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Ultrasonic Implantation and Imaging of Sound Sensitive Theranostic Agents for the Treatment of Arterial Inflammation

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KEYWORDS: Therapeutic Ultrasound, Ultrasound Contrast Enhancement, Atherosclerosis, Anti-Inflammatories, Dexamethasone

ABSTRACT

For site-specific diseases such as atherosclerosis, it is desirable to non-invasively and locally deliver therapeutics for extended periods of time. High intensity focused ultrasound (HIFU) provides targeted drug delivery, yet remains unable to sustain delivery beyond the HIFU treatment time. Furthermore, methods to validate HIFU-enhanced drug delivery remain limited. In this study, we report on HIFU-targeted implantation of degradable drug-loaded sound-sensitive multi-cavity PLGA microparticles (mcPLGA MPs) as a theranostic agent for the treatment of arterial lesions. Once implanted into the targeted tissue, mcPLGA MPs eluted dexamethasone for several days,

thereby reducing inflammatory markers linked to oxidized lipid uptake in a foam cell spheroid model. Furthermore, implanted mcPLGA MPs created hyperechoic regions on diagnostic ultrasound images, and thus non-invasively verified that the target region was treated with the theranostic agents. This novel and innovative multifunctional theranostic platform may serve as a promising candidate for non-invasive imaging and treatment for site-specific such as atherosclerosis.

1. Introduction

Cardiovascular diseases (CVD) account for the highest mortality rates worldwide and incidences have been dramatically increasing due to lifestyle-related factors and aging populations.¹ Among CVDs, atherosclerosis accounts for a majority of the reported morbidity and mortality.² Atherosclerosis is an inflammatory disease caused by hardening of the arterial walls and narrowing of the arteries resulting from the accumulation of lipids and fibrous tissues within the arterial intima.³ These plaques are formed from the build-up of foam cells that develop from monocytes attracted to the subendothelial part of the arteries that are rich in lipids. The monocytes then differentiate into macrophages and excessively uptake and metabolise modified lipoproteins, and in turn further differentiate into foam cells.⁴ These foam cells accumulate over time and combine into a plaque that slowly becomes at risk for ruptures that occlude blood vessels and cause serious health implications.⁴⁻⁶ Inflammation plays a major part in the progression of atherosclerosis, and thus anti-inflammatory drugs are under intense investigation as a therapeutic.

Dexamethasone (Dex) is one of the most used glucocorticoids for the treatment of atherosclerosis. Depending on the concentration, Dex may mitigate inflammation in two ways – (i) by forming a complex with glucocorticoid receptors (GR) and binding to nuclear factor kappa B (NFκB), blocking the downstream transcriptional activation of inflammatory genes, and (ii) by specific inhibition of proteins that stabilise mRNA of pro-inflammatory genes leading to lower expression of these genes.^{7,8} In addition to its anti-inflammatory properties, Dex has shown to be beneficial against cholesterol accumulation in mice by inhibiting foam cell formation⁹ and reducing cholesteryl ester accumulation in *in vitro* and *in vivo* atherogenic mice aorta models.¹⁰⁻¹²

At present, Dex is administered orally or intravenously. However, these delivery methods have limited efficiency because of the short circulation half-life and non-specificity of Dex. As a result,

these limitations necessitate repeated administration, risking systemic exposure to potentially toxic drug concentrations and reduced patient compliance. Due to the reliance on drug diffusion from circulation, drugs may not reside within the diseased tissues at therapeutically effective levels. Considering these issues and the chronic nature of atherosclerosis, there has been a shift towards targeted drug delivery with sustained delivery systems, thereby maximising therapeutic impacts while keeping side effects to a minimum.^{13,14}

Drug-loaded vesicles have potential in achieving sustained and localised drug delivery. Such vesicles include liposomes, polymer micro/nanospheres, and ultrasound-sensitive micro/nanostructures. The small size, large surface area to volume ratio, and mobility in tissue of drug-loaded vesicles make them an ideal choice for targeted therapy by aiding in (i) the penetration across blood-tissue barriers, (ii) circulation, and (iii) internalization.¹⁵ Yet, current drug delivery methods are limited due to the low efficiency in targeting, image-guided positioning accuracy, and distribution of therapeutics throughout the lesion sites. There remains a need for targeted drug delivery of drug-eluting devices that sustain the delivery for a prolonged duration of time after administration to treat chronic diseases.

High-intensity focused ultrasound (HIFU) is a minimally invasive therapeutic technique used for tumour ablation,¹⁶ drug and gene delivery,¹⁷ and blood-brain barrier (BBB) opening.¹⁸ Ultrasound-mediated drug delivery is often driven by acoustic cavitation, i.e., the dynamic oscillations of gas or vapor bubbles, to improve drug distribution¹⁹ and localized release.^{20,21} Yet, nucleate cavitation requires substantial acoustic energy capable of damaging off-target healthy tissue. Therefore preformed cavitation nuclei such as small gaseous particles (microbubbles) are widely used to enable cavitation at reduced acoustic pressure amplitudes.^{22,23} As such, microbubbles have been shown to nucleate cavitation and improve the local delivery of therapeutic

agents across cancers, cardiovascular diseases, and neurological disorders.^{24,25} Despite the many benefits microbubbles provide, microbubbles have disease specific limitations owing to their large size and rapid destruction²⁶ in an acoustic field. Furthermore, these particles do not extravasate beyond the blood vessel network. Thus solid cavitation nuclei such as mesoporous silica,²⁷ polymeric nanocups,²⁸ and gold nanocones²⁹ have been put forward with advantages of prolonging cavitation activity, penetrating beyond blood vessels, and be embedded into tissue. Similarly, non-biodegradable solid cavitation nuclei were shown to sustain cavitation across minutes and improved the distribution of a co-administered drug.²⁸

