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Title: Mapping Microbubble Viscosity Using Fluorescence Lifetime Imaging of Molecular Rotors

Short title: Molecular rotors measure microbubble viscosity using FLIM

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Encapsulated microbubbles are well established as highly effective contrast agents for ultrasound imaging. There remain, however, some significant challenges to fully realise the potential of microbubbles in advanced applications such as perfusion mapping, targeted drug delivery and gene therapy. A key requirement is accurate characterisation of the viscoelastic surface properties of the microbubbles; but methods for independent, non-destructive quantification and mapping of these properties are currently lacking. We present here a strategy for performing these measurements that utilizes a small fluorophore termed a ‘molecular rotor’ embedded in the microbubble surface, whose fluorescence lifetime is directly related to the viscosity of its surroundings. We apply Fluorescence Lifetime Imaging (FLIM) to show that shell viscosities vary widely across the population of the microbubbles and are influenced by the shell composition and the manufacturing process. We also demonstrate that heterogeneous viscosity distributions exist within individual microbubble shells even with a single surfactant component.

The potential utility of surfactant stabilized microbubbles (Fig. S1A) as contrast agents for ultrasound and more recently multi-modality imaging combined with therapeutic delivery has made them the subject of intensive study (1). A wide variety of experimental techniques and theoretical modeling have been applied to characterize their behavior and new manufacturing techniques developed to provide improved control over their properties (Fig. S1B). The surfactant coating not only stabilizes bubbles against diffusion of encapsulated gases, it can also be used to functionalise the bubble surface for a given application, for example, it can provide a scaffold to which targeting molecules or drugs are attached. Importantly, its properties can also be selected to “tune” the response of a microbubble to ultrasound (2). The exact role of the physico-chemical parameters of the coating, however, is still not fully understood and they are known to vary considerably across a microbubble population (3).

Currently, only indirect and quasi-static methods exist to measure the viscoelastic properties of the microbubble coating. These include: micropipette aspiration (4), ultrasound scattering and attenuation measurements (5), Fluorescence Recovery After Photobleaching (FRAP) (6) and atomic force microscopy (7). Estimates have also been successfully obtained through fitting theoretical models to acoustic and/or high speed camera measurements of single microbubbles (8). Importantly, this requires the microbubble coatings to be treated as a continuum (6), including coatings of mixed compositions, e.g. phospholipids and emulsifiers (9). There is now firm evidence from numerous studies, e.g. by TEM and fluorescence imaging (10, 11) that partitioning and domain formation occurs in multi-component bubble shells. These domains govern the surface microstructure (12) and affect the viscoelastic properties of the microbubble shell and hence their functional behavior.

To address these problems we have adopted an alternative strategy, utilizing a detection method which not only allows the effective viscosity of microbubble coatings to be quantified but also any spatial variations to be precisely mapped. Our method applies Fluorescence Lifetime Imaging Microscopy (FLIM)(13) to exploit the viscosity sensitive photophysical properties of a ‘molecular rotor’, the meso-substituted 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY-C10) (Fig. S2). This fluorescent dye can be seamlessly incorporated into structures such as a surfactant coating (Fig.S1C), in this case in the “tail” region of the phospholipid layer surrounding a microbubble. Since it is not a “tag” that is directly bound to the surfactant molecules it does not affect their behavior (Fig. S3). BODIPY-C10 behaves as a ‘molecular rotor’, due to the rotation of the phenyl ring in and out of the plane of the BODIPY chromophore (Fig. 1). As a result of this rotation, the non-radiative decay pathways are activated, leading to a reduction in the fluorescence quantum yield and the lifetime of the rotor compared with that in a viscous environment (14).

For the purposes of microbubble imaging we elected to use fluorescence lifetime to detect viscosity. Unlike fluorescence intensity, which changes as a function of the dye concentration, the fluorescence lifetime can provide a viscosity measure which is not affected by inhomogeneous distribution of the
rotor in a given region of the bubble coating. We experimentally determined the relationship between
the fluorescence lifetime and viscosity for BODIPY-C10 for a wide range of viscosities (0.6-1140 cP)
(Fig. 1A), and this was found to be well described by the Förster-Hoffmann equation (15) (Eq. 1-4).
This provided the means to produce a calibration graph (Fig. 1B) for converting lifetime into a
quantitative measure of viscosity. FLIM can detect individual lifetimes with the spatial resolution of a
multi-photon microscope (ca. 200 nm). Thus FLIM of the molecular rotor BODIPY-C10 provides a
minimally invasive technique that can directly quantify and image viscosity at a macro- or microscopic
resolution from lipid membranes of individual microbubbles. Importantly, the rate of intramolecular
rotation in BODIPY-C10 also corresponds to bubble oscillations at ultrasonic frequencies. It thus
provides a more relevant measure than quasi-static measurements and has the potential to be utilized
during ultrasound exposure, to determine also how coating properties may vary during bubble
oscillation; although this was not exploited specifically in this study.

In this study we investigate the impact of the microbubble preparation method, coating composition
and size on the magnitude and spatial distribution of the effective surface viscosity of individual
bubbles; and compare this with the variation in their response to ultrasound excitation.

Results and Discussion

Global microbubble analysis.

Initially, we used a single phospholipid 1,2-Distearoyl-sn-glycero-3-phophocholine (DSPC) to prepare
microbubbles using both a conventional sonication method and a microfluidic “T”-junction device. The
latter enables the preparation of bubbles with a high degree of control over their size and uniformity,
whilst the former produces bubbles with a broad size distribution. The sonication method has the
advantage of producing bubbles in high yield and also with superior stability. However, the underlying
reasons for the difference in stability are not fully understood. By applying the molecular rotors
approach, we were able to determine the mean viscosity of T-junction microbubbles as 663±119 cP
(3.40±0.26 ns), which was found to be significantly lower than for the sonicated bubble population at
857±110 cP (3.83±0.22 ns) (Fig. 2A). This is consistent with lower diffusivity and hence the greater
stability of the sonicated microbubbles. To the best of our knowledge this is the first instance of the
direct physical characterisation of the effect of preparation method upon microbubble structure which
could explain the differences in their behavior.

