fluorescently labeled by injecting 2 μ L of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, 1 mL/L, Invitrogen Molecular Probes, Eugene, Or, λ_{ex} 549 nm, λ_{em} 565 nm) into the vial.

Four different gases and gas mixtures were purged into the headspace of the vial. The gases tested were- 50:50 decafluorobutane (DFB, C₄F₁₀): octafluoropropane (OFP, C₃F₈), DFB

alone, 50:50 DFB: dodecafluoropentane (DDFP, C_5F_{12}), and DDFP alone. The boiling point of each gas is shown in Table 1.

The colloidal dispersions were agitated for 45 seconds in a modified dental amalgamator (VIALMIX™, Lantheus, New York, New York) at 4,000 RPM. The perfluorocarbon gas core of the microbubbles was then condensed to the corresponding nanodroplets by initially cooling the microbubbles in a NaCl/ice bath (-7°C to -10°C) followed by applying air pressure (85–130 psi) to the vial of microbubbles. Figure 1 shows the transition from a single vial of microbubbles (1–5 μm) that appear to be a creamy solution to nanodroplets (100–750 nm) which appear clearer as these are liquid-phase agents. The nanodroplets are roughly 5–6 times smaller than the original microbubbles. The condensation of the microbubbles to nanodroplets reduces the total volume of the solution. The size distribution of nanodroplets mirrors the size range of their corresponding microbubbles as would be expected when those microbubbles were condensed by our method. We have demonstrated previously that nanodroplets produced by condensation of microbubbles produce microbubbles of similar size to that contained in the precursor microbubble distribution (Sheeran et al. 2011), a finding which was also confirmed with intracellularly detected microbubbles following ultrasound application (Marshalek et. al. 2016).

The nanodroplets then remained in the NaCl/ice bath (-7°C to -10°C) for 15 minutes to promote nanodroplet formation prior to their addition to the cell culture.

Cell Preparation:

One to two days prior to PCCA formulation, poly d-lysine coated dishes (MatTek Corp., Ashland, Ma) were seeded with MDA-MB-231 cells along with 5 mL RPMI-1640 (Roswell Park Memorial Institute-1640) mixed with 10% fetal bovine serum and 1% antibiotics (100 $\mu\text{g}/\text{mL}$ penicillin-streptomycin). The MDA-MB-231 cells were seeded at a concentration of 2.0×10^6 cells/mL and were grown to roughly 50% confluency. Not reaching complete confluency was done purposely as it allowed for greater differentiation between individual cells or cell clusters which was needed to quantify the number of microbubbles per cell. Following PCCA formulation, the cell culture dish was washed with phosphate-buffered saline (PBS) for approximately 30 seconds. The PBS was aspirated and then 5 mL of folate-free RPMI was added to the cell culture dish. Next, 5 μL of calcein AM ($\lambda_{\text{ex}} = 495 \text{ nm}$ $\lambda_{\text{em}} = 516 \text{ nm}$, Life Technologies Molecular Probes, Eugene, OR) was added to the solution to stain living cells. Calcein AM is able to demonstrate cell viability and assists in visualization of microbubbles localized within cells, which appear as a “void” in the cytoplasm.

One-hundred μL (2 vol %) of the targeted PCCAs were then dispensed onto the cell culture dish. PCCAs in this size range are denser than the aqueous environment and naturally sink and settle against the plated MDA-MB-231 cells. PCCAs were incubated with the cells at 37°C for 10 minutes or one hour.

Confocal Imaging and Ultrasound Exposure:

Following incubation, the media was aspirated and the cells were washed three times with PBS in order to remove any unbound cells and unbound PCCAs. The cells were then