Many imaging modalities, such as ultrasound imaging, X-ray computed tomography (CT), and magnetic resonance imaging (MRI) are used for guiding or validating HIFU-based therapies. Among these imaging modalities, ultrasound imaging has several advantages such as real-time monitoring, high penetration depth, and low cost. By coupling HIFU and diagnostic ultrasound with a sound-sensitive microparticle enable a theranostic approach to administer therapy with real-time guidance and treatment validation. Conventional ultrasound imaging use microbubbles to improve acoustic scattering to create hyperechoic regions in the ultrasound image. The contrast enhancement stems from the gas within these microbubbles, which are highly compressible and are thus more echogenic than the surrounding tissues, **thereby enhancing** the backscattered ultrasound signal by ~10–30 dB.³⁰ **However, this** enhancement in contrast typically is limited to the blood vessels and capillaries carrying these microbubbles.

It is therefore desirable to combine the ultrasound contrast enhancement of microbubbles with **prolonged cavitation and extravasation potential of** solid cavitation nuclei to address the specific needs for treating and imaging chronic site-specific diseases such as atherosclerosis. Recently, mcPLGA MPs-based HIFU cavitation agents have been developed by our group,³¹ which

demonstrated the site-specific implantation of drug-loading particles into porcine arterial tissue by HIFU. However, drug loading into these particles was not performed; the therapeutic effect and potential for ultrasound contrast-enhanced imaging from these particles post implantation have therefore not yet been investigated and is the focus of this report. Here, we substantially advance on our previous work to re-engineer an all-in-one degradable ultrasound theranostic agent comprised of Dex-loaded mcPLGA MPs (Dex/mcPLGA MPs) with surface-stabilised gas nanobubbles. These mcPLGA MPs are capable of being implanted into tissue in response to HIFU and enhance the echogenicity of the tissue after implantation in response to diagnostic ultrasound. Once implanted, the microparticles hydrolytically degraded to sustain the release of a therapeutic agent at the site of the diseased tissue. We also employed an *in vitro* 3D foam cell spheroid model, to study the anti-atherogenic potential of Dex/mcPLGA MPs. Our study indicated reduced secretion of pro-inflammatory cytokines by the spheroids in response to HIFU+Dex/mcPLGA MPs, which further emphasized the therapeutic benefits of the ultrasound sensitive drug carriers.

2. Experimental Section

Materials: Resomer RG504 H (lactide:glycolide 50:50, Mw 38,000-54,000), Poly(vinyl alcohol) (PVA) (80% hydrolyzed, Mw 9,000-10,000), phosphate buffered saline (PBS) tablet, Dexamethasone (BioReagent, $\geq 97\%$), Rhodamine B (RhB), Dichloromethane (anhydrous, $\geq 99.8\%$), Fluoroshield™ with DAPI, and Phorbol 12-myristate 13-acetate (PMA) were purchased from Merck. Agarose was purchased from Vivantis Technologies. Human oxidized Low Density Lipoprotein (oxLDL) was obtained from Alfa Aesar by Thermo Fisher Scientific. Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). Aggregate culture of foam cells were seeded into Perfecta3D® 96-well hanging drop plate (3D Biomatrix™, USA).

Fabrication of mcPLGA MPs: mcPLGA MPs were prepared using a double emulsion method described in our previous work.³² In brief, 2 ml of PLGA in DCM (25 mg/ml) was mixed with 0.1 mL 20x PBS and sonicated over ice at 100 W for 30 s to form the primary W/O emulsion. To form the W/O/W emulsion, 10 ml of 5wt% PVA was to the primary emulsion and homogenized at 12000 rpm for 5 min. The obtained W/O/W emulsion was stirred for 2h in an open container to allow for evaporation of the organic solvent and pores formation. For RhB/Dex loaded mcPLGA MPs, the payload was added into the organic phase before sonication and homogenization following the method described above.

Characterization of mcPLGA MPs: FE-SEM and TEM were used to assess the size and morphology of mcPLGA MPs. Samples for SEM and TEM analysis were prepared as described previously.³² Size distributions were performed on a Malvern Instruments Zetasizer Nano-ZS instrument.

Encapsulation efficiency and release profile: The quantity of Dex present and release profile as a function of time was measured by UV-absorbance in solution.³² The encapsulation efficiency was calculated by the actual amount of drug entrapped (total drug added – free non-entrapped drug) divided by the total amount of drug added initially during preparation. The release profile was determined by the amount of drug delivered (M_t) to the amounts of effectively encapsulated drug (M_0), as a function of time.³³

HIFU Setup: Cavitation potential from mcPLGA particles were measured using a covential HIFU setup (1.1 MHz, 20 cycles, 10 Hz pulse repetition frequency, or PRF).³¹ Afterwards, the acoustic emissions were collected and post processed using power FFT to get power spectral density curves. Following that, cavitation threshold was determined by 6 dB difference between the received signal from mcPLGA MPs and water reference.

Diagnostic Ultrasound Setup: Clinical ultrasound imaging was acquired using a clinical ultrasound system (E-Cube 12-R, Alpinion Medical Systems, South Korea).³⁴ Scanning was performed with B mode operating at 10 MHz with a mechanical index (MI) of 1.0. Afterward contrast to tissue ratio (CTR) analysis was performed by normalizing the mean squared acoustic power of the backscattered signal from the mcPLGA MPs to the signal of tissue.