The composition of the microbubble coating is known to significantly affect both the stability and
response of microbubbles to ultrasound (12). We compared fluorescence lifetimes detected from
microbubbles produced by sonication from lipid-dye films containing DSPC, L-alpha-
Phosphatidylcholine (L-alpha PC) and DSPC+PEG40-stearate in different ratios (see Methods and
Supplementary Information). The DSPC and L-alpha-PC microbubbles were found to exhibit a similar
range of viscosities (Fig 2B) as might be expected given the similarity in their molecular structure (Fig.
S1). The addition of PEG to DSPC, however, had a marked effect, with viscosity being significantly
reduced with increasing PEG ratio: PEG 1:1 DSPC 337±57cP (2.49±0.2 ns), PEG 1:4 DSPC 426±57
cP (2.78±0.2 ns), PEG 1:9 DSPC 506±73.9 cP (3.01±0.21 ns) (Fig. 2C). For all compositions,
comparison of the observed lifetime for microbubbles of different size indicated that there was no
statistically significant correlation between shell viscosity and microbubble size (Fig. S4).

There was, however, considerable variation across each population in the measured viscosity for all
shell compositions (Fig. 2). A significant advantage of the molecular rotor-based viscosity
determination is the possibility of spatially resolved viscosity imaging, which to the best of our
knowledge is currently not possible with any other available technique.

This large viscosity variation was reflected in the ultrasound measurements. The mean power of the
scattered pressure detected from individual bubbles decreased with increasing proportion of PEG40
stearate in the coating, corresponding to a reduction in the measured viscosity as would be predicted
There was, however, a very large standard deviation in the measurements even though the bubble size distributions were matched between the different compositions and batches (Fig. 2 and Fig. S5). Theoretical simulations (Fig. 2D) further indicated that the scattered pressure is also sensitive to variations in effective elasticity of the microbubble coating, and this would be expected to vary with composition as well as viscosity. This reinforces the need for independent methods for determining coating characteristics such as that presented in this study.

**Individual microbubble analysis.** To further examine the differences between bubbles within a single population, we determined the fluorescent lifetimes in each pixel of individual DSPC microbubble images, Fig. 3. Fig. 3A shows two bubbles of a similar size, but with different lifetime distributions, as identified by two separate Gaussian peaks in the lifetime histogram (Fig. 3B). We applied a seismic color palette to the lifetime histogram (assigned a single color to each lifetime peak), which clearly differentiated the two Gaussian distributions. This operation confirmed that the lifetimes in each peak originated from the two separate bubbles which could be assigned viscosities of 600±140 cP (3.26±0.33 ns) and 1112±191 cP (4.32±0.33 ns) according to the calibration graph (Fig. 1B).

In addition, we produced lifetime maps over the surface of single bubbles. Even though the lifetime decay was found to be mono-exponential in every pixel, significantly different viscosities were observed across the same bubble (Fig. 3C) and without external stimuli. For example, in Fig. 3D, we resolved the average lifetimes in two separate regions of the same bubble, A = 3.43±0.11 ns and B = 3.63±0.11 ns, corresponding to viscosity differences of 670±50 cP and 761±54 cP, respectively (see Fig. S6 for further analysis). These data clearly demonstrates that even within a single bubble made up of a pure DSPC lipid vastly heterogeneous viscosity distribution could be observed. Our experiments were conducted at an ambient temperature which was much lower than the gel transition temperature of DSPC (55°C) and lack of membrane fluidity may be partly responsible for this inhomogeneity. In addition, the regions of higher apparent viscosity may correspond to regions of lamellae within the coating. These observations confirm that microbubbles should not be modeled as having a continuous shell, even for single lipid component systems.

The same methodology could be applied to determine the effects of interactions between bubbles on the surface characteristics (Fig. 4A). In Fig. 4B we used the TRI2 lifetime software (16) to extract and analyze the histogram information from individual bubbles in contact with their neighbors. As a collective for all the bubbles in the field of view, the histogram (Fig. 4B grey line) shows no distinguishing features. However, when each bubble’s histogram was individually analyzed, distinct subsections could be easily identified and associated to each bubble. It was also clear that each histogram in Fig. 4B had a non-Gaussian shape, unlike the two bubbles in Fig. 3B. The lack of symmetry suggested a non-homogeneous viscosity distribution similar to that observed in Fig. 3D (Fig. S6). We have applied the peak fitting analysis to the individual histograms of bubbles 2 and 3 in Fig. 4 and identified that there are essentially two lifetime populations with a broad intersection range between 3.1 ns and 3.4 ns (Fig. S7). Using this cross over band to differentiate areas of low and high viscosity we applied a seismic color plot to all bubbles. It can be clearly seen that bubble areas in contact with the neighboring bubbles appear as blue (lower viscosity, lifetimes below 3.1 ns), whilst areas of bubbles that are contact free are red (higher viscosity, lifetimes above 3.4 ns). Visual inspection of the image suggests that bubble contact lowers viscosity. Changes in refractive index due to contact would not be sufficient to account for these differences. Potentially, formation of bilayer/multilamellar structures with lower viscosities in the contact region (14) or shielding of the lipid phosphate groups from the surrounding water, could be responsible although further work is required to verify these hypotheses. These observations have practical implications for microbubble applications in vivo where they are initially subject to contact during injection at high concentration in bolus form ~10⁹ bubbles/ml and subsequently from other structures formed from lipidic materials, e.g.
plasma membranes of endothelial cells. Currently, these interactions are poorly understood and not accounted for in theoretical models.