prepared for confocal imaging using a Leica SP5 confocal microscope (AZCC Cancer Imaging Shared Resources). The environmental chamber surrounding the confocal microscope was equilibrated to 37° C. Z-stack images were collected with a 63x oil-immersion objective. The Z-stack images were taken from 10–11 planes, approximately one micron apart from each other in the z-direction. This produced 10–11 images over the entire diameter of the cell (approximately 10 microns), such that the entire three-dimensional structure of the cell could be assessed for microbubbles. Cells were imaged before applying ultrasound to assess any thermally-induced vaporization of nanodroplets. Pre-ultrasound imaging was performed three times and on different locations of the culture dishes in order to provide representative information about the cells. Imaging locations on the culture dish were selected at random to avoid biasing our results. To achieve acoustic activation of the PCCAs, the transducer of a Zonare portable ultrasound machine (ZONARE Medical Systems, Mountain View, CA) was immersed in the culture media and was initially scanned across the culture dish four times in two directions orthogonal to each other at a rate of 0.67cm² per second; each scan taking approximately four seconds. For later experiments, the number of scans was increased to 12. For all experiments, the Zonare L14–5sp linear transducer was used and all ultrasound parameters remained consistent; mechanical index= 1.0, ultrasound frequency= 9.0 MHz, dynamic range= 70.0, gain= 70.0, field of view= 26.0 mm, and pulse repetition frequency= 13.0 Hz.

Similar to the pre-ultrasound optical imaging, post-ultrasound optical imaging was carried out 3 times and on different locations of the culture dish. Cells treated with 4 repeated ultrasound scans were compared against those that underwent 12 repeated ultrasound scans in order to observe any possible changes post-ultrasound from added exposure. Following ultrasound application and microscopy, images were analyzed using the LAS AF software (Leica Microsystems, Buffalo Grove, IL) to assess PCCA internalization and acoustic activation.

As stated, for each culture dish tested (N=23), images were collected from three random sections pre-ultrasound and three sections post-ultrasound. To verify the presence of an intact intracellular microbubble, three sets of co-registered criteria were used; 1) a circulate DiI signal (red, greater than 600 nm) that co-registered with either; 2) a microbubble found in the transmitted light channel with an intact lipid shell, and/or; 3) a void in the green channel (calcein). In order to conclude that a microbubble was present intracellularly, and not extracellularly, the microbubble had to be entirely inside of the cell, and not bound on the cell border or surface, in all three channels as confirmed by Z-stack imaging. In initial experiments, all four gaseous mixtures were evaluated in order to determine the percentage of microbubbles per cell both Pre- and Post- US as well the ratio of intracellular microbubbles Pre- to Post-US, as shown in Table 2. This helped determine which PCCAs performed optimally under initial conditions. In each random region of interest that was analyzed, the average number of microbubbles/cell was calculated for the entire group of cells in the image, regardless of whether any microbubbles were present within individual cells as seen in Tables 3 – 5. Further statistical analysis was performed to quantify the average number of microbubbles/cell when only considering cells with at least one microbubble (Table 6). From this data, t-tests were performed to determine significance.

Results

As shown in Figure 2, Channel A indicates the calcein channel demonstrating cell viability. Channel B represents the DiI channel; a visualization of the lipid membranes of the microbubbles via the fluorescent channel imaging. Channel C is the transmitted light channel (Brightfield) that is capable of showing the cytoplasm and lipid membrane layer of the microbubbles. In Figure 2, note the single cell with five microbubbles present in one cross-sectional plane of the cell. Microbubbles ranged in size from just under one micron to a maximum of 5 microns. The microbubbles in Figure 2 are seen in all channels, thereby verifying their presence.

Figure 3 displays all three channels presented within single planes during Z-stack imaging. With Z-stack acquisition, imaging planes were captured at 1 micron intervals throughout the depth of the cell. 10–11 separate planes were assessed in each cell as this allowed for analysis of the entire thickness of most cells imaged. When passing through different planes at 1 micron intervals, it is clear the microbubbles are visible through the depth of the cell and through the appropriate number of planes. The number of planes in which a microbubble was detectable was dependent on its size. Furthermore, Z-stack imaging occurred at the same depth in all three channels, confirming co-localization. This imaging technique allowed us to see as many microbubbles within each individual cell as possible, and demonstrated how microbubbles with larger diameters were seen in an increased number of image planes. Analyzing the entire cell provided confirmation that PCCAs were endocytosed by the cells and were responsive to ultrasound-stimulated vaporization.

