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Manipulating Nanoscale Features on the Surface of Dye-Loaded Microbubbles to Increase their Ultrasound-Modulated Fluorescence Output

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Abstract

The nanoscale surface features of lipid-coated microbubbles can dramatically affect how the lipids interact with one another as the microbubble diameter expands and contracts under the influence of ultrasound. During microbubble manufacturing, the different lipid shell species naturally partition forming concentrated lipid islands. In this study the dynamics of how these nanoscale islands accommodate the expansion of the microbubbles are monitored by measuring the fluorescence intensity changes that occur as self-quenching lipophilic dye molecules embedded in the lipid layer change their distance from one another. It was found that when the dye molecules were concentrated in islands, less than 5% of the microbubbles displayed measurable fluorescence intensity modulation indicating the islands were not able to expand sufficiently for the dye molecules to separate from one another. When the microbubbles were heated and cooled rapidly through the lipid transition temperature the islands were melted creating an even distribution of dye about the surface. This resulted in over 50% of the microbubbles displaying the fluorescence-modulated signal indicating that the dye molecules could now separate sufficiently to change their self-quenching efficiency. The separation of the surface lipids in these different formations has significant implications for microbubble development as ultrasound and optical contrast agents.

Keywords

Fluorescence; Microbubble; Nanostructures; Surfaces; Ultrasound

1. Introduction

Microbubbles of perfluorocarbon gas have been used for many years as contrast agents to allow ultrasound to visualize the vasculature.[1, 2] The difference in compressibility between the gas of the microbubble and the surrounding water allows the microbubble to radially oscillate in size when exposed to the compression and rarefaction portions of an ultrasound pulse.[3–6]. This compressibility difference enables populations of microbubbles to be highly reflective to ultrasound.[1, 7] A common stable formulation for the gas used in contrast agent microbubbles consists of an air/perfluorocarbon mixture.[8] The perfluorocarbon gas has low solubility in water and helps stabilize the microbubble more effectively than pure air or nitrogen.[8, 9]

The small radius of curvature of the gas microbubble results in a high water surface tension that creates a net inward radial force increasing the pressure on the microbubble.[10] This higher-than-ambient pressure on the microbubble accelerates the gas dissolution into the water and can rapidly shrink the microbubble. This surface tension can be greatly reduced by coating the gas surface with a monolayer of lipids resulting in a significant microbubble lifetime increase.[11, 12] The type and ratio of lipids in this monolayer can have dramatic impacts on the flexibility and fluidity of the monolayer as a whole.[13] When exposed to ultrasound, the surface area of the microbubble undergoes expansion and contraction. The lipid shell is compliant to microbubble gas expansion, resulting in expansion of the monolayer.[10] In this expanded state, the distance between individual lipid molecules increases. If self-quenching lipophilic dye molecules are embedded in the lipid monolayer they too should separate during microbubble expansion. When separated, the dye molecules can emit a greater number of fluorescent photons than in the compression phase where the dye molecules come close together increasing their ability to self-quench. This would create an intensity modulated (or “blinking”) fluorescence signal which is modulated at the frequency of the applied ultrasound energy as illustrated in Figure 1.

Recently our group has demonstrated this fluorescence blinking effect in dye-loaded microbubbles exposed to ultrasound.[14] A self-quenching lipophilic dye [15–17] was incorporated into the lipid monolayer. Specialized detection systems were developed to successfully monitor this fluorescent blinking effect in-vitro.[14, 18] As shown in Benchimol et al., [14] this modulated fluorescence signal was directly correlated with the microbubble’s ultrasound-induced expansion and contraction, as monitored by side-scatter of the excitation light. The intensity peaks of the modulated fluorescence signal were observed to correspond in time with microbubble expansion in the rarefaction phase, when the fluorescent dye molecules in the shell were further apart. Congruently, the intensity minima of the fluorescence signal were seen to correspond with microbubble compression and the closer proximity of the self-quenching dye molecules in the shell. Only a small subpopulation of microbubbles from each batch were observed to produce fluorescence

modulations. It was hypothesized that the nanoscale lipid partitioning on the surface of the microbubble might be affecting the microbubble blinking ability.