Conclusion

We have shown that viscosity mapping using molecular rotor fluorescence lifetime imaging provides a direct means of quantifying the spatial distribution of viscosity in the microbubble coating. We have confirmed that inclusion of the dye within the lipid shell produced no observable effects on the measured viscosity (Fig. S3 and S8) or surface tension (Fig. S8) and that the bubbles were unaffected by irradiation at low laser powers, sufficient to obtain FLIM images (Movie S1-2). We have applied this technique to examine the effects of varying composition and production methodologies upon microbubble coating properties. The results have provided the first direct evidence that there can be a large variation in viscosity across a microbubble population, independent of individual bubble size, and that is correlated to the variability measured in their ultrasound response. We have also demonstrated that varying the coating composition can significantly alter the viscosity, in this case reducing it by the addition of a surfactant (PEG40-stearate). Again this was in agreement with the measured acoustic response of the microbubbles, with those having less viscous coatings showing an increase in amplitude of oscillation.

We believe that molecular rotors coupled with FLIM provide a unique tool for understanding the structure and physical properties of microbubble coatings at the microscopic scale. This type of data can provide invaluable information for characterizing and predicting microbubble behavior, removing the need for many simplifying assumptions e.g. shell homogeneity or indirect measurements involving the fitting of several interdependent parameters to a single data set. Practically, this method provides direct feedback for optimizing microbubble composition and manufacturing processes; and in the future, for accurately determining the effects of time, temperature, bubble concentration, the properties of the surrounding medium and ultrasound exposure on microbubble characteristics. It may also offer a powerful new method for investigating the interaction between microbubbles and biological structures under ultrasound excitation.

Materials and Methods

Viscosity calibration of fluorescence lifetime of BODIPY-C10. Spectroscopic grade methanol/glycerol mixtures prepared at twelve concentrations between 0-100% (w/v) glycerol and maintained at room temperature. The dynamic viscosity ($\eta$) of each binary mixture was measured using a Stabinger viscometer (SVM 3000, Anton Paar) with an accuracy and precision of ±0.35% and ±0.1%, respectively. BODIPY-C10 was dissolved at a concentration of 2.5 $\mu$M in the MeOH-glycerol mixtures and fluorescence lifetimes (Fig. 1A) were recorded in quartz cuvettes on a time correlated single photon counting (TCSPC) Jobin Yvon IBH data station (5000F, HORBIA Scientific Ltd.). Samples were excited at 467 nm using a 1 MHz pulsed NanoLED (N-467, HORBIA Scientific Ltd.) with a pulse width of 200 ps and a detector monochromator set to 515±5 nm. Decays in all instances were recorded until peak counts were >10,000 counts and at a maintained temperature of 22°C using a thermostatic circulating chiller (RE104, Lauda Technology Ltd.). The solution viscosity was correlated to the decay traces using the modified form of the Förster Hoffmann equation. This equation 1 describes the relationship between quantum yield ($\Phi_f$) and viscosity ($\eta$) where $\alpha$ and $\alpha$ represents constants. The quantum yield of a fluorophore can be related to the fluorescence lifetime ($\tau_f$) by the radiative ($k_r$) and non-radiative rates ($k_{nr}$), as in Eq. 2.

$$\Phi_f = z\eta^\alpha$$  \[1\]

$$\Phi_f = \frac{k_r}{k_r + k_{nr}} = \tau_fk_r$$  \[2\]
Substitution of Eq. 2 into Eq. 1 defines the relationship between the lifetime and viscosity, Eq. 3.

\[ \tau_f = \frac{z\eta^\alpha}{k_r} \]  

[3]

For calibration purposes it is useful to present this equation in the logarithmic form, Eq. 4.

\[ \log \tau_f = \alpha \log \eta + \text{const} \]  

[4]

We observed a linear relationship between (log \( \tau_f \)) and (log \( \eta \)) in a large viscosity range 7.7-1140 cP. This calibration plot (Fig. 1b) allows the conversion of experimentally measured lifetime (including those from microscopy FLIM experiments) to viscosity. It is important to note that the nature of the "viscosity" in membranes as measured by molecular rotors is not as straightforward as for bulk samples. At present the precise correlation to conventional measures (i.e. shear or dilatational) of membrane viscosity is not entirely clear (17). The range of action of the molecular rotor is such that the lengthscale on which the viscosity is measured is that of the molecule itself. Glycerol/methanol solutions provide a convenient means of determining the functional relationship between the fluorescence lifetime and viscous nature of the surrounding medium and have been widely used in previous studies utilizing molecular rotors for membrane characterization. This does implicitly assume however that the “macroscopic” three dimensional shear viscosity of the solutions as measured by other means is proportional to the dissipative mechanisms on the scale of the rotor believe this to be a reasonable assumption but are keen to highlight the distinction.

**Microbubble sample preparation.** Stock solutions of Boron-dipyrromethene (BODIPY-C10), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) (850365P, Avanti Polar Lipids Inc., USA), and L-a-Phosphatidylcholine hydrogenated (L-alpha-PC) (P4139, Sigma-Aldrich Ltd., UK), see Fig. S9 for lipid structures, were prepared in chloroform to concentrations of 4.2 mM, 75.9 mM and 60 g/l (approx. 76 mM, estimated given unknown precise MW), respectively. A stock solution of Polyoxyethylene(40) stearate (PEG) (P3440, Sigma-Aldrich Ltd., UK) was prepared in distilled water (24.45 mM). Lipid/dye films (900:1 molar ratio) were prepared by mixing the chloroform stock solutions of a corresponding lipid and BODIPY-C10 followed by solvent evaporation. Following this, 2 ml of aqueous solution, of either distilled H2O or PEG40-stearate stock solution in water, was added to create a final dye concentration of 4.2 \( \mu \)M. Microbubble samples were prepared from one of five compositions: DSPC, L-alpha-PC, PEG 1:9 DSPC, PEG 1:4 DSPC and PEG 1:1 DSPC, and two manufacturing procedures. The two methods of microbubble production (see Supplementary Methods) were used for this study: sonication and microfluidic T-junction. Both methods required dried lipid/dye films prior to processing, prepared as described above.