Table 2 displays the results from these early phase experiments (N=14) which showed that DFB (2.43) and DFB:DDFP 50:50 (3.29) exhibited the largest ratios of ultrasound activation, as determined by the ratio of intracellular microbubbles post-ultrasound / pre-ultrasound. These microbubble compositions also appeared at the highest percentage within cells post-ultrasound (DFB 66%, DFB:DDFP 50:50 70%). Note that DDFP showed the lowest percentage of spontaneous PCCA activation (35%) and the lowest percentage after ultrasound activation (62%). This is likely due to the higher stability of DDFP compared to its counterparts. The least stable formulation, OFP:DFB 50:50 produced the highest percentage of spontaneous PCCA vaporization, as determined by the percentage of cells with microbubbles pre-ultrasound (44%).

Table 3 demonstrates how the increase in the number of scans led to greater activation of DFB:DDFP 50:50 PCCAs and thus a greater number of microbubbles, but results differed for the DFB PCCAs as increased scans showed a lower presence of DFB microbubbles. These results led to further testing of DFB:DDFP 50:50 PCCAs.

Prior to the experiments displayed in Table 4, all PCCAs had been incubated for 1 hour. Table 4 shows the differences in spontaneous activation and ultrasound-mediated activation for DFB:DDFP 50:50 PCCAs when receiving 1 hour versus 10 minutes of incubation. Results show that there is less Pre-US (pre-ultrasound) spontaneous activation with shorter incubation time, but this shortened time also displayed a lower presence of microbubbles within cells post-ultrasound. However, the difference between ratios of intracellular

microbubbles Post-US (post-ultrasound) versus Pre-US was not significant between 10 and 60 minutes, suggesting that a shorter incubation time may be sufficient to maintain a similar Post to Pre-US microbubble formulation when compared with the longer duration of incubation.

The summarized results displayed in Table 5 appear to indicate that DFB:DDFP 50:50 PCCAs show the highest activation of microbubbles within MDA-MB-231 cells. Furthermore, increasing the number of scans using the ultrasound transducer seems to assist in greater activation of these PCCAs.

Table 6 is able to show that with only 4 scans of ultrasound, 50:50 DFB:DDFP activated microbubbles appeared more abundantly in cells at a significantly greater rate than its counterparts post-ultrasound. Although greater ultrasound exposure led to greater 50:50 DFB:DDFP MB/cell, it was not significant.

Discussion

It has been demonstrated that the folate receptor is overexpressed in breast cancer (Wang et al. 2008) and overexpression of the folate receptor alpha is implicated with increased uptake of folate and increased breast cancer cell development and growth (Marshalek 2016; Necela 2015). Previous studies verified that binding folate to PCCAs increases the frequency of intracellular microbubble observation within MDA-MB-231 breast cancer cells by a factor of roughly 45 compared to untargeted PCCAs (Marshalek et al. 2016). Given these findings, this study presents data that suggests how to better optimize PCCA formulations and experimental setup for detection of MDA-MB-231 breast cancer cells *in vitro*.

The objective of the study was to assess conditions for optimizing PCCA intracellular delivery and activation as a function of incubation time, number of ultrasound scans to induce activation, and PCCA condensed perfluorocarbon ratio. However, we were also aware that PCCAs are subject to thermal activation, which is predicted to increase with decreasing boiling point of the condensed perfluorocarbon (OFP, DFB, DDFP). Thus, one of the study goals was aimed at minimizing the number of microbubbles appearing within the cells resulting from thermal methods prior to ultrasound activation. We also avoided the possibility of creating competitive inhibition of folate receptors through the use of folate-free cell culture media. Another technique to avoid biased results was the use of calcein AM dye, which confirmed cell viability by only staining the cytoplasm of living cells.

Of the four initial PCCAs examined, DFB and 50:50 DFB:DDFP exhibited the greatest amount of ultrasound-mediated activation. It has been shown that nanodroplet design can have a dramatic effect on nanodroplet persistence *in vitro*, and that choice of perfluorocarbon will directly impact the *in vitro* half-life of the nanodroplets, which will lead to better or worse performance in internalization aims (Sheeran et al. 2018). Given this, results in these studies may have occurred in this fashion as the 50:50 DFB:DDFP PCCAs offered a stability that allowed them to maintain their liquid phase for a period of time that could yield high amounts of cellular internalization. For the experiments involving DFB PCCAs, 41% of cells showed evidence of microbubbles prior to ultrasound with an average