The work described here looks to study the effect of lipid partitioning on the ability of lipids to separate from one another during ultrasound-induced microbubble expansion. The typical manufacturing processes for lipid-coated microbubbles involve the use of a probe sonicator or rapid shaker which simultaneously pushes the air/perfluorocarbon gas mixture into the water phase to create bubbles while breaking up lipid micelles in the water and allowing them to recondense onto the gas/water interface of the microbubble.[19] The probe sonicator has the additional effect of raising the temperature of the fabrication solution which then slowly cools to room temperature after the sonicator power is removed. The different lipids and lipophilic dyes used to create the monolayer have been shown to naturally aggregate into islands of various configurations when allowed to slowly cool to room temperature.[11, 20] There is considerable interest in determining how these variations in lipid partitioning affect the dynamics of the surface as the lipid surface accommodates the expanding and contracting size changes of the microbubble in response to ultrasound. Kwan and Borden have studied the lipid shell mechanics during bubble growth and dissolution due to gas exchange which occurs on a much slower time scale than the individual ultrasound-induced expansion and contraction events.[21]

By monitoring the intensity of the fluorescence-modulated signal produced by self-quenching dye-loaded microbubbles, the real-time ultrasound driven expansion and contraction of these lipid structures can be monitored. Understanding which lipid species and structures are involved in allowing microbubble expansion is important information for applications that involve manipulating the surface properties of the microbubble through crosslinking or other methods to control the ultrasound reflectivity properties.

In this work different lipid surface structures were created by applying various temperature treatment regimens to the fluorophore surface-loaded microbubbles. The nanoscale surface structure of these bubbles was investigated using 3D structured illumination super-resolution microscopy (3D-SIM). An acousto-fluorescence detection system was used to understand how the observed structures expanded and contracted in response to the applied ultrasound through analysis of the acquired fluorescence and scattered-light signals.[14, 18]

Increasing the blinking signal incidence in a population of microbubbles can have important medical applications, particularly in medical imaging diagnostics. Generating a characteristically blinking light inside the body has the potential to overcome many of the challenges currently facing optical imaging in deep tissue. Current methods of optical analysis of tissue involve illuminating the body surface with light to allow the photons to penetrate into a region of interest and then detecting the photons that scatter their way back to the tissue surface. The photons are highly scattered by the tissue making their actual paths unknown for depths greater than 2–3 mm.[22] This makes it difficult to optically probe a specific tissue region of interest because it is unknown if detected photons actually passed through that region. There is also a large background of detected photons that did not pass through the region of interest.

Ultrasound can be focused to small, millimeter-scale volumes within the body [23] to interact with these dye-loaded microbubbles to produce a localized blinking signal. This signal would act as a beacon of light at a known location within the deep tissue. Detecting the blinking signal at the body surface would allow information to be gained about the optical properties of the specific deep tissue region in the ultrasound focal zone. Blinking will only occur in the focal zone of the ultrasound allowing the spatial location of this modulated signal to be precisely known from within deep tissue. The excitation light that is applied at the body surface can be filtered out at the detectors to allow collection of only the fluorescent photons. Lock-in amplification electronics can be used to remove the non-blinking fluorescent background generated from microbubbles not in the ultrasound focal zone by locking on to the blinking frequency and measuring only the blinking signal. The frequency of blinking will match that of the driving ultrasound. This approach can be used to overcome the spatial resolution limitations of optical imaging in deep tissue due to strong optical scattering. Use of this hybrid technique has many valuable clinical applications including the detection of tumor-associated biochemical information from suspected lesions to improve cancer diagnosis accuracy. An imaging system utilizing this ultrasound-modulated fluorescent microbubble probe would enable the extraction of optical biochemical information with millimeter-scale resolution in highly scattering environments.