**Sonication** involved pipetting 2 ml of aqueous solution on top of dried films in glass vials and probe sonication at 6W for 30 sec (XL-2000, Qsonica LLC, probe tip: CML-4) followed by mechanical shaking for 30 sec to form microbubbles.

**T-junction** microbubble preparation required a final working volume of 5-7 ml of aqueous solution to be added to the dried films. Prior to loading in the syringe driver (PHD 4400, Harvard Apparatus) the lipid/dye aqueous solution was bath sonicated to ensure homogenous dispersion of the lipid/dye mixture. A syringe driver forced the lipid/dye aqueous solution at a flow rate of 0.4 ml/min into the specially designed microfluidic polymethylmethacrylate (PMMA) block (18), whilst simultaneously flowing nitrogen gas at a constant pressure of 700 mbar, measured using a digital manometer (2026P, Digitron). Polyether ether ketone (PEEK) capillary tubing (inner diameter 75 \( \mu \)m) was connected via standard high-pressure liquid chromatography (HPLC) connectors and ferrules (Gilson Scientific Ltd.) to connect the input and exit ports of the T-junction block (Fig. 1B).
**Microbubble imaging protocol and imaging equipment.** Prepared microbubbles were transferred to glycerol coated coverslips and imaged. Initially, microbubbles were localized using brightfield and confocal microscopy using TCS SP5, Leica Microsystems Ltd. with a 488 nm excitation from an internal Argon ion laser through an x63 (N.A. 1.2) HCX PL APO CS water immersion objective lens with correction collar (11506279, Leica Microsystems Ltd.). Lifetime images were obtained using a time-correlated single photon counting (TCSPC) module (SPC830, Becker&Hickl) and internal FLIM detector (PMH-100, Becker&Hickl GmbH). The module was coupled and synchronised to the inverted scanning confocal microscope and Ti-Sapphire pulsed laser source (680-1080 nm, 80 MHz, 140 fs, Chameleon Vision II, Coherent Inc.).

Two-photon excitation of microbubbles was performed at 800 nm and emission captured for lifetime/intensity at a band pass of 500-660 nm, laser power was maintained <400 mW prior to entering the microscope to avoid bubble damage by intense irradiation (Movies S1 and S2). FLIM acquisition was continued until a pixel peak count of >100 was obtained, while the image format and ADC were set to 256x256 and 256, respectively. FLIM data was exported and analysed in TRI2 software (Version 2.4.1.1, Gray Institute for Radiation Oncology and Biology) (16) where a mono-exponential model was fitted, using the Levenberg-Marquardt algorithm, to each pixel lifetime decay. Pixels were binned to maintain a minimum peak count of 100 counts/pixel and only lifetimes with reduced chi-squares $\chi^2_{r}<1.2$ were accepted in final images; thresholding was used to remove background noise. A false rainbow colour scale was assigned to each fluorescence lifetime value (blue for a short lifetime and red for a long lifetime) to provide lifetime maps. Average microbubble lifetimes were calculated by masking the bubble of interest and fitting a mono-exponential decay to all the binned pixels (19). Further image analysis was completed by exporting fitted data to Origin Scientific solutions (OriginPro 8.5, OriginLab Corporation) and Matlab (R2009b, The MathWorks Inc.) (Fig. S6-7).

In order to determine the optimal concentration of BODIPY-C10 in lipid coating we investigated bubbles prepared at [lipid]/[dye] ratios from 200:1 to 3600:1. We did not detect any effect of dye concentration on measured viscosity or surface tension (Fig. S8) of lipid coating in this range.

**Microbubble acoustic response measurements and setup.** Microbubbles from the different DSPC+PEG ratio mixtures were prepared via sonication and fractionated by allowing them to stand at 4°C for 3 hours. Microbubbles were hydrodynamically isolated and streamed using a pair of co-axially aligned needles into the focal region of a pair of transducers (Fig. S5A). The microbubbles were interrogated by exciting the transmitting transducer (2.25 MHz focused, A306S, Panametrics-NDT) with a Gaussian-windowed 5 cycle 2.25 MHz sinusoid pulse, generated by an arbitrary function generator (33220A, Agilent). The signal was then amplified (50dB) by an RF power amplifier (325LA, Electric and Innovation) at a pulse repetition rate of 100 Hz. The scattered pressure was detected at 90 degrees using a 3.5 MHz focused transducer (V382, Panametrics-NDT) where the signal was amplified (42dB) using a pulser/receiver (DPR300, JSR Ultrasonics) and digitized with an oscilloscope (600 MHz, Xi64-A, Waverunner, LeCroy).