of 0.66 microbubbles per cell. Following ultrasound application, the number of cells that contained intracellular microbubbles increased to 66% and the number of microbubbles per cell increased to 1.61 when considering all cells evaluated versus 2.43 MB/cell when considering only cells with at least one microbubble. PCCAs consisting of 50:50 DFB:DDFP showed only 37% of cells containing microbubbles pre-ultrasound, with an average of 0.58 microbubbles per cell. Following ultrasound, 70% of cells showed evidence of microbubbles with an average of 1.91 microbubbles per cell when considering all cells evaluated vs. 2.73 MB/cell when considering only cells with microbubbles present intracellularly. DFB and 50:50 DFB:DDFP were tested further, using 12 scans instead of 4. 50:50 DFB:DDFP appeared significantly more than all other microbubbles tested, but DFB was also evaluated to serve as a comparison.

DFB PCCAs resulted in fewer MB/cell after 12 scans of ultrasound exposure versus four (1.61 microbubble/cell post-US after 4 scans vs 1.19 microbubbles/cell after 12 scans). This may reflect ultrasound induced disruption of some of the activated microbubbles following their formation. This is not surprising as Sheeran and coworkers, using ultra high speed videos, noted that their droplets would vaporize and subsequently fragment and dissolve as a function of higher mechanical indices (2011). However, with 50:50 DFB:DDFP, different results prevailed. With only four scans there was an average of 1.91 microbubbles per cell post-ultrasound, while with 12 scans there was an average of 2.35 microbubbles per cell post-ultrasound. It is not well established how different gas compositions affect cavitation in the setting of in vitro cellular imaging. Thus, it is difficult to conclude why increased ultrasound exposure decreased the number of DFB microbubbles observed intracellularly whereas it increased the number of 50:50 DFB:DDFP microbubbles. Studies by Chomas and coworkers have shown that smaller microbubbles are more likely to fragment when subject to acoustic pulsation (2001) and perhaps the greater vaporization threshold provided by DDFP, versus DFB alone, allows the microbubbles to expand larger in size versus quickly vaporizing. Studies by Dayton and coworkers have shown that application of appropriate acoustic intensity will drive droplet internalization and binding (Dayton et al. 2006). Perhaps DDFP offers greater resistance to ultrasound-induced disruption and instead uses the ultrasound energy to drive greater cellular binding. Another significant statistic was observed with the fraction of cells containing intracellular 50:50 DFB:DDFP microbubbles post-ultrasound as it increased to 82% from 70% when using 12 scans versus 4 scans, respectively. Applying 12 scans of the Zonare transducer to the 50:50 DFB:DDFP PCCAs also showed the highest ratio of microbubbles per cell post-ultrasound / pre-ultrasound at 3.46. The fact that these PCCAs showed the highest activation ratio most likely comes from their optimized stability as they avoid vaporization prior to ultrasound, are sensitive to acoustic vaporization, and are stable enough to resist cavitation. It is noted that for all experiments, the ultrasound frequency was set to 9.0 MHz, a mechanical index of 1.0, dynamic range of 70.0, gain of 70.0, field of view of 26.0 mm, and a pulse repetition frequency of 13.0 Hz. Maintaining consistent parameters assisted in ruling out any outside variables that may have contributed to intracellular uptake and ultrasound mediated activation of PCCAs. With the optimized stability of these 50:50 DFB:DDFP PCCAs, it is possible that more pulsing may have further enhanced acoustic droplet vaporization, while still being fairly resistant to fragmentation. Further experimentation is needed to confirm this

hypothesis. It is noted that these experiments were performed manually, so scanning time may not have always been exactly the same in each experiment. Consequently, discrepancies could occur secondary to changes in the scanning rate (which is a possibility), and thus all of the PCCAs may not have been exposed to the exact same amount of pulses. However, numerous experiments were performed (N=23) so as to ensure reproducibility and the transducer pulse repetition frequency output remained constant across all experiments.