2. Methods and Materials

2.1 Materials

Distearoyl phosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and distearoyl phosphatidylethanolamine-methyl poly(ethylene glycol) MW5000 (mPEG-DSPE 5k) was purchased from Laysan Bio, Inc. (Arab, AL). Dulbecco's phosphate buffered saline (DPBS) was purchased from Hyclone Laboratories Inc. (Logan, UT). The lipophilic carbocyanine dye, DiI, was purchased from Biotium, Inc. (Hayward, CA). Perfluorohexane (PFH) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Microbubble Manufacturing

50 μ l of 20 mg/ml DSPC in chloroform, 20 μ l of 50 mg/ml mPEG-DSPE 5k in chloroform, and 30 μ l of 1 mM DiI in chloroform were added successively to a 4 ml glass vial under vortex to ensure proper mixing. DiI was added at a ratio of 2 mol %, which was previously shown to be an effective fluorophore loading concentration to achieve fluorescence modulation at diagnostic ultrasound pressures.[14] The chloroform was evaporated under an argon or nitrogen stream while mixing on the vortexer to make a thin film of lipid along the inner surface of the vial. 500 μ l of DPBS was added to the vial and the lipids were resuspended by vortexing for 15 seconds with subsequent heating at 75°C for 1 minute. This cycle of vortexing and heating was repeated until the lipids were well suspended and no residue was left on the vial walls. The sample was allowed to cool to room temperature.

The vial top was covered with parafilm to create a gas barrier to the external atmosphere. 1 ml of PFH was drawn into a 5 ml syringe with a 22 gauge needle. The syringe was rotated to coat the walls with liquid PFH several times and encourage vaporization. The syringe was allowed to sit for at least 3 minutes and the PFH vapor was allowed to come to equilibrium

with the liquid PFH and atmospheric gases in the syringe at atmospheric pressure. The needle of the syringe was bent at 130° into a hook configuration and inserted through the parafilm into the vial headspace. The syringe was then pumped 65 times into the vial headspace while being careful to prevent any liquid PFH from entering the vial. This saturated the air in the vial headspace with a PFH/air mixture. The tip of the XL-2000 probe sonicator (QSonica LLC., Newtown, CT) was immediately inserted through the parafilm and positioned 1 mm below the gas/water interface. The probe sonicator was then turned on at 25 W for 3 seconds. The resulting microbubbles were allowed to sit for at least 5 minutes before further processing.

The above mixture of PFH vapor and air was used in the fabrication process in order to incorporate PFH vapor inside the bubble at a concentration that stabilized the bubbles at atmospheric pressure and gas composition. Microbubbles fabricated without PFH would experience more rapid gas dissolution and collapse.[8, 9] Pure PFH exists as a liquid at room temperature and thus cannot be used as the sole gas component of the microbubble. The microbubbles in this study were fabricated and stabilized at atmospheric pressure and gas content as these were the conditions the bubbles were exposed to during the course of the study.

Excess lipid and dye in the sonicated solution was washed from the microbubbles. The microbubbles were transferred to a microcentrifuge tube and centrifuged at 1000 rpm for 3 min to float the bubbles. The supernatant was partially removed and replaced with DPBS. This process was repeated 1–2 additional times.

Microbubbles were imaged using conventional brightfield and fluorescence microscopy using a Nikon upright microscope fitted with a high numerical aperture objective (Nikon 60× oil immersion NA=1.4).

2.3 Microbubble Temperature Treatment

The washed microbubble samples were temperature treated according to the following five temperature regimens which were adapted from Borden et al.[20] Heating was performed at 75°C which is above the 56°C lipid transition temperature of the DSPC shell component.

1. “Rapid-cooling”: 3 minutes of heating at 75°C, followed by 5 minutes of cooling at 0°C. Rapid is used in reference to the rate of degrees/min at which the lipids pass through the transition temperature.
2. “Slow-cooling”: 3 minutes of heating at 75°C, followed by 5 minutes of cooling at 45°C.
3. “Rapid-cooling, short intervals”: 1 minute of heating at 75°C, followed by 1 minute of cooling at 0°C.
4. “Slow-cooling, short intervals”: 1 minute of heating at 75°C, followed by 1 minute of cooling at 45°C.
5. “No treatment”: washed microbubbles were kept at room temperature.

To achieve these temperature regimens the microbubbles were drawn into an 18 inch long 20 gauge metal needle and the end of the needle was then blocked by insertion into a rubber stopper. The needle was fully immersed into water baths at the indicated temperatures and durations described above, with an immediate full-immersion transition between the different temperature baths. The treated microbubbles were then transferred into microcentrifuge tubes.