Cell Culture and spheroid formation: THP-1 cells, a human monocytic cell line (ATCC, Rockville, MD) were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Pan Biotech, Germany) supplemented with 100 IU/ml of penicillin, 100 µg/ml streptomycin (Pan Biotech, Germany), 2 mmol/l L-glutamine, 10% (vol/vol) FBS (Gibco, USA) and incubated in a humidified atmosphere of 5% CO₂ in an incubator at 37 °C. The medium was replaced every 2–3 days by centrifuging these suspended cells at 125 g for 5 min. For differentiation of THP-1 monocytes into macrophages, THP-1 cells were seeded at a density of 7×10^5 cells/ml with differentiation medium, growth medium supplemented with 50 ng/mL PMA, for 3 days to obtain macrophages. Subsequently, for foam cell induction, macrophages were incubated with 100 µg/ml oxLDL in the differentiation medium for 2 days.

A three-dimensional (3D) foam cell spheroid model was developed by modification of the hanging drop method.³⁵ Foam cells were seeded into Perfecta3D® 96-well hanging drop plate (3D Biomatrix™, USA) at a density of 1.25×10^6 cells/mL and incubated for 4 days at 37 °C with 5% CO₂. The foam cell spheroids formed were collected and subjected to treatment immediately.

HIFU implantation of mcPLGA MPs into Spheroid Studies and ultrasound imaging: To create a spheroids embedded sample chamber for HIFU exposure, spheroids were first embedded into alginate beads. Then the beads were washed and mounted along the channel of agarose chamber mentioned above. Then RhB/DAPI-mcPLGA MPs were suspended in PBS (1 mg/ml) and pumped

through the channel (0.2 ml/min) for ultrasound exposure (1.1 MHz, 3.2 MPa, 5% duty cycle). After exposed to ultrasound for 5 min, the sample chamber was washed with PBS to remove the remaining mcPLGA MPs and imaged using the therapeutic ultrasound setup described above or collected for further analysis.

The distribution of mcPLGA MPs throughout the spheroids and the release of therapeutics were analysed using confocal microscope (Zeiss LSM 800). To determine the histological and physiological effects of HIFU mediated delivery of Dex loaded mcPLGA MPs to foam cell spheroids, the spheroids were sectioned (10 μm) using a Leica CM1950 Cryostat, mounted on glass slides, and stained with Oil Red O to visualize lipid under inverted microscopy (AxioVert 200, Carl Zeiss).

Cytokine Array: In order to examine the inflammatory cytokines secreted by foam cell spheroids, cell culture supernatants were collected and subjected to a cytokine array (Human Cytokine Antibody Array (Membrane, 42 Targets); Abcam, USA) according to the manufacturer's instructions. Signal intensities were quantified using the Image Quant software and normalized to the untreated samples.

Statistical analysis: Data presented were representative of 3 independent experiments and quantitative results were expressed as mean \pm standard deviation (SD) unless otherwise specified. Q. Data were analysed by One-Way and Two-Way ANOVA with post hoc Tukey's analysis using Origin Pro 2015 (OriginLab, USA) or student's T-test. A p value below 0.05 was considered statistically significant.

3. Results and Discussion

The mcPLGA MPs were fabricated following a modified double emulsion method that used PBS as a porosigen.³² The obtained mcPLGA MPs exhibited a mean size of $2.5 \pm 0.5 \mu\text{m}$ with a

multitude of surface cavities (**Figure 1a-c**). The rough cavity inner surface facilitates gas trapping required for ultrasound imaging and ultrasound enhanced drug delivery. Gas trapping into the cavities was accomplished by a drying and resuspension process.²⁸ No agglomerations were observed as indicated by the polydispersity index (PDI), PDI=0.13, in either deionized water or PBS (**Figure 1d**).

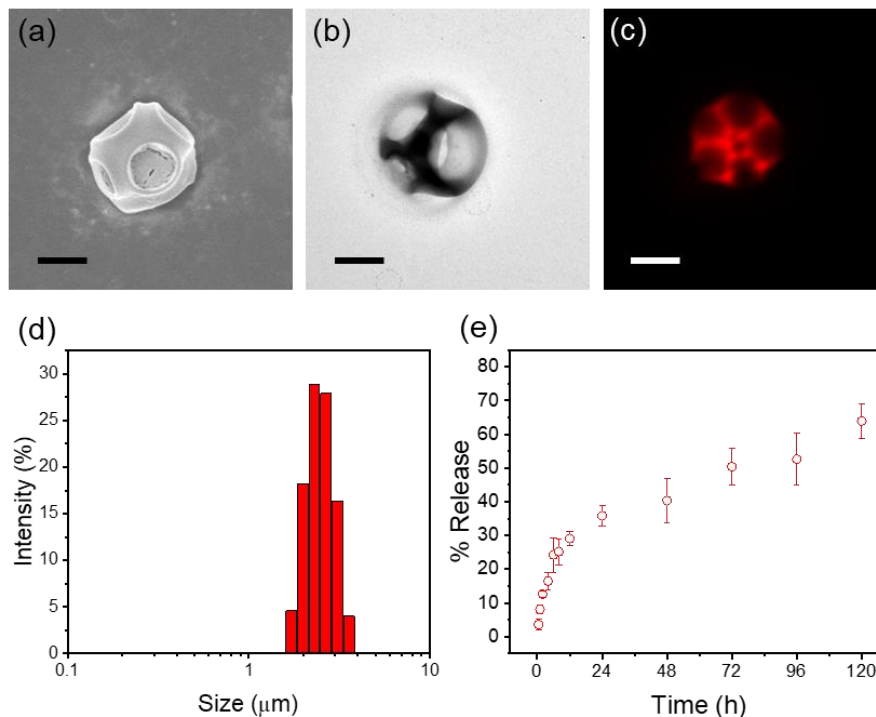


Figure 1. Representative SEM (a), TEM (b) and fluorescence (c) image of Dex-loaded mcPLGA MPs (Dex/mcPLGA MPs). Rhodamine B (RhB) was also co-loaded to track the location of particles (c). (d) Size of mcPLGA MPs as measured by Dynamic Light Scattering (DLS). (e) **Dex release profile *in vitro***. Scale bars represent 1 μm. 3 independent experimental sets were performed.