Syringe pumps (AL-1000, World Precision Instruments) were used to control the flow rates in the inner and outer needles of the coaxial flow. Deionised water was pumped through the outer needle (910 μm inner diameter) and the microbubble solution was delivered through the inner needle (190 μm inner diameter). A third syringe pump was used to maintain a stable flow by providing suction through a tube (2 mm Inner dia.) located 2 cm away from the center of the inner needle. The transducers were aligned by maximizing the received backscatter from millimetre sized air bubbles pumped through the inner needle. Precise alignment of the transducers and needles was achieved by separately mounting the components onto linear xyz manually adjustable stages. The transducers and coaxial flow were securely mounted into a degassed and deionized water tank. The incident pressure at the confocal region was measured using a calibrated 75 μm needle hydrophone (Precision Acoustics Ltd.) the peak negative pressure was detected to be 284 kPa, which was
comparable with medical ultrasound pressures (Fig. S5B-C). Confirmation and evaluation of scattering events was performed by comparing the frequency spectrum from flow without microbubbles prior to each experiment. Microbubble flow was also visualized using a high-speed camera (Memrecam GX-8, NAC) to confirm that microbubbles were isolated and that the inner flow was confined to a jet approximately 100 μm in diameter travelling with a central line velocity of 0.12 ms⁻¹.

The scattered pressure from individual microbubbles was detected by capturing data only when a certain amplitude threshold had been exceeded. The stored data were transferred from the oscilloscope to a PC where they were analyzed using Matlab (v.11a, The MathWorks Inc.). The power Fourier transform for each signal was taken and summed within the 6dB frequency bandwidth to obtain an estimate of the scattered power corresponding to the driving frequency. The scattered power depends not only on the viscosity of the microbubble coating but also the effective elasticity (see below), as well as the size of an individual microbubble. In addition to the high speed camera imaging therefore, size distributions of the different composition populations were determined prior to the scattering measurements in order to ensure they were matched as closely as possible. Optical images of the microbubbles were taken using brightfield light microscopy (DM500, Leica Microsystems Ltd.) with a 40x objective (0.8 N.A.) (LUMPLFLN 40XW, Olympus Corp.). The size distributions were subsequently determined using purpose written code in Matlab (20). This provided an average Log-normal size distribution of 5.5 μm for all compositions (n>300) (Fig. S5D). These data were used in theoretical simulations as described below to determine the expected sensitivity of the scattered pressure to the coating viscosity. Finally, the entire experiment was repeated 3 times and the trend seen in this work was consistent.

**Comparison of theoretical simulations of microbubble response to experimental results.**

Simulations were performed to determine the sensitivity of the microbubble acoustic response to the bubble size distribution and to variations in the coating characteristics. The theoretical results were then compared with those obtained experimentally. The response of a population of microbubbles having the same size distribution and driving pressure as measured in the experiment, were simulated using a modified-Rayleigh-Plesset equation (Eq. 5) describing encapsulated microbubbles with linear viscoelastic coatings characterized by parameters viscosity (μ_σ) and shear modulus (G_s) (21). This model was selected in order to maintain consistency with the calibration of the molecular rotor on the basis of shear viscosity. As discussed above, however, there is some uncertainty as to the relationship between macroscopic shear viscosity, as measured using conventional methods, and that determined by molecular rotors. It is appropriate therefore to consider μ_σ and G_s as effective viscosity and elasticity parameters, rather than quantities which could be measured on a sample of the bulk coating material.

It is also important to note that Eq. 5 describes the microbubble coating as a thin shell and is valid for small amplitude oscillations. Alternative equations of motion, wherein viscous dissipation is described in terms of a 2D membrane dilatational viscosity, have also been proposed in the literature and are likely to be more appropriate for a surfactant monolayer (22, 23). Since the results of this study and others have indicated that the microbubble coating may not be a simple monolayer, however, we elected to retain the linear viscoelastic shell treatment.

\[
\ddot{R}R + \frac{3}{2} \dot{R}^2 - \frac{P_0 - P_L + P_I(t)}{\rho} = \frac{R}{\rho c} \ddot{P}_L = 0 \quad [5]
\]
\[ P_z = -4\mu \left( \frac{\dot{R}}{R} \right) - \Delta T_s + P_0 \left( \frac{R_0}{R} \right)^3 \]  \[ \text{[6]} \]

\[ \Delta T_s = \frac{12ds}{R_0} \left( \frac{1}{1+x} \right)^4 \left( G_s x + \mu s \dot{x} \right) \]  \[ \text{[7]} \]

\[ x = \frac{R}{R_0} - 1 \]  \[ \text{[8]} \]

The Eq. 6-8 were solved using ODE45, an explicit 4th order Runge-Kutta algorithm, in Matlab (v.11a, The MathWorks Inc.) using a variable time step and parameter values (see Table S1).

The mean shell viscosities measured in the experiments were used for \( \mu_r \) while the modulus was varied between 10 and 70 MPa. It was not possible to vary the effective elasticity independently of the coating viscosity or to measure it directly. It was assumed, however, that it would scale in a similar manner to viscosity as it is will be similarly dependent upon the surface molecular concentrations of the different coating components (23). The model was run for bubbles following the same size distributions as measured in the experiments (Fig. S5D) and the scattered power at the fundamental frequency (6dB) plotted for comparison against experimental results (Fig. 2D). As may be seen, there was good agreement between the theoretical and experimental results with large standard deviations in the data being produced by variations in either the bubble size and/or the coating parameters. This further demonstrates the advantage of using a direct measurement technique such as FLIM to measure coating parameters rather than relying on acoustic methods where the measured variable is dependent upon several parameters whose effects cannot readily be decoupled.

**Further lifetime analysis of individual microbubble lifetimes.** When analyzing the lifetime data for individual microbubbles, we have detected heterogeneity in the lifetime distribution within shells. The analysis of the asymmetric histograms was simplified by extraction of the histogram lifetime data to Origin software (Origin Pro 8, Origin Lab). The peak fitting in Origin provided the justification for deciding on the color scheme in the FLIM images.

*Two non-contact DSPC microbubbles of a similar size (Fig 3A):* Two distinct Gaussian peaks are clearly apparent in Fig 3B therefore no hidden peak fitting was required to assign the seismic color hue mid-point for the individual bubbles.

