Microbubbles containing lysolipid enhance ultrasound mediated blood-brain-barrier breakdown *in vivo*

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Graphical Abstract

Abstract

Ultrasound and microbubbles (MBs) offer a non-invasive method of temporarily enhancing blood-brain barrier (BBB) permeability to therapeutics. To reduce off-target effects, it is desirable to minimize the ultrasound pressures required. We have shown that a new formulation of MBs containing lysolipids (Lyso-MBs) can increase the cellular uptake of a model drug *in vitro*. Our aim in this study was to investigate whether Lyso-MBs can also enhance BBB permeability *in vivo*. Female BALB/C mice were injected with either Lyso-MBs or control MBs and gadolinium-DTPA (Gd-DTPA) and exposed to ultrasound (500kHz, 1Hz pulse repetition frequency, 1ms pulse length, peak-negative pressures 160-480kPa) for 2 minutes. BBB permeabilisation was measured via magnetic resonance imaging (7.0T) of Gd-DTPA extravasation and subsequent histological examination of brain tissue to assess serum immunoglobulin G (IgG) extravasation (n=8 per group). A ~2 fold enhancement in BBB permeability was produced by the Lyso-MBs at the highest ultrasound pressure compared with the control. These findings indicate that modifying the composition of phospholipid-shelled microbubbles has the potential to improve the efficiency of BBB opening, without increasing the ultrasound pressure amplitude required. This is particularly relevant for delivery of therapeutics deep within the brain.

Introduction

The blood-brain barrier (BBB) is a selective barrier between the systemic circulation and the cerebrospinal fluid that regulates brain homeostasis and prevents uncontrolled flow of substances from the blood to the brain.^{[1][2]} As a morphological and physiological barrier, the BBB exists to protect the brain.^[3–5] Consequently, however, it presents a significant challenge for drug delivery to the brain as the vast majority of large molecule drugs and over 98% of small molecule drugs cannot cross it.^[6] In addition, the components of the BBB continuously adapt in response to various physiological changes in the brain and these adaptations can act to further frustrate drug delivery.^[7] For example, multiple major trans-membrane efflux pumps are upregulated at disease sites^[8–11] and studies have shown that this directly limits the efficacy of drug delivery,^[12,13] particularly in brain tumours.^[14]

Ultrasound-mediated blood-brain barrier disruption (US-BBBD) is a promising minimally-invasive approach for increasing the permeability of the blood brain barrier to therapeutics in a localised and targeted manner. As this technique is not specific to the therapeutic agent delivered, it has the potential to transform the treatment of a wide range of brain diseases and disorders by allowing free passage of molecules up to 2000 kDa into the brain.^[15,16] Demonstration of US-BBBD was first published almost immediately after the clinical approval of the ultrasound contrast agent Optison.^[17] The combination of microbubbles (MBs) and ultrasound provided repeatable BBB opening without damage to surrounding tissue. This could not be accomplished with focused ultrasound alone.^[17,18]

The safety of US-BBBD has been assessed in multiple models, including rats,^[19] rabbits,^[20,21] primates^[22,23] and humans,^[24] Under appropriate exposure conditions, US-BBBD has been shown to be a transient and reversible process. The time reported for the BBB to close after sonication does, however, vary considerably between animal models and even between different studies in the same model. For example, times of 3-8 hours^[25,26] have been reported in rabbits, 12 minutes-72 hours in mice^[27,28] and rats^[19,29] and up to 6 days in primates^[23]. Alternative criteria have also been used to

assess safety, including formation of histological lesions, changes in behavioral and/or motor function and changes in brain function assessed via Electro-encephalography (EEG), Positron Emission Tomography (PET) or Magnetic Resonance Imaging (MRI) Imaging. Most studies have reported only mild side-effects. It should be noted however that there have been more serious side-effects reported in some patients in recent clinical trials^[30].

US-BBBD has been reported to cause between a 30 and 500% increase in the uptake of chemotherapeutics in the brain, a measure that has been widely associated with increased survival. Enhanced delivery of multiple drugs, including doxorubicin,^[19,31–35] paclitaxel,^[36] temozolomide,^[37,38] and carboplatin^[39,40] has been shown. This has led to improved tumour control in animal models being reported in multiple studies^[32,34,37,41–43] and both progression-free and overall survival of human patients being dramatically increased.^[44] US-BBBD has also been used to enhance the delivery of other classes of therapeutics, including antibodies^[15,45] and genes.^[46,47]

Ultrasound contrast agents, consisting of MBs with a gas core surrounded by a lipid, protein or polymer coating,^[48–50] have been the most widely studied mediators of US-BBBD.^[51] At clinically relevant ultrasound frequencies, the pressures required to produce therapeutic effects are typically less than 1 MPa. This is notably lower than the majority of other cavitation promoting agents.^[51] Moreover, MBs are ‘theranostic’ agents, providing simultaneous and co-localized imaging and therapeutic delivery.^[52]