During fabrication, Dex resided in the oil phases of the emulsion alongside the PLGA polymer. As the oil phase was removed, Dex was trapped in the PLGA matrix with an encapsulation

efficiency of $70.3 \pm 6.9\%$. **Figure 1e** shows the release of Dex from mcPLGA MPs in PBS across 5 days. Initially, $29.1 \pm 5.1\%$ of Dex was released within 12 hours. However, it required 5 days to release $63.9 \pm 7.8\%$ of Dex from mcPLGA MPs, which was consistent with previous observations.³²

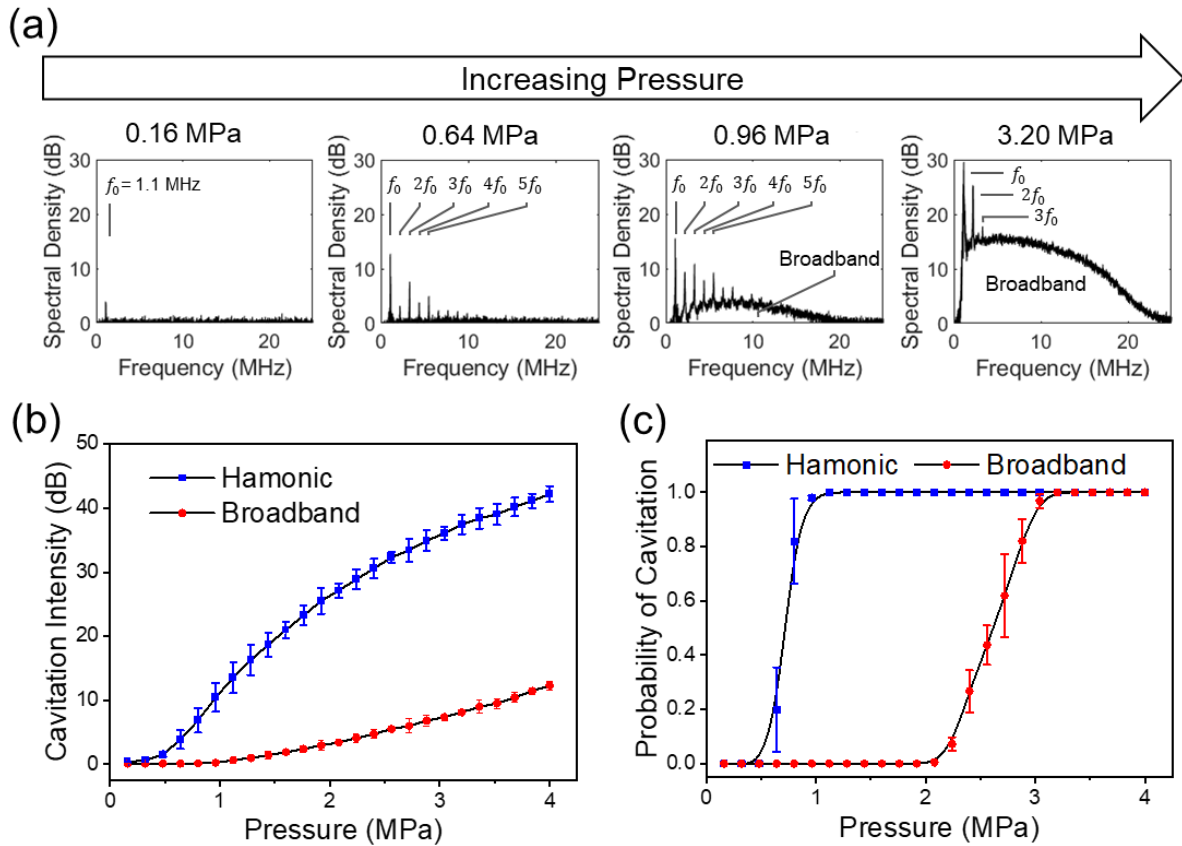


Figure 2. Acoustic response of mcPLGA MPs exposed to 1.1 MHz HIFU. (a) Representative spectral density curves of mcPLGA MPs under different exposure pressures. (b) Cavitation intensity and (c) probability of cavitation of mcPLGA MPs.

The acoustic responses from mcPLGA MPs were measured using a 1.1 MHz HIFU transducer.^{28,31} During exposure to HIFU across a range of pressure amplitudes (0.16-4.0 MPa), we detected both harmonic and broadband signals (**Figure 2a and S1**). Backscattered signal centered around the fundamental and harmonic frequencies is often attributed to the non-linear dynamics of cavitation bubbles, indicating the inception of stable cavitation regime.³⁶ In contrast, broadband