Given the above data, more testing was performed on the 50:50 DFB:DDFP PCCAs. The last variable was comparing different incubation times of the 50:50 DFB:DDFP PCCAs- one hour versus ten minutes. This difference in incubation time study aimed to maximize PCCA uptake and activation using the same ultrasound parameters as used in previous experiments (mechanical index remained at 1.0). The PCCAs that received ten minutes incubation combined with 12 scans from the ultrasound transducer (instead of four) based upon the observation that the 50:50 DFB:DDFP PCCAs that received 12 scans produced superior results (Table 4). With ten minutes of incubation time, prior to ultrasound, only 31% of the cells contained microbubbles with an average of 0.42 microbubbles per cell. These pre-ultrasound numbers are lower than the numbers seen with one hour incubation of the 50:50 DFB:DDFP PCCAs which showed 41% of cells with microbubbles and an average of 0.68 microbubbles per cell. This trend continued post-ultrasound. The PCCAs that were incubated for ten minutes were able to internalize and vaporize into microbubbles post-ultrasound in 64% of cells with an average of 1.32 microbubbles per cell. When incubated for one hour, 82% of cells showed evidence of microbubbles post-ultrasound with an average of 2.35 microbubbles per cell.

From the above results it became evident that, of the formulations tested and with the ultrasound parameters used, the 50:50 DFB:DDFP PCCAs appeared optimal for targeting and internalizing within MDA-MB-231 breast cancer cells. This conclusion comes from several factors. First, it exhibited the least amount of thermal activation prior to ultrasound application. The only formulation to show lower spontaneous PCCA activation was DDFP PCCAs, but these PCCAs did not exhibit as much ultrasound activation as the 50:50 DFB:DDFP PCCAs. This is likely due to their increased vaporization threshold. In other words, the 50:50 DFB:DDFP PCCAs provided enough stability to show low amounts of spontaneous activation, which allowed these PCCAs to stay in droplet form giving them more time to internalize within the cells, as well as still show significant activation upon acoustic pulsing. Secondly, upon ultrasound activation, these PCCAs showed the highest percentage of cells with microbubbles and also exhibited the highest number of microbubbles per cell compared to the other PCCAs, regardless of whether the cells were scanned 4 or 12 times. These experiments were performed *in vitro*, and results may differ when insonating through tissues *in vivo* due to greater attenuation of the ultrasound signal.

Finally, shorter incubation time presented interesting results which indicated decreased numbers of microbubbles found intracellularly in all categories. The decreased incubation time led to less cells with at least one microbubble both pre- and post-US and less MB/cell both pre- and post-US. When considering these outcomes, it is necessary to weigh the drawbacks of each method: lower overall microbubble presence (shorter incubation time) versus greater spontaneous activation pre-US (longer incubation time). This result may be

viewed in two ways when translating to the clinical setting: 1) If these PCCAs are being used as an imaging agent only, ten minutes of incubation may be the better option as less contrast would be seen prior to ultrasound application thereby not causing possible erroneous non-specific targeting; 2) In contrast, the one hour incubation showed more microbubbles per cell following ultrasound application. Thus, longer incubation times could be employed for therapeutic applications, where microbubble cavitation within the cell may be more desirable with the potential for intracellular drug delivery and directed intracellular apoptosis (Matsunaga et al. 2012).

Conclusion

This study was able to demonstrate internalization and ultrasound-mediated activation of targeted PCCAs within MDA-MB-231 breast cancer cells. We evaluated four different perfluorocarbon gas combinations within the microbubble formulation and determined the rates of spontaneous activation as well as the differences in responses to ultrasound mediated activation between each formulation. Furthermore, this study also evaluated PCCA response to greater ultrasound exposure and it was found that greater activation from increased exposure may be dependent on the formulation of the PCCA. This study also demonstrates the differences seen when applying differing amounts of incubation time. Ten minute incubations as well as one hour incubations of the targeted PCCAs with MDA-MB-231 breast cancer cells both proved to be sufficient time for PCCAs to internalize within the cells.

Acknowledgments

This work was supported by National Institutes of Health grant no. CA185684 (to T.O.M). The authors also acknowledge the use of the confocal microscope operated by the Tissue Acquisition and Cellular/Molecular Analysis Shared Resource (TACMASR) facility supported by the University of Arizona Cancer Center Support Grant, NIH CA023074. Lastly, the authors acknowledge Professor Arthur Gmitro, Dr. Andrew Rouse, and Pier Ingram for their insightful technical support.