A significant challenge for the clinical application of US-BBBD is creating an ultrasound field of sufficient intensity to cause therapeutic effects at locations deep within the brain without producing harmful effects in the overlying tissue, e.g. pre-focal skin and tissue heating or off-target BBBD. Whilst many studies have been published that optimise the ultrasound parameters for different drug delivery applications,^[53–57] the studies that have compared MB formulations have predominantly compared size distributions,^[58,59] with few investigations examining the influence of MB composition.^[60]

Recently, however, it has been shown that lipid molecules transfer from microbubbles to cell membranes both before and during ultrasound exposure, and that this transfer modifies the structural properties of the cell membranes.^[61,62] This effect has been exploited to improve drug delivery by developing microbubbles (Lyso-MBs) containing lysolipids, whose transfer causes an increase in cell membrane permeability.^[62] These Lyso-MBs have been shown to cause a ~5-fold increase in the cellular uptake of a model drug *in vitro* compared to MBs containing no lysolipid (Control-MBs).^[62] Importantly, these Lyso-MBs were also shown to be non-toxic in the absence of ultrasound.^[62] The detailed mechanisms of US-BBBD have yet to be conclusively established, but both transcellular transport and tight-junction disruption have been proposed^[59] and hence lysolipids might be expected to have a similar effect upon BBB permeability; by enabling either endothelial cell membrane poration or disruption of the lipid structures comprising tight junctions. The aim of this study was to determine whether or not Lyso-MBs are capable of increasing US-BBBD when compared to Control-MBs.

Materials and Methods

Overview of experimental procedure

All animal experiments were approved by the University of Oxford Clinical Medicine Ethics Review Committee and the UK Home Office (Animals [Scientific Procedures] Act 1986), and conducted in accordance with the University of Oxford Policy on the Use of Animals in Scientific Research and the ARRIVE Guidelines under Project Licence number PC4932237.

US-BBBD was investigated by measuring both gadolinium-diethylenetriamine pentaacetate (Gd-DTPA) uptake into the brain parenchyma and serum immunoglobulin G (IgG) extravasation via magnetic resonance imaging (MRI) and histology respectively. Female BALB/c mice (8-11 weeks old, Charles River, UK) were anaesthetized using isoflurane (1.5-2.0%) in 30% O₂: 70% N₂O. The

top of the scalp was shaved and the mouse was then placed in a purpose built MRI cradle that was compatible with the ultrasound set-up used (Figure 1B). The position of the mouse skull relative to the MRI cradle during ultrasound exposure was kept constant via a stereotaxic frame, enabling comparison of the pre- and post- ultrasound MRI scans and thus allowing quantification of US-BBBD from image hyperintensity caused by Gd-DTPA uptake into the parenchyma. This set-up also enabled anaesthesia to be maintained throughout ultrasound exposure.

Animals were first placed in a 7.0 T horizontal bore magnet (Agilent Technologies Inc., US) and a first set of MRI scans acquired (see below). Following this, animals were transferred to the ultrasound setup before 3 minutes of ultrasound exposure (0.5MHz, 1Hz pulse repetition frequency (PRF), 10 ms pulse length, various pressures (Table 1)) to account for the highly variable endogenous acoustic response. Immediately prior to the injection of $\sim 10^8$ microbubbles (either Lyso-MBs or Control-MBs), 30 μ l of Gd-DTPA was injected via a tail vein. Ultrasound exposure continued for a further 2 minutes whilst the microbubbles were circulating. The cradle was then placed back into the magnet and a second MRI acquisition performed using the same settings as for the first scan. Mice were then sacrificed using an overdose of pentobarbitol administered via intraperitoneal injection, followed by transcardial perfusion fixation to preserve the brain for histological analysis. Comparison of the pre- and post- ultrasound MRI scans allowed quantification of Gd-DTPA uptake in the brain parenchyma. A summary of this timeline can be found in Figure 1A.

Ultrasound exposure

Ultrasound exposure was carried out using a purpose built setup that allowed reproducible treatment of mice whilst in an MRI cradle (Figure 1B). The set-up consisted of a 500 kHz transducer (H107, Sonic Concepts, US), mounted directly below a holder for the MRI cradle. Transducer alignment was conducted prior to the animal experiments by maximising the reflection signal from an alignment target located at the position where the mouse's head would be positioned. The MRI cradle was mounted on a 3-axis bed positioner which was locked in place once alignment was completed.