noise which covers all frequencies is a result of shockwaves generated by occurrence of the violent inertial collapse of bubbles at large acoustic intensities and is indicative of inertial cavitation.²⁸ From the frequency spectrum (Figure 2a), only the fundamental frequency (1.1 MHz) was detected by the passive cavitation detection (PCD) at 0.16 MPa. This signal was likely due to weak reflection of the transmitted sound from the water-gel interface and not due to cavitation. Harmonics were detected from 0.32 MPa and were more pronounced at 0.64 MPa, which were indicative of stable cavitation typically observed from microbubbles.³⁷ When the pressure was increased to 0.96 MPa, the PCD detected both the broadband noise and harmonics, which may be due to a mixture of stable cavitation and inertial cavitation occurring from the cavitation agent population. At 3.2 MPa, broadband emissions became dominant, showing that the bubbles violently collapsed and underwent intense inertial cavitation.³⁸ These violent collapses tend to emit shockwave pulses that are detected as broadband noise, hence the rise in baselines at the higher-pressure amplitudes.³⁹ As previously mentioned, stable cavitation emitted higher harmonic emissions. Those harmonic emissions exponentially increased with an initial rapid that slowed as the pressure amplitude increased (Figure 2b). In contrast, broadband emissions increased following a power function. The stable and inertial cavitation thresholds (i.e., a 50% probability for an event to occur) were measured at 0.7 MPa and 2.6 MPa, respectively. The probability for cavitation reached 100% at 1.1 MPa for stable cavitation and 3.2 MPa for inertial cavitation (**Figure 2c**). Therefore, the cavitation response of mcPLGA MPs may be adjusted to achieve the desired mechanical effect by changing the input acoustic pressure.

Samples of mcPLGA MPs were tested for contrast enhancement **using** a diagnostic ultrasound setup at 10 MHz with a mechanical index (MI) of 1.0. As can be seen in **Figure 3**, the mcPLGA MPs displayed the highest contrast enhancement with a CTR (contrast to tissue ratio) value of 22.9

± 3.2 dB, which is comparable to commercially available ultrasound contrast agents.³⁰ Nonporous hollow spherical PLGA microparticles (i.e., polymer microbubbles) with the same size (hsPLGA MPs, **Figure S2**) did not achieve substantial contrast enhancement (CTR = 7.2 ± 2.9 dB). One explanation for this discrepancy may be that while the multi-cavity particles have surface stabilised gas bubbles that can nucleate at comparatively lower input pressures, the hollow spheres have a rigid polymer shell that needs higher pressures to either induce volumetric oscillations or rupture the shell to release the gas. Hence, at the pressure range tested, the multi-cavity variant provided a higher backscattered signal from these surface stabilised gas bubbles which are missing in the hollow variant. These results indicate that mcPLGA MPs were capable of nucleating cavitation to provide sufficient contrast enhancement for clinical imaging. Given that the mcPLGA MPs are of a clinically relevant size (1-5 μm) and that their response both to HIFU and diagnostic ultrasound is comparable to commercial contrast agents, these particles may be suitable as a theranostic agent.^{30,40}

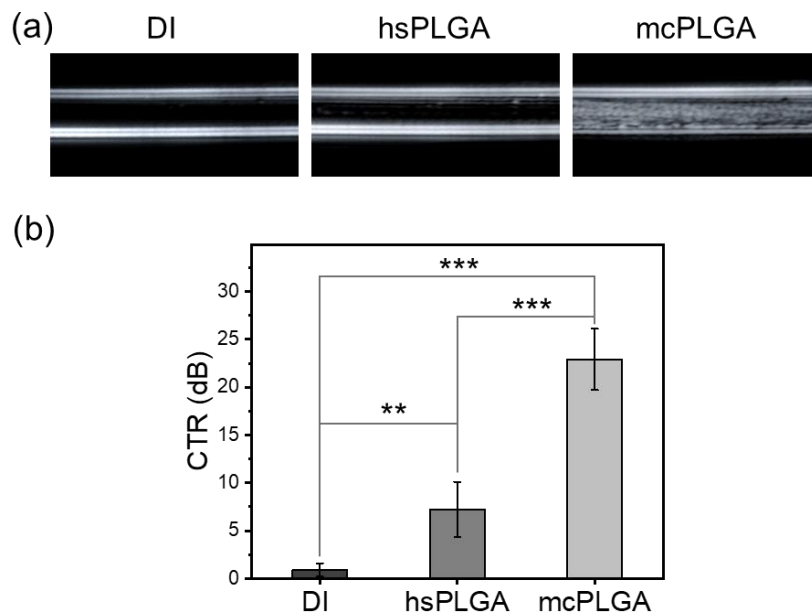


Figure 3. Contrast enhancement from mcPLGA MPs. (a) B mode ultrasound imaging with the diagnostic ultrasound scanner of DI water and 1 mg/ml suspensions of hollow sphere PLGA

(hsPLGA) microparticles and mcPLGA MPs. (b) Corresponding CTR values of DI water, hsPLGA, and mcPLGA MPs (mean \pm SD, n = 3). ** denotes a $p < 0.01$ and *** denotes a $p < 0.001$.

After confirming the cavitation threshold, we demonstrated targeted implantation of mcPLGA MPs into atherosclerotic models using acoustic cavitation. Considering that the arteries of a mouse are orders of magnitude smaller than that of a human and too small for the HIFU beam focal volume, we deemed a murine model is impractical to use. Larger animals (e.g., pig models) are challenging to use because building a robust atherosclerotic model remains difficult and costly. Instead, *in vitro* foam cell spheroid models were selected as the arterial model because foam cells are responsible for the progression of atherosclerosis. Studying these foam cells will also elucidate the molecular processes that help determine the treatment efficacy of the therapeutic.⁴¹ The foam cell spheroids were encapsulated in alginate beads (~3 mm) and then mounted next to the lumen of an agarose flow chamber prior to exposure to HIFU (**Figure 4a**). Before HIFU treatment, the foam cell spheroid structure was imaged with diagnostic ultrasound (10 MHz at 1.0 MI), which showed a predominantly hypoechoic region. Thereafter, the foam cell spheroid structure was treated with HIFU irradiation for 5 min (3.2 MPa, 5% duty cycle, 2 Hz RPF). Under these conditions, the mcPLGA MPs were site specifically implanted into spheroid structures (**Figure S3**). These parameters are in line with *in vivo* ultrasound-mediated studies treating tumours²⁸ and opening Blood-Brain Barrier,¹⁸ and results have shown minimal damage to the surrounding tissues. After HIFU treatment, the lumen was flushed of mcPLGA MPs and the spheroids were imaged using a conventional diagnostic ultrasound transducer (10 MHz at 1.0 MI). As can be seen from **Figure 4b**, the foam cell spheroids embeddings post HIFU treatment were hyperechoic under conventional B-mode imaging, with an image contrast 15.2 ± 1.6 dB higher than pre-HIFU image.