2.4 3D Structured illumination super-resolution microscopy (3D-SIM)

Microbubble nanostructures were visualized using a 3D structured illumination super-resolution microscopy (3D-SIM) technique on the OMX system (Applied Precision Inc., Issaquah, WA) with a 100× oil immersion objective. Microbubbles were immobilized in glycerol immediately prior to imaging to avoid motion artifacts during image acquisition.

2.5 Acousto-fluorescence detection system and signal acquisition

Fluorescence and scattered-light modulation signals were acquired from insonated microbubbles using a previously described in-house designed acousto-fluorescence detection system.[14, 18] Briefly, a 532 nm laser, 2.25 MHz US transducer and an optical signal collection objective were aligned mutually orthogonally and confocally to form a water bath interaction zone. The field of view of the optical signal collection objective was approximately 2 mm in diameter at the site of the interaction zone. The 2.25 MHz ultrasound was pulsed at a 1 Hz repetition rate with 15 sine wave cycles per pulse and a pressure of 1.0 MPa. The 2.25 MHz frequency is within the resonance frequency range of the 1–5 μm microbubbles. The ultrasound was focused to an approximately 1 mm^3 voxel, creating the region of microbubble size modulation. A syringe pump was used to deliver a dilute solution of microbubbles in a thin fluid stream into the interaction zone to simultaneously interact with both the ultrasound pressure wave and excitation laser. To load microbubble samples into the system, bubbles were loaded into a 60 ml syringe pre-filled with water and mixed thoroughly to obtain sufficiently dilute solutions to achieve approximately 1 bubble per signal detection. To detect fluorescence, the light collected from the objective was collimated and passed through a series of wavelength interference filters to filter out the scattered light, detecting only fluorescence emission. The only difference between the setup used here and the setup used in Benchimol et al.[14] is that only one photomultiplier tube (PMT) was used to collect both fluorescence and scatter signals. Scattered light oscillations were obtained by removal of the fluorescence filters from the optical path. All the microbubbles were observed to scatter light but only a fraction of those actually displayed measurable fluorescence intensity modulation. The flow rate of microbubbles through the system was kept constant over the entire monitoring period. Signals were acquired from the PMT using a LabVIEW acquisition program which generated both time and frequency domain data. Each microbubble sample was monitored by acquiring signals during insonation in the scattered-light detection configuration for a period of about 5 minutes, yielding 300 signals. Analyzing these 300 signals for modulations using the analysis program described in section 2.6 established the detection rate of microbubbles for a given sample. Previous work established that microbubbles passing through the system consistently produced detectable side scattering signals.[14] The syringe pump was kept running and an additional 300 signals were collected immediately afterward

with the fluorescence filters inserted into the system. The number of detected fluorescence modulations was compared to the number of detected scattered light modulations for the same sample on the same day to compute the fluorescence modulation percentage for that specific sample.

2.6 Acousto-fluorescence signal analysis program

A custom program was developed in LabVIEW 8.2 to systematically analyze acquired signals for the presence of fluorescence and scattered-light modulations at the applied ultrasound frequency. The program determined the presence of a modulation in a collected signal by sampling the magnitude of the acquired Fourier transform at the 2.25 MHz frequency and comparing it with the background noise level of the signal, excluding very low frequency noise. A signal detection threshold level of 1.25 times the background level was determined and validated by testing on control microbubbles prepared with no fluorescent dye which did not generate ultrasound-modulated fluorescence signals. At this detection threshold level, no false positive signals were generated by the analysis program and this threshold was used to evaluate all microbubble treatments for incidence of modulation signals. By applying this threshold, the number of microbubbles that scattered the excitation laser light was counted for a set of 300 acquired scatter-configuration signals. The same threshold was then applied to count fluorescence modulations for the corresponding set of 300 acquired fluorescence-mode signals. Triplicate batches of microbubbles were prepared using the four above-described temperature regimens as well as no treatment. The three batches of all five treatments were prepared and analyzed on separate days.