Animals were held in position in the cradle using cheek bars for the duration of all experimentation so that the US focus was aligned with the brain at the mid-point between bregma and lamda. Animals were inverted to allow ultrasound to pass through the top, rather than the bottom, of the skull and coupled to the mylar window using degassed ultrasound gel. In water, the focal volume had lateral and axial full width half amplitude dimensions of 4.1 mm and 25.2 mm respectively (as measured using a needle hydrophone, Precision Acoustics, Dorset, UK). Some attenuation and distortion of the field upon propagating through the skull and internal anatomical structures would be expected. Choi et al.^[63] reported a ~18% reduction in pressure at a frequency of 1.5 MHz; whilst Ilovitsh et al.^[64] reported ~0 transmission loss at 250kHz. Thus attenuation of <10% would be expected at 500 kHz, which is within the measurement uncertainty of the hydrophone used to calibrate the transducer. Similarly attenuation due to transmission through the water bath, coupling gel and mylar window would be expected to be comparatively negligible.

Figure 1: Methods and materials. (A) Experimental timeline of the experiments comparing US-BBBD potential of Lyso-MBs and Control-MBs. (B) Schematic diagram of the key components in the set-up used to expose mice to ultrasound. (C) Diagram showing key elements of a representative PCD trace. To reduce the influence of acoustic emissions arising from elsewhere in the animal, the background PCD noise was subtracted (a power spectrum subtraction) from the acoustic signature from the microbubble injection. The acoustic signature of the animal often had a greater power amplitude than the subsequent microbubble injection.

MB preparation

Two MB formulations were used in this study (Figure 3). Control-MBs consisted of 1,2-dibehenoyl-sn-glycero-3-phosphocholine (DBPC) and polyethylene glycol (40) stearate (PEG40S) in a 9:1 molar ratio. Lyso-MBs contained DBPC, PEG40S and 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (LysoPC) in a 9:1:4 molar ratio.

Control-MBs

DBPC, PEG40S
9:1

Lyso-MBs

DBPC, PEG40S, LysoPC
9:1:4

Figure 2: The constituents and their molar ratios in the two MB formulations used in this study. Control-MBs are formed from DBPC and PEG40S. Lyso-MBs are formed from DBPC, PEG40S and LysoPC.

The MBs were manufactured using a previously reported protocol.^[62] Briefly, the lipids (Avanti Polar Lipids Inc., US) and PEG40S (Sigma Aldrich, US) were added to a glass vial in the relevant molar ratios such that the total weight of DBPC and PEG40S was 20 mg. This mixture was then heated on a hotplate at 58°C for 12 hours, to allow the chloroform (in which the lipids are supplied) to evaporate. This lipid film was then suspended in 5 mL PBS for 1 hour on a hotplate at 85°C under constant magnetic stirring. Lipids were then dispersed for 90 seconds using a sonicator (XL 2000, probe diameter 3 mm, 20 W, 22.5 kHz, Misonix Inc., US) with the tip completely immersed in the lipid-PBS solution (power setting 4). The glass vial was then placed in an 85°C water bath for 60 seconds

to keep the solution above the melting point of the lipids. Finally, the solution was sonicated for a further 30 seconds at power setting 4 to ensure homogeneous dispersion.

MBs were then formed by placing the sonicator tip at the air-water interface under constant sulphur hexafluoride (SF₆) flow (The BOC Group plc, UK) and sonicating for 30 seconds (power setting 14). Immediately after production, the vial containing the MB suspension was capped and placed in ice for at least 20 minutes. For the experiments described in this study, no further processing of the MBs was undertaken, as the previous *in vitro* work indicated that centrifugal washing of the bubbles had a negligible effect on sonoporation; and similarly neither free lysolipid nor micelles formed by lyso-MB destruction had a statistically significant effect on membrane permeability compared to Control-MBs.^[62]

Measuring MB size and concentration

For quantification of microbubble size and concentration, 10 µL of the microbubble suspension was transferred onto a Neubauer improved cell counting chamber (Hausser Scientific Company, US) under a 24 mm × 24 mm glass coverslip (VWR International, US). At least 10 images of microbubbles were acquired at 40x magnification using a Leica DM500 microscope (Leica Microsystems GmbH, Germany) coupled with a CCD camera (MicroPublisher 3.3 RTV, QImaging, Canada). Microbubble sizing and counting was performed using a purpose-written code in MATLAB® (The MathWorks Inc., US).^[65] Dilution for concentration matching was always performed immediately prior to experimentation to limit the effects of dilution on MB size distribution, concentration, and stability. Further details on Lyso-MB characterization may be found in Aron et al. ^[62]

MRI analysis of Gd-DTPA uptake into the brain parenchyma

All MRI scans were performed using a horizontal wide-bore superconductive 7.0 T MRI system (Agilent Technologies Inc., US) with a 26mm inner diameter transmit/receive birdcage RF coil (Rapid Biomedical, Germany). All mice were imaged pre- and post- Gd-DTPA injection/ultrasound

exposure using a T₁-weighted spin echo multi slice (SEMS) sequence. The parameters were repetition time (TR) = 500ms, echo time (TE) = 8ms, number of averages = 4, field of view = 22.5 x 22.5 x 22.5 mm³ and acquisition matrix = 128 x 128 x 32 (each MRI voxel had dimensions = 0.176 x 0.176 x 0.7mm). The total scan time was 4 minutes and 24 seconds.