References

- Cao W, Zheng W, Chen T Ruthenium Polypyridyl Complex Inhibits Growth and Metastasis of Breast Cancer Cells by Suppressing FAK signaling with Enhancement of TRAIL-induced Apoptosis. *Scientific Reports* 2015;5:9157. [PubMed: 25778692]
- Chomas JE, Dayton PA, May DJ, Ferrara KW Threshold of fragmentation for ultrasonic contrast agents. *JBO* 2001;6:141–151.
- Couture O, Bevan PD, Cherin E, Cheung K, Burns PN, Foster FS A model for reflectivity enhancement due to surface bound submicrometer particles. *Ultrasound Med Biol* 2006;32:1247–1255. [PubMed: 16875958]
- Dayton PA, Zhao S, Bloch SH, Schumann P, Penrose K, Matsunaga TO, Zutshi R, Doinikov A, Ferrara KW Application of Ultrasound to Selectively Localize Nanodroplets for Targeted Imaging and Therapy. *Mol Imaging* 2006;5:160–174. [PubMed: 16954031]
- Kripfgans OD, Fowlkes JB, Miller DL, Eldevik OP, Carson PL Acoustic droplet vaporization for therapeutic and diagnostic applications. *Ultrasound Med Biol* 2000;26:1177–1189. [PubMed: 11053753]
- Landmark KE, Johansen PW, Johnson JA, Johansen B, Uran S, Skotland T Pharmacokinetics of perfluorobutane following intravenous bolus injection and continuous infusion of sonazoid in healthy volunteers and in patients with reduced pulmonary diffusing capacity. *Ultrasound Med Biol* 2008 34(3): 494–501. [PubMed: 18096304]