*A single DSPC microbubble with heterogeneity within the lipid shell (Fig 3C):* The heterogeneity in the shell of a single DSPC bubble Fig 3C showed some zonal differences that required further investigation, however the differences in lifetimes were not as obvious from the lifetime histogram as in Fig 3A. However, by eye the lifetime histogram was asymmetric (Fig. S6), which suggested that there was more than one distinct population within the shell. Using a Gaussian fitting algorithm we confirmed that the histogram was indeed asymmetric, Fig S6, and that two lifetime peaks were observed at 3.4 and 3.7 ns, corresponding to the distinct viscosities of 670 and 760 cP, respectively.

*Multiple DSPC microbubbles in contact - Touching bubbles (Fig. 4):* The multi-peak analysis was applied to the lifetime histograms for bubbles 2 and 3 in Fig. 4B. For bubble 2 two peaks can be clearly seen, with maxima at 2.9 and 3.6 ns, which originate from two lifetime (viscosity) populations within that microbubble (Fig. S7A). Likewise, bubble 3, although less distinct, reveals two peaks within the lifetime histogram with maxima at 2.9 and 3.9 ns (Fig. S7B). Importantly, there is a common cross over space, between 3.1 and 3.4 ns for these separate lifetime populations in bubbles 2 and 3, Fig S7. This common cross over space suggested to us that there are two very distinct regions of viscosity, corresponding to the lifetimes below 3.1 ns (lower viscosity) and those with lifetimes above...
3.4 ns (higher viscosity). We applied the red/blue color hue (with separator at 3.1-3.4 ns) to the whole image in Fig 4. It can be clearly seen that microbubble areas in contact with the neighboring bubbles appear as blue (lower viscosity, lifetimes below 3.1 ns), whilst areas of bubbles that are contact free are red (higher viscosity, lifetimes above 3.4 ns) (Fig. 4A). In spite of the fact that domains of varied viscosity ranging between 300 and 1200 cP were detected in this study, we note that it is possible that the rotor selectively avoids some lipid domains of extremely tight packing.

References


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**SI Materials and Methods**

**BODIPY-C10 synthesis and spectroscopic characterization.** BODIPY-C10 was synthesized by minor modifications to the previously reported procedure (1). Spectroscopic data: \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta 7.93\) (s, 2H), \(7.61 – 7.49\) (m, 2H), \(7.03\) (ddd, \(J = 18.0, 9.9, 3.4, 4H\)), \(6.56\) (dd, \(J = 4.1, 1.5, 2H\)), \(4.06\) (t, \(J = 6.5, 2H\)), \(1.93 – 1.79\) (m, 2H), \(1.60 – 1.21\) (m, 17H), \(0.89\) (td, \(J = 6.8, 2.1, 4H\)). Mass spectroscopy time-of-flight electron spray (TOF-ES)+ identified the mass as \((M+H^+)\) 424.2507; calculated \(C_{25}H_{31}N_2OF_2B\) \((M+H^+)\) 424.2498. The absorption spectrum of BODIPY-C10 in MeOH possesses a sharp peak at 495 nm \((\epsilon = 6.4x10^5\ M^{-1}\ cm^{-1})\), typical of BODIPY dyes, Fig. S2. Its emission spectrum is characterized by a broad featureless band \((\lambda_{\text{max}} = 515\ nm)\), Fig S2. Excitation spectrum matches the absorption spectrum of BODIPY-C10 in MeOH. Absorption spectra were recorded with a UV-Visible spectrophotometer (HP8453, Agilent Technologies Ltd., UK). Molar absorption coefficient \((\epsilon)\) was calculated by applying the Lambert-Beer law to the absorbance spectra \((A_{\text{max}} < 1.5)\) of the compound. Steady-state photoluminescence spectra were measured in right angle mode with a HORIBA FluoroMax®-4 using 1 cm quartz cuvettes (Starna Scientific Ltd.). The concentration of the air-equilibrated sample solutions was adjusted to obtain absorption values \(A < 0.1\) at the excitation wavelength. Emission quantum yields were determined using fluorescein \((\Phi_f = 0.95)\) (2) as standard.

**Evaluation of lipid/dye and dye-dye interaction.** In order to determine the optimal concentration of BODIPY-C10 in a lipid shell we performed a series of lifetime and intensity measurements on DSPC lipid microbubbles containing different molar ratios of [lipid]/[dye]. By maximizing the concentration of dye in the lipid shell we were able to obtain brighter images, reduce acquisition time and minimize laser exposure to the sample. At the same time, we had to ensure that the high concentrations of the dye did not lead to aggregation, self-quenching and other competing photochemical processes which might compromise viscosity determination according to the calibration graph.

We prepared the solutions of microbubbles with lipid/dye ratios ranging between 3600:1 to 28:1 using the T-junction production method as described in the methods section (main text) and recorded fluorescence lifetime and intensity images of individual microbubbles. A x20 (0.7 N.A.) HCX PL APO CS dry objective lens (11506513, Leica Microsystems Ltd.) was used for collecting. The PMT gain and offset were fixed for the duration of the experiment, as was the z-position of the objective in the axial plane to maintain identical settings.
Concentrations of less than 2 μM (>2000:1) were not suitable for effective visualization, failing to produce reasonable signal/noise in the image within the experimental collection time of a few minutes. We recorded a linear increase of the average image intensity until 17 μM [BODIPY-C<sub>10</sub>], (200:1 [lipid]/[dye]), Figure S3, which is expected when aggregation or quenching is absent. Above 17 μM [BODIPY-C<sub>10</sub>], no further intensity increase was observed. At the same time, at dye concentrations greater than 17 μM bi-exponential decays were observed, indicative of quenching and interactions of neighboring dyes (Fig. S3). To avoid these effects all further experiments were carried out at a 900:1 [lipid]/[dye] ratio. During production of these microbubbles, T-junction parameters for flow and pressure where maintained indicating that the increasing dye concentration had little or no effect on the microbubble surface tension.