The brain was manually masked in the pre- and post-Gd-DTPA scans. To analyse differences in BBB breakdown over the entire brain, grey intensity on the pre- versus post- treatment scan was measured and compared using a purpose written Matlab® (Mathworks, US) script. Average background intensity per slice was calculated from the pre-treatment dataset and these averages were corrected for the contrast change induced by Gd-DTPA injection in the absence of BBB breakdown (calculated from naïve animals pre- vs. post-Gd-DTPA images). On the post-treatment images, voxels with values greater than 3 standard deviations above the corrected mean of the pre-treatment scan (thus indicating enhanced Gd-DTPA uptake into the parenchyma) were considered to be hyperintense voxels.

To generate the US-BBBD heat maps (Figure 3), Matlab® was used to spatially align the centres of the brains in the sagittal, transverse and coronal planes. As all animals were the same age at the time of treatment, the variation in brain sizes was no greater than ± 1 MRI voxel in any plane. Once the brains were aligned, the number of hyperintense voxels could be summed for each projection. Thus, the heatmaps in Figure 3 show the total number of hyperintense voxels across all animals for each projection.

Histological analysis of extravasated serum IgG

Following perfusion fixation with 0.9% saline followed by periodate-lysine-paraformaldehyde (PLP) containing 0.1% glutaraldehyde (PLP_{light}), brains were removed from the skull and kept in PLP_{light} for 4 hours, after which they were placed in a 40% sucrose solution until they sank to cryoprotect. Brains were frozen in isopentane and stored at -20°C. Each brain was sectioned at 20 μ m thickness,

across the region of the brain containing the striatum, and every 10th section was stained, as below, at 200 µm intervals.

Slides were then air dried and rehydrated in PBS, placed in 1% H₂O₂ in methanol for 20 minutes, and washed in PBS and PBS-tween. After blocking in 10% Tris-NaCl-blocking buffer (TNB), slides were washed in PBS, and then exposed for 45 minutes to a secondary antibody (biotinylated anti-mouse IgG) at a dilution of 1:100 in PBS. They were then washed, incubated with an Avidin-Biotin Complex solution (Vector Labs, UK) and washed again. Slides were then exposed to 3,3-diaminobenzidine (DAB) peroxidase substrate solution (Sigma Aldrich, US) in 0.1M phosphate buffer, counterstained using cresyl violet, dehydrated in an increasing concentration of alcohol followed by Xylene, and mounted in dibutylphthalate polystyrene xylene (DPX).

Images of the histology sections were analysed using Aperio ImageScope® (Leica Biosystems, Germany) using a previously published method.^[66] The digital images were visualised using the Imagescope® software and manually annotated to outline the entire section as a region of interest. DAB staining was quantified using the in-built Positive Pixel Count v.9 algorithm, with pixels having a value between 0-100 in the red channel (corresponding to the areas with dark DAB staining) considered to represent serum IgG extravasation. The number of these strong-positive intensity brown pixels was then summed for each section to determine the corresponding area of BBB breakdown.

Acoustic emissions monitoring

Acoustic emissions monitoring was achieved using a single element passive cavitation detector (PCD) with a 3.5 MHz centre frequency (Olympus, US) and sensitivity down to 1 MHz, co-aligned with the ultrasound transducer. This allowed subsequent analysis of any acoustic emissions from the brain. PCD signals were recorded on a streaming digital oscilloscope (model HS3, TiePie, The Netherlands) that was triggered by the function generator producing the ultrasound exposure signal. Prior to signal recording, the PCD signal was passed through a 500 kHz notch filter (Allen Avionics,

US) to remove scattering at the ultrasound fundamental frequency and, thereby, allow substantially improved use of digitizer dynamic range.

For offline processing, each recorded voltage trace from the PCD was truncated in the time domain to remove the portion of the signal prior to the first reflection from the mouse. A Hanning window was applied to the voltage trace to reduce edge effects. Matlab® was used to compute the fast Fourier transform (FFT) and the power spectral density (PSD) for each voltage trace. The harmonic and ultraharmonic signal powers were determined by integrating the power spectral density with respect to frequency over 30 kHz bands at each harmonic and ultraharmonic from 1 to 8 MHz. The broadband power was determined by integrating the power spectral density with respect to frequency over the remaining frequencies between 1 and 8 MHz. The total energy of acoustic emissions for each sample was calculated by integrating the powers (harmonic, ultraharmonic and broadband) with respect to time. Subtraction of the background PCD noise (recorded immediately prior to microbubble injection) was conducted to remove inter-animal variations in acoustic response (Figure 1C).