- Low PS, Kularatne SA Folate-targeted therapeutic and imaging agents for cancer. *Curr Opin Chem Biol* 2009;13:256–262. [PubMed: 19419901]
- Luo W, Wen G, Yang L, Tang J, Wang J, Wang J, Zhang S, Zhang L, Ma F, Xiao L, Wang Y, Li Y Dual-targeted and pH-sensitive Doxorubicin Prodrug-Microbubble Complex with Ultrasound for Tumor Treatment. *Theranostics* 2017;7:452–465. [PubMed: 28255342]
- Marshalek JP, Sheeran PS, Ingram P, Dayton PA, Witte RS, Matsunaga TO Intracellular delivery and ultrasonic activation of folate receptor-targeted phase-change contrast agents in breast cancer cells in vitro. *J Control Release* 2016;243:69–77. [PubMed: 27686582]
- Matsunaga TO, Sheeran PS, Luo S, Streeter JE, Mullin LB, Banerjee B, Dayton PA Phase-Change Nanoparticles Using Highly Volatile Perfluorocarbons: Toward a Platform for Extravascular Ultrasound Imaging. *Theranostics* 2012;2:1185–1198. [PubMed: 23382775]
- Mountford PA, Borden MA On the thermodynamics and kinetics of superheated fluorocarbon phase-change agents. *Advances in Colloid and Interface Science* 2016;237:15–27. [PubMed: 27574721]
- Mulder WJM, Strijkers GJ, van Tilborg GAF, Griffioen AW, Nicolay K Lipid-based nanoparticles for contrast-enhanced MRI and molecular imaging. *NMR Biomed* 2006;19:142–164. [PubMed: 16450332]
- Necela BM, Crozier JA, Andorfer CA, Lewis-Tuffin L, Kachergus JM, Geiger XJ, Kalari KR, Serie DJ, Sun Z, Moreno-Aspitia A, Aspita AM, O'Shannessy DJ, Maltzman JD, McCullough AE, Pockaj BA, Cunliffe HE, Ballman KV, Thompson EA, Perez EA Folate receptor- α (FOLR1) expression and function in triple negative tumors. *PLOS ONE* 2015;10:e0122209. [PubMed: 25816016]
- Rapoport N Phase-shift, stimuli-responsive perfluorocarbon nanodroplets for drug delivery to cancer. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2012;4:492–510. [PubMed: 22730185]
- Sheeran PS, Streeter JE, Mullin LB, Matsunaga TO, Dayton PA. Toward ultrasound molecular imaging with phase-change contrast agents: an in vitro proof of principle. *Ultrasound Med Biol* 2013;39:893–902. [PubMed: 23453380]
- Sheeran PS, Matsuura N, Borden MA, Williams R, Matsunaga TO, Burns PN, Dayton PA Methods of Generating Submicrometer Phase-Shift Perfluorocarbon Droplets for Applications in Medical Ultrasonography. *IEEE Trans Ultrason Ferroelectr Freq Control* 2017;64:252–263. [PubMed: 27775902]
- Sheeran PS, Luo S, Mullin LB, Matsunaga TO, Dayton PA Design of ultrasonically-activatable nanoparticles using low boiling point perfluorocarbons. *Biomaterials* 2012;33:3262–3269. [PubMed: 22289265]
- Sheeran PS, Luo S, Dayton PA, Matsunaga TO Formulation and Acoustic Studies of a New Phase-Shift Agent for Diagnostic and Therapeutic Ultrasound. *Langmuir* 2011;27:10412–10420. [PubMed: 21744860]
- Sheeran PS, Wong VP, Luo S, McFarland RJ, Ross WD, Feingold S, Matsunaga TO, Dayton PA Decafluorobutane as a phase-change contrast agent for low-energy extravascular ultrasonic imaging. *Ultrasound Med Biol* 2011;37:1518–1530. [PubMed: 21775049]
- Sheeran PS, Yoo K, Walker WR, Williams R, Tremblay-Darveau C, Burns PN Impact of encapsulation on in vitro and in vivo performance of volatile nanoscale phase-shift perfluorocarbon droplets. *Ultrasound Med Biol* 2018; 8:1836–1852.
- Sirsi S, Borden M Microbubble Compositions, Properties and Biomedical Applications. *Bubble Sci Eng Technol* 2009;1:3–17. [PubMed: 20574549]
- Smith RA, Manassaram-Baptiste D, Brooks D, Doroshenko M, Fedewa S, Saslow D, Brawley OW, Wender R Cancer screening in the United States, 2015: a review of current American cancer society guidelines and current issues in cancer screening. *CA Cancer J Clin* 2015;65:30–54. [PubMed: 25581023]
- Unger EC, Porter T, Culp W, Labell R, Matsunaga TO, Zutshi R Therapeutic applications of lipid-coated microbubbles. *Adv Drug Deliv. Rev* 2004;56:1291–1314. [PubMed: 15109770]
- Wang ZJ, Boddington S, Wendland M, Meier R, Corot C, Daldrup-Link H MR imaging of ovarian tumors using folate-receptor-targeted contrast agents. *Pediatr Radiol* 2008;38:529–537. [PubMed: 18357444]

- Wilson KE, Wang TY, Willmann JK Acoustic and photoacoustic molecular imaging of cancer. *J Nucl Med* 2013;54:1851–1854. [PubMed: 24187042]
- Ye P, Zhang W, Yang T, Lu Y, Lu M, Gai Y, Ma X, Xiang G Folate receptor-targeted liposomes enhanced the antitumor potency of imatinib through the combination of active targeting and molecular targeting. *Int J Nanomedicine* 2014;9:2167–2178. [PubMed: 24855354]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.
Condensation of microbubbles (left) into nanodroplets (right).

Fig. 2.
Post-ultrasound image shows intracellular activated microbubbles at a variety of sizes.

Fig. 3.
Z-stacking imaging of microbubbles within a cell when passing through multiple planes.
Each plane in this image had a width of 1 μm .

Table 1.

Displays the boiling point of each gas evaluated throughout all experimentation.

Gas	Boiling Point (C)
Dodecafluoropentane (DDFP)	29°
Decafluorobutane (DFB)	−1°
Octafluoropropane (OFP)	−37°

Table 2.

Summary of the results from the initial experiments $\pm$ SEM (standard error of mean) scans with Zonare transducer, 31 hour incubation, N=14).