**Evaluation of viscosity dependence on the microbubble morphology and size.** Previous studies (3) have shown that microbubble ultrasound response is size-dependent and it has been suggested that this is not due merely to the effect upon the resonant frequency of the bubbles but that the coating properties may themselves be size dependent. We have therefore investigated whether the shell viscosity, as measured by molecular rotors, can be correlated to the microbubble size. Microbubble size was determined using Leica LAS software. These measurements were initially verified by importing a brightfield image into Fiji (4) and a line plot profile was drawn across the image (Fig. S4A). The graph produced two minima locations associated with the microbubble edge (Fig. S4B); the distance between these two minima matched exactly the measurements by Leica LAS. All further microbubble diameters were analyzed using the minima positining in the LAS software and values then correlated to viscosity (Fig. S4D).

The relationship between microbubble diameter and viscosity was tested using the Spearman correlation test, which found that there was no correlation between viscosity and microbubble size for all composition except DSPC (p=0.046) and PEG 1:1 (p=0.007), and here the level of correlation was low (0.39). All statistical analysis was completed using Origin scientific software (OriginPro 8.5, OriginLab Corporation). As could be seen from Figure S4, we did not detect any correlation between the shell viscosity of the microbubbles of various compositions and the microbubble size.

**Evaluation of bulk viscosity and surface tension dependence on the dye concentration.**

The meaningful measurements of viscosity was only possible above 100:1 [lipid]/[dye] ratio (Fig. S3), where a monoexponential fluorescence decay was detected for BODIPY-C<sub>10</sub>. We have compared the lifetimes of BODIPY-C<sub>10</sub> between 200:1 and 3600:1 ratios and found that they are identical. Thus in this broad concentration range there was no discernible effect of dye on the measured effective viscosity in the lipid shell. While no measurements are possible without the probe, we believe that it is reasonable to extrapolate this trend to zero concentration of the dye and assume that the dye has no effect on the viscosity of the microbubble shell.

We also conducted direct viscosity and surface tension measurements on bulk samples for lipid/dye ratios between 3600:1 and 28:1, using a force tensiometer (Sigma 702, Attension Biolin Scientific) and micro viscometer (516 10/I, Ostwald), respectively. The surface tension remained stable at 35.33 (s.d. 0.9) mN/m and the viscosity was constant at 4.17 (s.d. 0.16) cP across the tested range of lipid/dye ratios, Fig. S8, confirming the insignificant effect of dye loading on the bulk properties of the microbubble solutions.

**Evaluation of effect of laser irradiation on bubble morphology**

We have established that high levels of two-photon irradiation cause bubble swelling and shedding of lipids from the shell (Movie S1). Thus we have restricted ourselves to <400 mW powers, which were found to have no adverse effect on bubble morphology (Movie S2).
L-α-PC is a naturally occurring lipid containing molecules with a range of chain lengths (Fig. S9A). It is used here in hydrogenated form in order to minimize the presence of double carbon bonds which would prevent close packing on the bubble surface. The structure of predominant species is shown below. This lipid is extremely close in structure to DSPC (Fig. S9B) but DSPC consists of molecules with a more uniform chain length. The gel transition temperatures for L-α-PC and DSPC are 45-65°C and 55°C respectively.