Experimental groups

Table 1 outlines each of the experimental groups for which US-BBBD was tested and the number of animals in each group. The no ultrasound, ultrasound only and lyso-PC groups were used to investigate any measurable effect on the BBB of the different MB formulations, the ultrasound alone and free lysolipid respectively.

Condition	Number of animals
No ultrasound	4 with Control-MBs, 4 with Lyso-MBs
160kPa PNP	5 with Control-MBs, 5 with Lyso-MBs
240kPa PNP	5 with Control-MBs, 5 with Lyso-MBs
320kPa PNP	8 with Control-MBs, 8 with Lyso-MBs
480kPa PNP	8 with Control-MBs, 8 with Lyso-MBs
US only (640kPa PNP)	4
Lyso-PC only*	4

*Table 1: A summary of the number of animals in each group. PNP = peak negative pressure. *The injected quantity of free lysolipid was 10 times that in the Lyso-MB suspension.*

Statistical Analysis

All data were analyzed using built-in analysis methods in GraphPad Prism 5.0 (Graphpad Software, San Diego, CA). Mann-Whitney test was used for statistical comparisons. P values were used to assess the significance of the data, where *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. All data with error bars were presented as mean $\pm$ standard deviation of the mean (SD).

Results

Figure 3 shows sagittal, transverse and coronal heat maps of the average distribution of US-BBBD for Lyso-MBs and Control-MBs at the highest pressure tested (as measured by MRI) together with representative T1 weighted images. Figure 4A shows the US-BBBD in each of the experimental groups outlined in Table 1, calculated from the post-Gd-DTPA hyperintensities; the total number of image voxels with an intensity greater than 3 standard deviations above background (hyperintensity) is shown. In agreement with the heat maps in Figure 3, these results show that Lyso-MBs caused significantly more US-BBBD than Control-MBs at 480 kPa ($p = 0.0026$). No significant differences occurred between Lyso-MBs and Control-MBs at any other pressures.

When comparing the results at 320 kPa and 480 kPa, the only significant difference in US-BBBD was seen with Lyso-MBs ($p = 0.0008$). Both MB formulations significantly increased US-BBBD at 240 kPa and 320 kPa, whereas neither MB formulation had a significant effect at 160 kPa and 240 kPa. Only Lyso-MBs showed a significant effect on US-BBBD between the no ultrasound and 160 kPa groups. No significant differences in BBBD were found between the ultrasound only, lysolipid only and MB only groups. Whilst not a direct measure of toxicity, the MRI measurements of BBBD show that Lyso-MBs alone (without ultrasound) resulted in negligible Gd-DTPA uptake across the BBB relative to Control-MBs alone.

Figure 3: Ultrasound-mediated blood brain barrier breakdown in all 8 animals for both Lyso-MBs and Control-MBs at the highest pressure tested (480kPa PNP). The upper plots show sagittal, transverse and coronal projections of the volumetric sum of hyper-intense voxels (as measured by MRI), summed across all 8 animals in each group. The colour scales are equal for each plot to aid visual comparison between the two groups (brighter represents more US-BBBD). An illustrative line is shown around each projection indicating the edge of the brain. These results should be analysed in combination with the statistics in Figure 4A. The lower images show representative MRI images from 3 animals in each group at the highest pressure tested (480kPa PNP).

Figure 4: US-BBBD for each of the conditions in Table 1. At the highest pressure tested (480kPa PNP), Lyso-MBs significantly increased US-BBBD when compared to Control-MBs ($n=8$; $p<0.05$, two tailed t -test) in both (A) and (B). At all other pressures tested, there was no significant difference between Control-MBs and Lyso-MBs. (A) US-BBBD measured by Gd-DTPA uptake into the brain parenchyma. Hyperintense voxels are those with a signal intensity greater than 3 standard deviations above background when comparing the pre-US and post-US images. (B) US-BBBD as measured by immunostaining for extravasated serum IgG. Error bars in both plots represent +1 standard deviation. Histological images can be found in Figure S2. (N.b. for the ultrasound only and lysolipid only groups, no MBs were used).

Figure 4B shows US-BBBD results obtained via immunohistochemical staining of extravasated serum IgG. This technique is complementary to the MRI/Gd-DTPA enhancement results in Figure 4A and the results show a similar trend. Figure 4B suggests that Lyso-MBs caused a significantly greater amount of US-BBBD than Control-MBs at the highest pressure tested, (58.9 ± 28.5 ($\times 10^6$) vs 28.4 ± 31.3 ($\times 10^6$), $n = 8$, $p < 0.05$) at the highest pressure, in agreement with Figure 4A.