Signals acquired from all three days were combined for each temperature treatment regimen and the number of fluorescence modulating microbubbles was divided by the number of scattering microbubbles to compute a percentage of fluorescence-modulating microbubbles for each treatment. The proportions of fluorescence-modulating microbubbles were analyzed for statistically significant differences using a chi-square test. A post hoc comparison was then conducted between groups using the Bonferroni adjustment. The percentage of fluorescence-modulating microbubbles was also calculated for each treatment for each of the three days (batches). The mean fluorescence modulation percentage of the three batches and standard deviation was calculated for each treatment to show batch-to-batch variability. The combined data from all three batches for each treatment was tabulated to show the bubble count that displayed fluorescence modulation and no modulation for each treatment (Table 1). The total bubble count for each treatment was determined by the total number of scattered light modulation detected signals. The fluorescence modulation count was determined by the total number of fluorescence modulation detected signals, and the no modulation count was determined by the difference between these two totals.

3. Results

3.1 Microbubble manufacturing and temperature treatment

Microbubbles surface-loaded with fluorescent DiI in the lipid monolayer were prepared by the manufacturing process described above and are shown prior to temperature treatment in

Figure 2a and b. The bulk solution of microbubbles with no temperature treatment is shown prior to washing in Figure 2c. Using conventional fluorescence microscopy with a high numerical aperture, all prepared bubbles were seen to have surface-loaded fluorescence, but no microstructural features or presence of discrete domains could be seen on the bubble surface with this microscopy technique. No visible difference in fluorophore surface patterning could be seen with this technique between bubbles that were not temperature treated and bubbles that were subjected to various times and regimens of thermal treatment. Microbubbles were found to be stable through the temperature treatment processes, persisting for several days, however the microbubble yields were lower after thermal treatment likely due to rupture of the least stable bubbles.

3.2 3D structured illumination super-resolution microscopy

Surface patterning of the lipids and fluorophore was not visible in the conventional fluorescence images shown in Figure 2b. This imaging system did not have the capability to resolve features below the diffraction limit and fluorescence from all sections of the microbubble appeared as a summed 2D projection creating what appears to be a uniform hazy distribution of fluorescence across each microbubble. The use of 3D structured illumination super-resolution microscopy (3D-SIM) allowed visualization of microbubble surface features below the diffraction limit. The axial sectioning capability of this technique allowed removal of the confounding fluorescence from out-of-focus layers to reveal the nanoscale surface structure and island partitioning of the fluorophore. Top surface and cross-section images of microbubbles exposed to various temperature treatments are seen in Figure 3. Under no temperature treatment, the fluorophore was seen to have partitioned into isolated island structures creating a speckled appearance, with most of the structures less than 500 nm in size. These structures were seen on the surface of the microbubble as well as its cross-section indicating that these structures were prevalent over the entire microbubble surface. For microbubbles that were surface-melted followed by “rapid-cooling” through the lipid transition temperature, these island structures disappeared and were replaced with a uniform fluorescence over the surface and cross-section of the microbubble. When the microbubbles were allowed to “slowly cool” through the transition temperature, the images displayed that the fluorophore was again partitioning into concentrated islands although to a lesser degree than the bubbles with no thermal treatment.

3.3 Acousto-fluorescence detection system signals

Time domain signals and frequency spectra of typical acquired scattered light and fluorescence signals are shown in Figure 4 for microbubbles of different thermal treatment regimens. Signals acquired from detected laser side-scatter resulting from microbubble interaction with the excitation laser are shown in Figure 4a with the corresponding spectra shown in Figure 4b. Both rapid-cooled and non-temperature-treated bubbles display a peak at the 2.25 MHz ultrasound driving frequency, indicating that both types of bubbles undergo size modulation in response to the applied ultrasound pressure. However, when the fluorescence filters are applied to look at signals resulting from ultrasound-modulated self-quenching of the surface-loaded fluorophore, there is a lack of detectable modulating signal for bubbles that did not undergo thermal treatment as shown in Figure 4c. The 2.25 MHz

peak resulting from fluorescence intensity modulation is clearly visible for the rapid-cooled microbubble shown in Figure 4d.