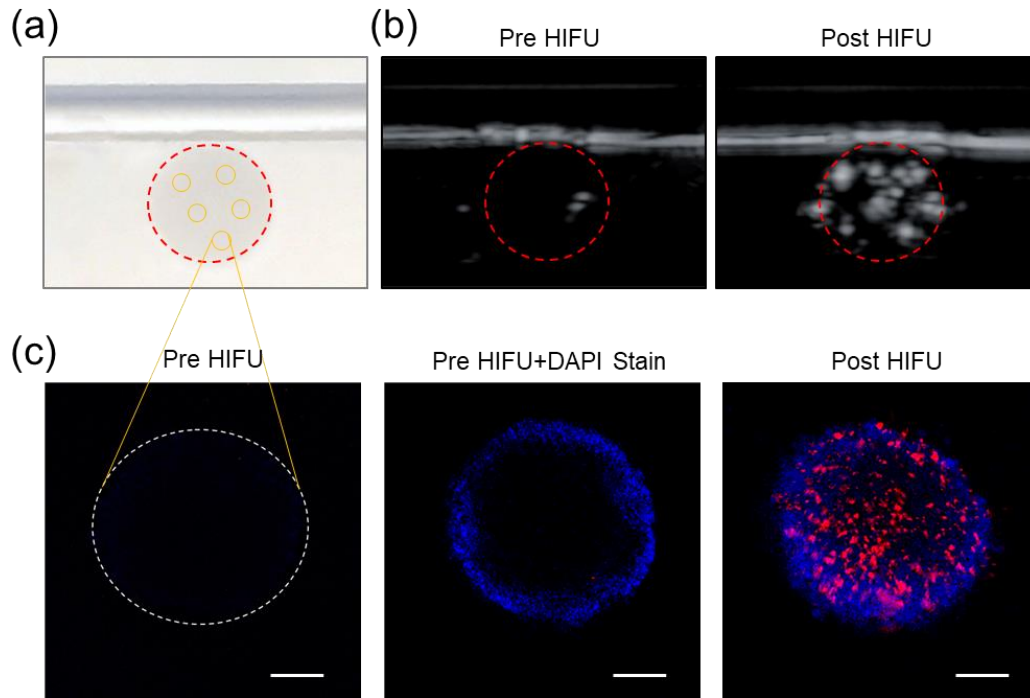


Figure 4. (a) Spheroids (indicated by the yellow cycles) embedded in alginate beads (indicated by the red dashed cycles). (b) B-mode ultrasound images of spheroids embedment before and after HIFU treatment. ROI is marked by red dashed cycles. (c) Confocal images of extracted foam cell spheroids. (Left) An image of a foam cell spheroid treated with 1 mg/ml mcPLGA MPs without HIFU exposure. (Middle) A similar foam cell spheroid treated with 1 mg/ml mcPLGA MPs without HIFU exposure but stained with DAPI to emphasize the diffusion limitation of cell spheroids. (Right) An image of a foam cell spheroid treated with 1 mg/ml mcPLGA MPs (loaded with DAPI and RhB) after HIFU exposure (right). The scale bars represent 100 μm . mcPLGA MPs were indicated by red and DAPI fluorescence by blue.

The foam cell spheroids were also extracted from the spheroid embedment. This was achieved by dissolving the alginate beads using sodium citrate. After extraction, the foam cell spheroids were imaged by confocal microscopy to evaluate the penetration and distribution of RhB/DAPI co-loaded mcPLGA MPs. **Figure 4c** showed fluorescent images of spheroids pre- and post-HIFU

mediated implantation. Control spheroids **without HIFU exposure** showed minimum presence of fluorescent particles. **Then** the non-HIFU treated spheroids were stained with DAPI. DAPI staining exhibited strong fluorescence only in the outer edges of spheroids, due to limited permeability to diffuse deep into the core of the spheroid. In contrast, mcPLGA MPs penetrated and distributed throughout the entire spheroid in the presence of HIFU (**Figure 4c and S4**).

Here, we **demonstrated the remote implantation** of a biodegradable drug delivery vehicle into spheroids and enhance contrast after implantation. Conventional cavitation **agents for drug delivery and imaging** (i.e., microbubbles) are **neither** able to be implanted into tissue nor enhance contrast after implantation. **Similarly, other solid cavitation nuclei decouple from the drug delivery vehicle, thereby** limiting the **penetration depth of the therapeutic and its dispersal into the tissue.**²⁸ **In our study,** by coupling the therapeutic **agent to** biodegradable solid cavitation nuclei, we **are able to** demonstrate precise targeting of **a** drug with HIFU and verifying **its** delivery with contrast-enhanced ultrasound imaging. As an ultrasound responsive vehicle actively driven by HIFU, these particles are able to be remotely implanted into the site of diseases, deliver therapeutics, and enhance contrast imaging of diseased tissue. Furthermore, the particles were not randomly distributed but were instead primarily accumulated within the beam focus of HIFU at the wall away from the HIFU transducer. This observation, **the same with literature using different solid cavitation agents,**^{28,29} may be attributed to the cavitation nucleated from gas trapped on the particle cavities. These cavitation events, which only occur at the HIFU focus, likely created local microstreaming that in combination with acoustic radiation forces from the HIFU will transported particles away from the HIFU transducer and embedded them into tissue. Here, diagnostic ultrasound is used to verify the location of the particles post HIFU treatment. There are also other

established methods, such as passive acoustic mapping, that may be used to monitor the treatment in real time.⁴³