Figures 5A, B and C show the average energy of the harmonic, ultraharmonic and broadband emissions of the animals in each group respectively, split by MB type (white bars represent Control-MBs, black bars represent Lyso-MBs). There were no statistically significant differences between the emissions of each MB type at any of the incident pressures tested. The apparent reductions in broadband energy at 240 and 320 kPa (Figure 5C) were due to high variability in the measurements and were not statistically significant.

Figure 5: An overview of the acoustic emissions from the animals at all pressures. Average harmonic (A), ultraharmonic (B) and broadband energy (C) of acoustic emissions. White bars represent Control-MBs, black bars represent Lyso-MBs (n=8; error bars indicate one standard deviation).

Discussion and future directions

As shown in Figure 4, Lyso-MBs were found to significantly increase US-BBBD above that caused by Control-MBs at 480 kPa PNP. This was not attributable to differences in microbubble size (Figure S1), microbubble concentration or microbubble cavitation activity as measured by acoustic emissions (Figure 5). In the previous *in vitro* study it was hypothesized that the observed increase in cell membrane permeability was due the incorporation of the lysolipid into the membrane and the corresponding reduction in the energy required for pore formation and/or increase in pore stability.^[62] As mentioned in the introduction, it is possible that a similar mechanism is responsible for the increase in US-BBBD, either through pore formation facilitating transcellular transport or tight junction disruption. It was, however, not possible to determine this from the histological work in this study and further investigation would be needed, e.g. in an *in vitro* BBB system.

Whilst no therapeutic effect was investigated in this study, the enhanced delivery of Gd-DTPA serves as a proxy for the enhanced delivery of small molecule therapeutics. Furthermore, extravasation of IgG indicates likely permeability to larger therapeutics of similar size (150 kDa), such as antibodies. It would be interesting to explore whether or not this enhancement in Gd-DTPA and IgG transfer translates to similar small molecule therapeutics and, if it does, whether it leads to an enhanced therapeutic effect of those small molecules in the brain. In addition to US-BBBD, it is possible that Lyso-MBs could improve delivery to other tissues in the body. This would be particularly desirable for those tissues that are deep within the body where delivering high ultrasound energies presents practical and safety challenges. Potential targets include the pancreas and the tight cellular membranes in the kidney.

The systemic toxicity of the Lyso-MBs was not measured directly in this study. No cellular damage or lesion formation was observed during histological analysis of any of the brains, but further analysis of the systemic toxicity, clearance routes and longer term effects of microbubbles containing lysolipid is required. Also, it was not possible within this study to examine BBB permeability at different time

points or to confirm the reversibility of the US-BBBD and these are clearly both very important considerations for future development.

Interestingly the IgG results (Figure 4B) suggest that both microbubble formulations had some effect on the BBB in the absence of ultrasound. To the best of the authors' knowledge this has not been previously reported and is itself worthy of further investigation. One possible explanation is the large quantity of sub-micrometre phospholipid micelles that co-exist with MBs within a suspension (either because they are not removed during manufacturing or subsequently formed by lipid shedding^[67]) and may interact with endothelial membranes modifying their permeability prior to US exposure. As indicated in the Methods section, the effect upon sonoporation of either centrifugal washing of MBs or direct exposure to lysolipid micelles was found to negligible *in vitro*, but the effect of blood components upon the formation of micelles and subsequent biological interactions *in vivo* is unknown and this mechanism should not be ruled out.

With regard to the effect of the lysolipid specifically, its presence in the MB suspension did not seem to affect IgG uptake in the absence of ultrasound. In fact, the Lyso-MBs promoted less IgG uptake than the Control-MBs (Figure 4B). The reasons for this are unclear but potentially relate to differences in circulation time. Since the Lyso-MBs contained a smaller proportion of PEG, they may have been cleared more rapidly and thus had less time in contact with the BBB. Again, further studies are needed to understand the pharmacokinetics of both MB formulations. What this result does suggest, however, is that the lysolipid is incorporated into the bubbles and does not interact significantly with the BBB prior to ultrasound exposure. This is in agreement with the previous *in vitro* results, in which the incorporation of lysolipid into MBs was seen to decrease the its effect on cells at equivalent concentrations. ^[62]

It is also consistent with the fact that a significant difference in US-BBBD between the Lyso-MBs and the Control MBs was only observed at peak negative ultrasound pressures promoting substantial cavitation activity, i.e. when both ultraharmonic and broadband emissions were of comparable

magnitude to the harmonic emissions (Figure 5). Under these conditions, MB destruction and hence local release of the lysolipid would be expected. A preliminary experiment to investigate this further was carried out using a fluorescently labelled lysolipid. The results also indicated that ultrasound was required to promote lysolipid uptake by cell membranes (Figure S5).