Type of PCCA	OFP : DFB 50:50	DFB	DFB : DDFP 50:50	DDFP
% of cells with MB Pre-US	44% $\pm$ 6.9% (169/380 cells)	41% $\pm$ 5.2% (116/282 cells)	37% $\pm$ 1.4% (113/358 cells)	35% $\pm$ 1.9% (58/164 cells)
% of cells with MB Post-US	65% $\pm$ 3.6% (275/420 cells)	66% $\pm$ 6.1% (205/311 cells)	70% $\pm$ 1.9% (276/393 cells)	62% $\pm$ 0.7% (94/152 cells)
Ratio of intracellular MB Post-US/Pre-US	1.95	2.43	3.29	2.21

Table 3.

Comparison of PCCA vaporization following more scans with the Zonare transducer $\pm$ SEM (N=6 for the PCCAs receiving 12 scans). Note that MB/cell considers the total number of microbubbles from all cells in an image divided by the total number of cells in that image.

Type of PCCA	DFB 4 scans (N=4)	DFB 12 scans (N=3)	DFB : DDFP 50:50 4 scans (N=4)	DFB : DDFP 50:50 12 scans (N=3)
% of cells with MB Post-US	66% $\pm$ 6.1% (205/311 cells)	62% $\pm$ 0.6% (148/238 cells)	70% $\pm$ 1.9% (276/393 cells)	82% $\pm$ 0.6% (152/187 cells)
MB/cell Pre-US	0.66 $\pm$ 0.49	0.72 $\pm$ 0.55	0.58 $\pm$ 0.40	0.68 $\pm$ 0.49
MB/cell Post-US	1.61 $\pm$ 0.78	1.19 $\pm$ 0.66	1.91 $\pm$ 0.81	2.35 $\pm$ 0.79
Ratio of intracellular MB Post- US/Pre-US	2.43	1.65	3.29	3.46

Table 4.

PCCA vaporization both pre and post-ultrasound with 1 hour versus 10 minute incubation $\pm$ SEM (N=3 for both 1 hour incubation and 10 minute incubation).

PCCA/Method	DFB : DDFP 50:50 1 hour incubation, 12 scans (N=3)	DFB : DDFP 50:50 10 minute incubation, 12 scans (N=3)
% of cells with MB Pre-US	41% $\pm$ 1.1% (111/272 cells)	31% $\pm$ 1.3% (97/311 cells)
% of cells with MB Post-US	82% $\pm$ 0.6% (153/187 cells)	64% $\pm$ 1.5% (140/218 cells)
MB/cell Pre-US	0.68 $\pm$ 0.49	0.42 $\pm$ 0.35
MB/cell Post-US	2.35 $\pm$ 0.79	1.32 $\pm$ 0.61
Ratio of intracellular MB Post-US/Pre-US	3.46	3.14

Table 5:

Statistics for all different types of PCCAs and parameters evaluated $\pm$ SEM. Note that the mean MB/cell in this table is considering all cells evaluated, whether a microbubble was present or not post-ultrasound.

Gaseous core	# of Scans	Incubation Time	Mean MB/cell Pre-US	Mean MB/cell Post-US
DFB:OFP 50:50	4	1 hour	0.61 ± 0.37	1.19 ± 0.51
DDFP	4	1 hour	0.58 ± 0.45	1.28 ± 0.66
DFB	4	1 hour	0.66 ± 0.49	1.61 ± 0.78
DFB	12	1 hour	0.72 ± 0.55	1.19 ± 0.66
DFB:DDFP 50:50	4	1 hour	0.58 ± 0.40	1.91 ± 0.81
DFB:DDFP 50:50	12	1 hour	0.68 ± 0.49	2.35 ± 0.79
DFB:DDFP 50:50	12	10 minutes	0.42 ± 0.35	1.32 ± 0.61

Table 6:

Statistics derived from two sample t-tests assuming unequal variances. The mean MB/cell and p values in this table are considering only cells that had at least one microbubble present post-ultrasound. Using this analysis criteria, there was an average of 2.73 50:50 DFB:DDFP MB/cell post-ultrasound (4 scans).

Gaseous core	MB/cell post ultrasound (only considering cells with at least one microbubble)	P values when compared to 50:50 DFB:DDFP (4 scans).
50:50 OFF:DFB	1.81	<0.0001
DFB	2.43	0.039
DDFP	2.07	<0.0001
50:50 DFB:DDFP (12scans)	2.85	0.406