Given that mcPLGA MPs are capable of generating cavitation and propelling themselves into spheroids, we next demonstrated that site-specific delivery of Dex enhanced the lipid reduction in foam cell spheroids. **Figure 5** shows representative images of spheroids stained with Oil Red O and haematoxylin to show lipid (red) and nuclei (blue) under different treatment conditions after extraction and further culturing. Compared with untreated and HIFU+mcPLGA MPs treated samples, there was an expected reduction of oil droplets for spheroids treated with Dex alone. However, oil droplets near the core of the foam cell spheroid were still present due to diffusion limitations similar to our observations with DAPI staining. Interestingly, HIFU propelled Dex/mcPLGA MPs produced a more dramatic and uniform reduction of oil droplets compared to all the other treatment groups.

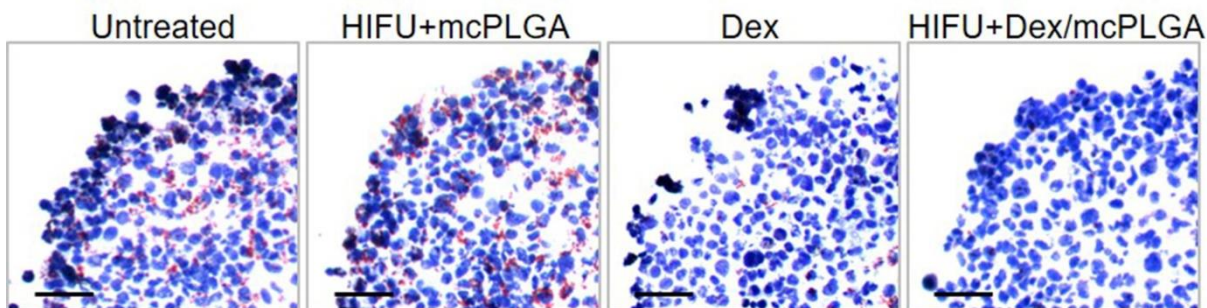


Figure 5. Oil Red O staining of foam cell spheroids under different treatment conditions. The scale bars represent 50 μm .

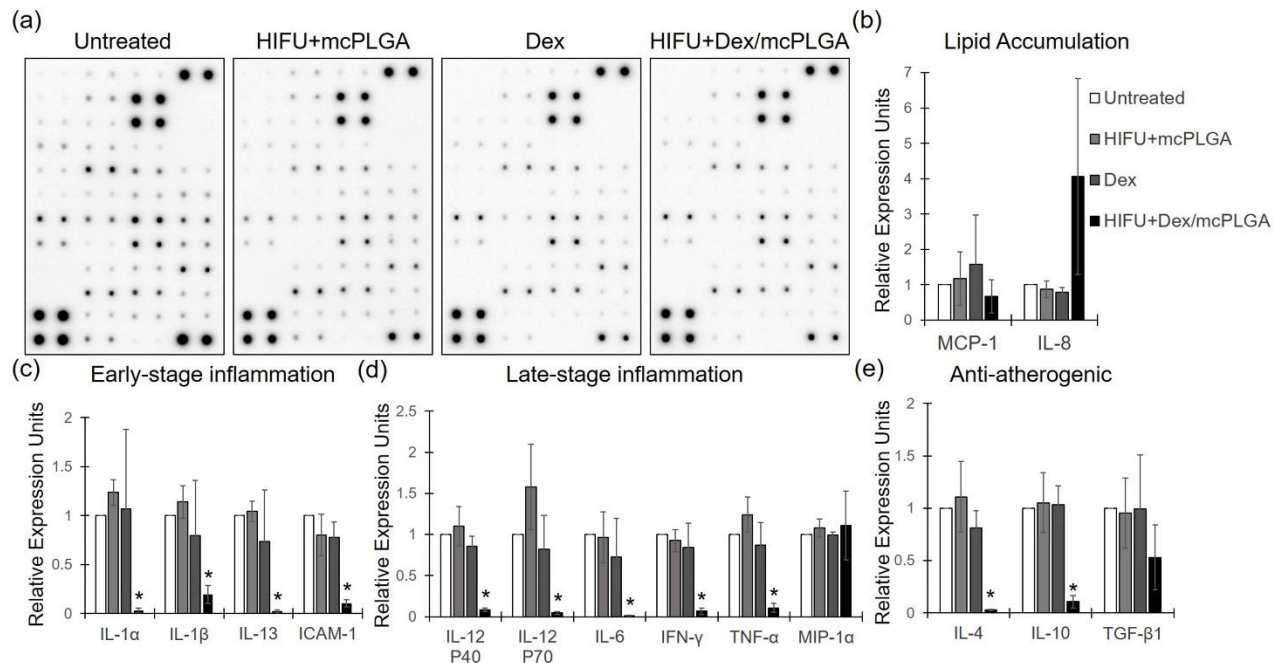


Figure 6. Estimation of cytokine release from foam cell spheroids. THP-1 derived foam cell spheroids exposed to HIFU+mcPLGA MPs, Dex alone, HIFU+Dex/mcPLGA MPs or untreated. Supernatants were collected and secreted cytokines were examined using a cytokine array (a) Representative chemiluminescent images of cytokine array untreated or exposed to HIFU+mcPLGA MPs, Dex or HIFU+Dex/mcPLGA MPs. Graphs showing quantification of the cytokine secretions categorized into three categories: (b) lipid accumulation, (c) Early-stage inflammation, (d) late-stage inflammation, and (e) anti-atherosclerotic cytokines. * denotes a $p < 0.05$ between the indicated groups and untreated controls.