Conclusion

The aim of this study was to determine whether Lyso-MBs, shown to enhance *in vitro* cellular delivery in previous work^[62] would also enhance US-BBBD *in vivo*. To this end, animals were injected with either Control-MBs (containing no lysolipid) or Lyso-MBs and exposed to ultrasound at a range of different peak negative pressures. Acoustic emissions were monitored using a single element PCD. US-BBBD was then measured by Gd-DTPA uptake into the brain parenchyma (via MRI) and by the extravasation of serum IgG (via immunohistochemistry). Lyso-MBs were found to significantly increase both Gd-DTPA and IgG uptake following 2 minutes of ultrasound exposure at a centre frequency of 500 kHz, 1 Hz pulse repetition frequency, 1 ms pulse length and a peak-negative pressure of 480 kPa. This was the only pressure tested at which significant cavitation activity was detected. There was no difference in size, concentration or acoustic emissions between the Lyso-MBs and the Control-MBs indicating that the effect was due to the presence of the lysolipid. No acute toxicity was observed over the course of the experiments but further work on MB pharmacokinetics, duration of BBB opening and longer term safety should be performed. These findings indicate that the composition of microbubbles can be adapted to enhance US-BBBD, and therefore demonstrates the *in vivo* relevance of a novel method of microbubble optimization, particularly for the delivery of drugs to the brain.

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Supplementary Information

Figure S1: Normalised average size distributions and representative microscope images of Control-MBs and Lyso-MBs. Images captured at 40x and microbubbles diluted by a factor of 50 prior to imaging.

Figure S1 shows the normalised average size distribution of Control-MBs and Lyso-MBs and representative images of both formulations. Control-MBs are formed of DBPC and PEG40S in a 9:1 molar ratio, Lyso-MBs are formed of DBPC, PEG40S and LysoPC in a 9:1:4 molar ratio. Microbubbles were formed by the sonication method described in the main paper. For the experiments in this study, 1×10^8 microbubbles were injected into the tail vein.

Figure S2: A representative histology section from each animal in the 480 kPa group. Extravasated serum IgG is visible in brown (DAB-peroxidase substrate solution). Differences in blue cresyl violet staining arise from differences in the local concentration of dye and do not affect measurements of US-BBBD.

Figure S2 shows images of a representative histology section from each of the animals in the 480 kPa groups. The blue colouring is cresyl violet (a cellular stain) and the brown colouring is a stain for extravasated serum IgG. Whilst it is visibly clear that, on average, the animals injected with Lyso-MBs have more widespread US-BBBD, the coronal distribution of US-BBBD is highly irregular in both groups. It is likely that these differences result from slight differences in positioning of the animals within the cradle and perhaps small differences in skull thickness leading to the establishment

of different intracranial standing wave patterns. Visual inspection reveals that the US-BBBD caused by Control-MBs appears more punctate than that caused by Lyso-MBs, suggesting that it has arisen primarily around major blood vessels in the ultrasound focus.

Figure S3: The average harmonic power of the acoustic emissions, split by microbubble type (green – Lyso-MBs and blue – Control-MBs) at each pressure over the duration of ultrasound exposure. Error bars indicate ± 1 standard deviation.

Figure S3 shows the average power of the harmonic acoustic emissions over the duration of ultrasound exposure for all animals, split by microbubble type and ultrasound pressure. These results, in combination with those in Figure 5 and Figure S4 show that there were no significant differences

in acoustic emissions between the microbubble types. This is particularly important at 480 kPa PNP which observed a significant difference in US-BBBD.

Figure S4: Scatter graphs showing the relationship between the energy of harmonic emissions and US-BBBD as measured by IgG histology for each pressure. Circles represent Control-MBs whereas triangles represent Lyso-MBs.

Figure S4 shows the correlation between the harmonic component of the acoustic emissions and the US-BBBD (as measured by histology) for each animal. The lack of clear correlation provides further evidence that the difference in US-BBBD cannot be attributed to increased cavitation activity

Figure S5: Representative images showing cells after exposure to either NBD-lyso-MBs or NBD-lyso-MBs plus US. Cell membranes are stained in Cellmask Deep Red (red) and the MBs have NBD-lyso-PE in the shell (green). $N = 3$ for both MBs and MBs + US conditions. Scale bar = $20\mu\text{m}$.