3.4 Ultrasound-modulated fluorescence signal analysis

The percentage of microbubbles that displayed a detectable fluorescence intensity modulation as defined by the signal analysis threshold is shown for the different temperature treatment groups in Figure 5a. Less than 5% of the microbubbles that did not undergo temperature treatment displayed a detectable fluorescence modulation signal. Microbubbles that were rapid-cooled (heating at 75°C for 3 minutes with subsequent cooling for 5 minutes at 0°C) showed a ten-fold increase in the generation of this signal, with 54% producing fluorescence modulations. A chi-square test conducted at the 0.05 significance level showed that the percentage of bubbles that displayed fluorescence modulation was not independent of the different temperature treatments. A post hoc comparison between groups using the Bonferroni adjustment found that the fluorescence modulation percentage of the rapid-cooled microbubble group was significantly different from all other thermal treatment groups ($p < 0.05$). The horizontal bars in Figure 5a denote treatment pairs where no significant difference was found between them ($p < 0.05$). Approximately 19% of the microbubbles that were slow-cooled through the transition temperature (heating at 75°C for 3 minutes followed by cooling for 5 minutes at 45°C) displayed a detectable fluorescence modulation signal. Microbubbles that were heated and rapidly-cooled for 1 minute time intervals (1 minute 75°C, 1 minute 0°C) displayed fluorescence modulation incidence of approximately 19% and microbubbles that were heated and slow-cooled for the same time intervals (1 minute 75°C, 1 minute 45°C) displayed fluorescence modulation incidence of approximately 7%. There was no significant difference between the microbubbles that were heated and slow-cooled for 1 minute intervals and those bubbles that received no treatment. These two groups had significantly lower fluorescence modulation incidence than the other treatment groups. A data summary for Figure 5a of the total bubble counts that displayed modulation and no modulation for each temperature treatment is given in Table 1. Batch-to-batch variability is shown in Figure 5b where the mean fluorescence modulation percentage of the three batches and standard deviation was calculated for each treatment.

4. Discussion

Microbubbles fabricated using the described probe sonication process displayed a lipid surface layer where the majority of the lipophilic dye was concentrated in islands and in large structures that extended beyond the spherical surface. These islands were either formed as a result of incomplete breaking of the micelles by the sonication probe or by recondensation of the lipid and fluorophore species into these structures as the sonicated samples slowly cooled to room temperature. The dye and associated lipids were observed to partition into different structures at different temperatures due to the fluidity of the monolayer. The transition temperatures of the species forming the shell are 55°C for DSPC and 74°C for DSPE. When heated to 75°C both lipid types showed increased fluidity and the islands of concentrated lipid dissociated and distributed more evenly throughout the monolayer. At room temperature (25°C) the monolayer's fluidity was greatly reduced and the lipids were solidified in place.

The different cooling rates from the 75°C melting temperature resulted in distinct final surface structures. When cooled rapidly from 75°C to 0°C the lipids and dye did not have the opportunity to recondense into islands and were “frozen in place” in the distributed state. When cooled slowly, the lipid monolayer passed through a temperature region where the different lipids were allowed to recondense back into the energetically favorable tightly packed island structures forming concentrated regions of the lipid species and dye. In bubbles of similar composition, carbocyanine dyes such as DiI have been observed to preferentially partition into the pegylated lipid domain.[24] The islands were still observed when the microbubbles were heated to 75°C for only 1 min and rapidly cooled supporting that the short heating time was insufficient to melt the islands and allow them to distribute.

Microbubbles subjected to all four described temperature regimens (as well as no treatment) were able to undergo oscillations when exposed to ultrasound as detected by the strong excitation laser side-scattering signals. Less than 5% of the microbubbles that did not undergo any temperature treatment displayed any detectable fluorescence modulation. This indicates that the dye molecules in the islands were not able to separate from one another far enough to reduce self-quenching and that the islands of DSPE-PEG 5k and dye did not expand significantly when exposed to ultrasound. Another possibility is that the dye molecules were too concentrated in the islands to undergo fluorescence modulation even if the islands did expand.

Over 50% of the microbubbles that underwent the 3 minute heating and subsequent rapid cooling treatment showed measurable fluorescence intensity modulation. This indicates that when the dye was distributed evenly across the surface of the microbubble the dye molecules were more likely to be able to move far enough from one another to change the amount of self-quenching that occurred as the microbubble expanded. Another potential contributor to the increase in signal incidence with rapid-cooling is that the observed lipids and fluorophore extending outward from the surface of the bubble could have been melted back onto the main gas sphere, providing more available fluorophore coupled to the size-oscillating microbubble. Higher fluorescence modulation percentages could potentially be achieved through an extended heating period, an even more rapid temperature quenching rate, or multiple cycles of rapid-cooling. A general trend was seen in Figure 5 that the more thermal manipulation the microbubbles received (longer intervals, more rapid cooling), the higher the incidence of fluorescence modulation.