**Statistical analysis.** Sample populations were found to be normal distributed with the exception of the DSPC microbubbles prepared via the T-junction method and sonicated DSPC:PEG 1:1 bubbles. Population means were tested with a parametric one-way ANOVA mean comparison Fisher test and non-parametric tested using a one-way Kruskal-Wallis ANOVA, both tests showed that the population means were significantly different ($\chi^2 << 0.001$, $p << 0.0001$; alpha 0.05). The only exception is the comparison between DSPC and L-alpha sonicated microbubbles (see Fig 2a main text). Scattered data were tested and shown to be not normal distributed, therefore a Kruskal-Wallis ANOVA test was applied to the populations, which were shown to be significantly different ($\chi^2 =0.0176$).
Fig. 1. BODIPY-C₁₀ fluorescence lifetime calibration vs. viscosity. (A) Selected fluorescence decay traces recorded in methanol/glycerol mixtures (30-95% glycerol) of different viscosity. (B) The logarithmic calibration plot of measured lifetimes vs. viscosity plotted according to the Förster-Hoffmann equation, which was fitted with a linear fit (dash-dot line) and found to be linear between 7.7 and 1140 cP. Chemical structure of BODIPY-C₁₀ is also shown.
Fig. 2. Quantification of microbubble shell viscosities. (A) Viscosity dependence on microbubble manufacturing method as measured by BODIPY-C<sub>10</sub> lifetime, comparing sonication (●) (n=28) to T-junction preparation (●) (n=19) ("p << 0.001). (B) Effect of microbubble lipid composition on viscosity for DSPC (●) (n= 28), L-alpha-PC (▲)(n= 27). (C) Viscosity dependence on microbubble coat composition: PEG 1:9 DSPC (▼) (n=82), PEG 1:4 DSPC (●)(n= 41), PEG 1:1 DSPC (♦)(n= 46); Boxes show 25-75% of microbubbles, whiskers are the standard deviation and thick bar indicates the
mean value (* p << 0.001). (D) Normalized scattered power at the fundamental frequency (2.25MHz) for ratios of PEG 1:9 DSPC, PEG 1:4 DSPC, PEG 1:1 DSPC (n= 150,80, 69) (* χ²< 0.05) are compared between experiment (closed symbol) and simulations (open symbol) using a modified Rayleigh Plesset equation. The simulations use an elastic modulus and viscosity respectively of 70 MPa and 507cP (1:9), 40 MPa and 426 cP (1:4), 10 MPa and 337 cP (1:1).
Fig. 3. In-depth individual microbubble analysis. (A) Brightfield (scale bar = 20 \( \mu \)m) overlaid with confocal fluorescence images of two individual DSPC microbubbles of a similar size containing BODIPY-C<sub>10</sub>. Also shown are the results of FLIM data analysis: fluorescence intensity (amplitude), chi square (goodness of fit, \( \chi^2 \) = 0.8-1.2) and lifetime distribution (\( \tau = 2.5 - 5 \) ns). (B) Analyzing lifetime histogram from bubbles in (A) shows two distinct peaks with maxima at 3.25 ns and 4.2 ns. Applying a
seismic color hue mid-point at 3.75 ns identifies that short (blue) and long (red) lifetimes are originating from the two different microbubbles. The lifetime distributions and viscosities for each bubble and the whole image are shown on a Box plot. (C) Individual microbubble with heterogeneous lifetime morphology across the shell. Same displayed information as in (A) (scale bar = 20 μm, \( \chi^2_r = 0.8-1.2, \tau = 3-4 \) ns). (D) Masking of high lifetime variation zones A (green) and B (blue) from the lifetime image (C) allows lifetime/viscosity determination in each area. Separately binning all pixels in zones A and B identifies two clearly distinct mono-exponential decays, as shown. The analysis of each decay along with the whole image is shown on a Box Plot.
Fig. 4. Investigation of microbubble interactions. (A) Brightfield (Scale bar = 20 μm) overlaid with confocal fluorescence images of multiple touching DSPC microbubbles containing BODIPY-C₁₀. Also shown are the results of FLIM data analysis: fluorescence intensity (amplitude), chi square (goodness of fit, $\chi^2$ 0.8-1.2) and lifetime distribution ($\tau$ = 2.6 - 4.4 ns). Lifetime map with seismic color hue centrally located between 3.1 - 3.4 ns. (B) Lifetime histograms and zoomed in FLIM images of the individual masked microbubbles. Individual histograms of four bubbles (black, red, green and blue) are displayed against histogram of all lifetimes in the image (grey). The rainbow legend on far right represents lifetime variation in the individual lifetime microbubble images. The orange band represents a border region between bubble regions with and without contacts, which is also shown as a white strip in the seismic image in (A).
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**Fig. S1.** Overview of microbubble processing and imaging. a, Lipid microbubble hypothesized structure and ultrasound response. Shown here are the responses to ultrasound of two bubbles determined using high speed video microscopy. b, A diagrammatic representation of the two microbubble preparation processes, T-junction microfluidic preparation and sonication (see Supplementary Methods). c, Brightfield/fluorescence image of a microbubble containing embedded BODIPY-C10 (top) with a lifetime image of BODIPY-C10 showing viscosity distribution in a microbubble (bottom).
Fig. S2: Spectroscopic characterization of BODIPY-C_{10}. Absorption (black) and steady-state fluorescence (red) and excitation (blue) spectra of BODIPY-C_{10} in MeOH.
Fig. S3: Determination of maximum dye loading into microbubble lipid shell. a, BODIPY-C10 intensity (*) and averaged lifetime (■) as a function of increasing dye/lipid ratio. b, Representative intensity images and associated lifetime decays of bubble shells containing 2, 17 and 68 μM of BODIPY-C₁₀.
Fig. S4: Relationship of microbubble diameter with viscoelastic properties. a-c, Determination of microbubble size. a, Brightfield image of microbubble with line drawn across it (Scale bar 20 μm). b, Intensity (Grey) scale of line plot across microbubble visualizing the minima points, identified by arrows, across the bubble. c, Line plot across microbubble obtained in Leica LAS software, using minima points as the reference for the edge of bubble. d, Variation of microbubble viscosity with size for different compositions and manufacturing protocols (DSPC/Sonicated ■, L-alpha PC ●, T-junction ▲, PEG 1:9 DSPC ▼, PEG 1:1 DSPC ◆, PEG 1:4 DSPC ◀).
Fig. S5: Experimental setup to measure scattered pressure from single microbubbles. a, A 2.25 MHz focused transducer is used to excite a microbubble passing through the coaxial flow. The scattered pressure is then detected using a focused 3.5 MHz transducer. Side on view shows a zoomed in area of the coaxial flow. b, The pressure waveform used to excite the bubbles as measured at the focal region with a calibrated needle hydrophone. c, Corresponding frequency power spectrum of pressure waveform showing narrowband response. d, Microbubble size distribution fitted to Log-normal distribution curve for solutions PEG 1:1 DSPC (n= 417; turquoise), PEG 1:4 DSPC (n = 276; blue), PEG 1:9 DSPC (n = 342; pink).
**Fig. S6:** Evaluation of lifetime histogram of a single non-contact DSPC microbubble with heterogeneous lifetime distribution in the shell. Two Gaussian peaks (green lines) have been fitted to the lifetime histogram (black line) of the full lifetime image from Fig.3c to separate the lifetime populations and produce cumulative peak fit (red line) that matches the lifetime histogram.
Fig. S7: Evaluation of lifetime histograms of two DSPC bubbles that are in contact with neighboring bubbles. Lifetime histograms (black line) of bubbles with applied peak fitting (green line) and the representative cumulative peak fit (red line). a, Bubble 2. b, Bubble 3.
Fig. S8: Effect on surface tension and viscosity of increasing the microbubble lipid/dye ratio in bulk samples; ■ surface tension (n=2, repeats =2) ● viscosity (n=3).
Fig. S9: Chemical structure of the phospholipids used to create microbubbles. (A) L-α-PC. (B) DSPC.