MBs containing a fluorescently tagged lysolipid (NBD-lyso-MBs) were also used to allow direct visualisation of lipid transfer between MBs and cells. Cells were imaged using dual wavelength microscopy before exposure to NBD-lyso-MBs, after exposure to just NBD-lyso-MBs and exposure to NBD-lyso-MBs plus US (180 kPa PNP, 500 kHz, CW, 60 s exposure). Due to commercial availability it was unfortunately not possible to exactly match the characteristics of the labelled and unlabeled lysolipids (18:1 NBD-lyso-PE vs. 16-lyso-PC). The data should therefore only be treated as preliminary (this is discussed further below). They do nevertheless provide evidence of lysolipid transfer from MBs to cell membrane and that ultrasound is required to promote this transfer in substantial amount.

Method – NBD-lyso-MBs were prepared as described in the main manuscript but using the labelled lysolipid. NBD-lyso-MBs were centrifugally washed prior to lipid transfer experiments to remove free fluorescent lysolipid from solution to enable visualisation. A-549 human lung cancer cells were cultured in 6.5mm Transwell plates (Corning, Germany) as detailed in Section 4.6.4. After washing once with PBS, 100µl of NBD-lyso-MBs (at 1×10^8 MBs per ml, outlined in Section 4.5.1 and manufactured as in Section 4.6.2) was added to the cells. This configuration was then exposed to ultrasound (180kPa PRP, 500kHz, CW, 60 seconds exposure) from a H107 transducer (Sonic Concepts, US). Cells were then washed twice more with PBS before being incubated for ten minutes with CellMask Deep Red Plasma Membrane Stain (Thermofisher Scientific, US). Dual wave fluorescence microscopy on a Zeiss LSM 780 confocal microscope (Carl Zeiss AG, Germany) allowed imaging of both the CellMask in the cell membranes and any NBD-lyso-PE present in the cell membranes. Images were collected of cells before exposure to NBD-lyso-MBs, after exposure to just NBD-lyso-MBs and exposure to NBD-lyso-MBs plus ultrasound. All images were captured with the same gain and exposure times, and were captured in the plane of maximum green fluorescence.

The predicted effects of the differences between the lysolipids on MB-cell transfer are:

Chain length: Lipids with a longer hydrocarbon chain length transfer less readily to cell membranes.⁴⁸⁰ The 18 carbon version of 16-lyso-PC (18-lyso-PC) would be expected to have less effect on the cell membrane fluidity than 16-lyso-PC due to the greater similarity in lipid chain length to endogenous lipids and based on the cell lipid order for different chain length lipids in Section 4.9.

Headgroup: 18:1 NBD-lyso-PE has a phosphatidylethanolamine (PE) head rather than the phosphatidylcholine (PC) headgroup that all of the other lipids analysed in this Chapter have. A lysolipid with a PE head would be expected to have a lower effect on cell membrane fluidity than a lysolipid with a PC head (and equivalent chain length) due to a PE head being smaller than a PC head (causing a reduction in the conical nature of the lysolipid).

Fluorescent tag: The NBD molecule tagged to 18:1 NBD-lyso-PE may have affected both lipid transfer and cell membrane fluidity. However, fluorescently tagging the lipid is the only way to directly observe lipid transfer and so any influence of the fluorophore is unavoidable. Previous work that has reported lipid transfer⁵⁴⁶ actually observed transfer of DiI, not the lipid itself. Transfer of DiI does not guarantee lipid transfer.

Saturation: 18:1 NBD-lyso-PE is an unsaturated lipid (with a carbon-carbon double bond in its hydrocarbon chain) whereas all other lipids in this study were saturated (do not have the carbon-carbon double bond). This double bond creates a kink in the hydrocarbon chain and removes the ‘conical’ shape. Unsaturated lipids have higher transfer rates and cause a greater increase in cell membrane fluidity than saturated lipids^{547,548} due to their irregular shapes. Therefore, 18:1 NBD-lyso-PE would definitely have a higher lipid transfer rate than 18:0 NBD-lyso-PE (the saturated equivalent) but due to the other differences outlined here, it is uncertain whether the transfer rates would be greater or lower than those of 16-lyso-PC.

In addition, monolayers or bilayers formed by unsaturated lipid molecules are much less tightly packed, and therefore less stable. The NBD-lyso-MBs were consequently larger and less concentrated than lyso-MBs (Figure S6). It is worth noting that this size difference almost certainly arises from the unsaturated nature of these lipids and is not due to either the NBD (fluorescent tag) or the PE headgroup; the MBs used for previous *in vitro* experiments^[62] formed with 1% NBD-DSPE (18:0 NBD-PE), a saturated lipid, exhibited an indistinguishable size distribution from that of lyso-MBs.

Figure S6: Size distribution and representative microscope images of NBD-lyso-MBs and lyso-MBs. The images, captured at 40x, show undiluted NBD-lyso-MBs and lyso-MBs that have been diluted by a factor of 50 prior to imaging.