Increasing the percentage of microbubbles that display detectable fluorescence intensity modulation is essential to improving the future efficacy of these microbubbles as optical imaging contrast agents. Imaging techniques using these microbubbles can allow extraction of tissue biochemical information with previously unattainable spatial precision by using the ultrasound-modulated signal to extract the signal generated from the ultrasound focal zone. The biochemical information obtained could allow for detection of tumor-associated hallmarks such as hypoxia [25, 26] and angiogenesis [27] and could lead to significant improvements in the accuracy of cancer diagnostic techniques. This detection method requires the consistent generation of a modulating-fluorescence signal, which is enabled through the use of a rapidly-cooled thermal regimen to produce more effectively modulating bubbles.

This microbubble temperature treatment method and the resultant dynamic bubble behavior under ultrasound have implications for applications that require the lipids of the microbubble monolayer to be well-distributed. For example, applications that require surface-crosslinking and manipulation of microbubble stiffness to change the acoustic properties will need to take island formation into account when designing the linking chemistry between the different lipid species. The crosslinking is more likely to be effective at constraining microbubble expansion if it is made evenly across the surface rather than just between islands. Evenly distributed crosslinking could also help increase microbubble stability when exposed to low level ultrasound to extend their effective imaging lifetime.

Evenly distributed lipid coatings are also important when covering the bubble surface with PEG or other coatings to prevent immune system recognition. When the pegylated lipids all congregate into island structures, this leaves regions where the PEG coating does not cover the surface potentially reducing the circulation half-life of the microbubble. This PEG layer also helps increase microbubble stability during storage as it helps prevent coalescence. The PEG prevents physical contact of lipid layers from neighboring microbubbles.

A uniform coating is also important when using targeting ligands on the microbubbles to cause preferential accumulation in specific tissues. Targeting ligands are most frequently coupled to the surface of microbubbles and liposomes by attachment at the ends of the same lengthy PEG chains used to avoid immune recognition. As an illustrative example, the use of cRGD peptide as a targeting ligand for the neovasculature marker $\alpha\beta3$ integrin [27, 28] could be made more efficient by controlling the distribution of the associated PEG. Targeting efficiency would be enhanced when the cRGD covers the entire surface of the microbubble evenly rather than being concentrated into islands which may or may not come into contact with cells as the bubble passes through the vasculature.

5. Conclusion

Lipid-coated microbubbles containing self-quenching lipophilic dye molecules were manufactured by a probe sonication method resulting in surface nanostructures that displayed islands of high dye concentration. The dye molecules in these islands were not able to separate sufficiently from one another during the expansion phase of microbubble interaction with ultrasound to modulate their self-quenching properties. Temperature treating these microbubbles to melt the lipid islands allowed the dye to more evenly distribute about the surface of the microbubble. Subsequent rapid cooling was applied to solidify the lipids in place preserving this distributed state. The even distribution allowed the dye molecules to separate sufficiently from one another to change their self-quenching efficiency resulting in a ten-fold increase of detectable fluorescence intensity modulations. This supports that the lipid islands were not able to expand sufficiently with the rest of the lipid monolayer. The enhanced efficiency of these contrast agents to generate ultrasound-induced fluorescence modulations enables the development of imaging techniques which make use of their precisely-controlled fluorescence signal and promise to improve optical detection of clinically relevant biochemical information in deep tissue.

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Figure 1.

Schematic of fluorescence intensity modulation (“blinking”) mechanism. Microbubbles undergo size oscillations in response to the ultrasound pressure wave. At low ultrasound pressure, the bubble expands, separating the dye molecules and reducing their ability to quench each other. At high ultrasound pressure, the bubble is compressed, and the dye molecules are quenched to a greater extent due to close proximity. This results in a fluorescence intensity modulation signal at the same frequency as the applied ultrasound wave.

Figure 2.

Conventional imaging of DiI surface-loaded microbubbles. (a) Brightfield illumination. Each microbubble appears as a circle with white and dark regions created due to interference resulting from a lensing effect caused by the index of refraction mismatch between the water and the gas. (b) Fluorescent image of the same field of microbubbles. Fluorescence from the center of individual microbubbles indicates presence of surface-loaded fluorophore. (c) Image of the bulk microbubble solution prior to washing showing the microbubbles as a turbid pink suspension.

Figure 3.

Super-resolution microscopy images of surface-loaded fluorescent microbubbles prepared with various temperature treatment regimens. Microbubbles that did not undergo temperature treatment displayed highly concentrated islands of fluorescence seen on both the top surface and in cross-sections. For microbubbles that were heated to 75°C for 3 minutes to melt the lipid monolayer followed by cooling at 0°C for 5 minutes (“rapid-cooling”), the fluorophore was seen to more uniformly coat the surface of the microbubble. This fluorophore distribution suggests that the concentrated fluorophore in the islands was melted to a more homogenous distribution during the heating phase and then when rapidly cooled this even distribution was solidified into place. When the microbubbles were heated to 75°C for 3 minutes followed by cooling at 45°C for 5 minutes (“slow-cooling”), island structures were able to re-form before the fluid motion of the lipids was restricted. The crosshatch patterns seen in some of the cross-sectional images are image reconstruction artifacts from the 3D-SIM technique.

Figure 4.

Example time and frequency domain signals acquired from rapid-cooled microbubbles and bubbles that were not temperature-treated. (a) Time domain signals acquired from side-scatter of the excitation laser resulting from microbubble size oscillations. Both rapid-cooled and non-treated bubbles display characteristic oscillations. (b) Frequency spectra of the acquired scattered light signals shown in panel a. Both rapid-cooled and non-treated bubbles display strong peaks at the 2.25 MHz ultrasound driving frequency. (c) Acquired time domain fluorescence signals. Rapid-cooled bubbles show a characteristic fluorescence oscillation that is not seen in a typical untreated bubble. (d) Frequency spectra of the acquired fluorescence signals shown in panel c. The rapid-cooled microbubble displays a clear peak at the driving frequency resulting from ultrasound-driven cyclic quenching of the surface dye. A typical untreated microbubble does not show any clear peak in the frequency spectra.

Figure 5.

The percentage of microbubbles that displayed detectable fluorescence modulation under different temperature treatment regimens. (a) Fluorescence modulation percentages from the combined signals acquired from all three microbubble batches, each of which included all five temperature treatments (combined bubble count data shown in Table 1). A ten-fold increase in fluorescence modulation incidence was seen in rapidly-cooled microbubbles (3 minutes 75°C, 5 minutes 0°C) as compared to bubbles that were left untreated. Other treatment regimens including slow-cooling (3 minutes 75°C, 5 minutes 45°C) as well as

shorter time intervals of heating and cooling (1 minute 75°C, 1 minute 0°C and 1 minute 75°C, 1 minute 45°C) were less effective than rapid cooling at generating fluorescence modulations. Horizontal bars are used to indicate treatment groups that were not found to be significantly different using a chi-square test with post hoc comparisons between groups using the Bonferroni adjustment ($p > 0.05$). (b) Batch-to-batch variability of the fluorescence modulation percentage for each temperature treatment. Data shown as the mean fluorescence modulation percentage and standard deviation of three batches of microbubbles prepared and analyzed on separate days. Microbubbles slow-cooled for 1 minute time intervals (1 minute 75°C, 1 minute 45°C) showed the highest batch-to-batch variation.

Table 1

Data summary for Figure 5a showing the bubble count that exhibited fluorescence modulation and no modulation for various temperature treatments.

	Treatment						Total
	No Treatment	3 min heat, 75 – 0 °C	3 min heat, 5 min cool 75 – 45 °C	3 min heat, 5 min cool 75 – 0 °C	1 min heat, 1 min cool 75 – 0 °C	1 min heat, 1 min cool 75 – 45 °C	
No Modulation	600	161	351	348	455	1915	
Modulation	28	191	83	83	35	420	
Total	628	352	434	431	490	2335	