After qualitatively establishing the site-specific delivery of Dex/mcPLGA MPs into foam cell spheroids, we studied the effects of Dex/mcPLGA MPs on the inflammatory pathway under HIFU excitation. In order to achieve this, foam cell spheroids were untreated or exposed to HIFU+Dex/mcPLGA MPs. Additionally, the spheroids were also exposed to HIFU+mcPLGA MPs or Dex alone, to eliminate the possibility of either one of the conditions being responsible for

the effect. Subsequently, the supernatants were collected and probed for secreted inflammatory cytokines (**Figure 6a**). After screening and analysis, the cytokines were grouped into 4 categories – (i) lipid accumulation, (ii) early-stage inflammation, (iii) late-stage inflammation, and (iv) anti-atherogenic cytokines. It was noted that there was no significant **difference in the** secretion profile of cytokines MCP-1 and IL-8 which are responsible for lipid accumulation^{35,44} by foam cells, upon exposure to the different treatments (**Figure 6b**). It is reported that oxidized low density lipoprotein (oxLDL) induces early phase pro-inflammatory cytokines, triggering the “priming” of the inflammatory pathway by binding to Toll-like receptors (TLRs) or cluster of differentiation 36 (CD36) receptors on macrophage surfaces.^{45,46} This step ensures activation of NFκB pathway leading to transcriptional activation of components of the NLR family pyrin domain containing 3 (NLRP3)-inflammasome complex which regulates **induction** of caspase-1, and catalyses the cleavage of pro-IL-1β and pro-IL-18, **stimulating** the secretion of the biologically active counterparts as well as IL-1α.^{47,48} Our results indicated that upon exposure to HIFU+Dex/mcPLGA MPs, there was a significant reduction in secretion of IL-1α and IL-1β (**Figure 6c**), which was not observed when the spheroids were exposed to either HIFU+mcPLGA MPs or Dex alone. Secretion of IL-1α and IL-1β marks the initiation of the early inflammation phase along with cytokines including IL-10 and IL-13, which induce the recruitment of macrophages. Secretion of these cytokines were also reduced upon administration of HIFU+Dex/mcPLGA MPs (**Figure 6c and e**). Furthermore, a reduction in the levels of adhesion molecule ICAM-1 which recruit macrophages⁴⁹ to the endothelial wall (**Figure 6c**) was also observed. Again, there was no statistically significant effect of HIFU+PLGA MPs or Dex alone.

IL-1β and IL-18 activate late stage pro-inflammatory cytokines. IL-1β induces secretion of IL-6, MIP-3α and TNF-α, while IL-18 induces IFN-γ.⁴⁵ The current study corroborates this with the

observed reduction of IL-12, IL-6, IFN- γ , TNF- α and MIP-1 α (**Figure 6d**) in HIFU+Dex/mcPLGA MPs treated conditions. The cytokines IL-6 and IL-12 work as a pair, whereby IL-6 promotes the release of tumor necrosis factor alpha (TNF- α) and IL-1R α and induce formation of fatty streaks.⁵⁰ At the same time, IL-12 regulates differentiation of TH1 cells marking the completion of early stage atherosclerosis.⁵⁰ The most important pro-atherogenic cytokines are Interferon- γ (IFN- γ) and TNF- α . IFN- γ promotes uptake of oxLDL and foam cell formation whereas TNF- α further promotes plaque formation and expression of monocyte chemoattractant protein-1 (MCP-1). MCP-1 and IL-8 are subsequently expressed in macrophages in response to cholesterol loading.^{45,50}

Transforming growth factor- β (TGF- β), IL-4 and IL-10 are anti-inflammatory cytokines.⁵¹ These cytokines inhibit the Th1, Th2 cells and macrophages **from proliferation, induction and differentiation**. However, our results indicate reduced secretion of IL-4 and IL-10 by foam cell spheroids exposed to HIFU+Dex/mcPLGA MPs, while there was no change in TGF- β levels (**Figure 6e**). Both Dex and HIFU+mcPLGA MPs exposed spheroids did not show any significant difference. IL-10 is an anti-inflammatory cytokine which enhances oxLDL-induced foam cell formation, thus playing a protective role.⁵² However, reduced levels of IL-10 may indicate reduced foam cells and thus reduced potential for plaque formation. Similarly, the role of IL-4 in atherosclerosis is not well understood and there are contradictory reports.⁵³⁻⁵⁵ Hence, further investigation of the role of HIFU+Dex/mcPLGA MPs on these anti-inflammatory cytokines and their overall impact on atherosclerosis will be beneficial, as our understanding of the **function** of these cytokines in terms of macrophage recruitment and foam cell development remains incomplete.

4. Conclusion

Here, we report on the HIFU-mediated implantation of a theranostic agent (mcPLGA MPs) for targeted drug delivery and contrast-enhanced ultrasound imaging. The degradable nature and unique surface structure of the designed mcPLGA MPs endowed the theranostic with distinctive advantages. After gas trapping, mcPLGA MPs nucleated both stable and inertial cavitation under exposure to HIFU at clinically relevant exposure conditions. We then showed that mcPLGA MPs were able to be remotely implanted into an in vitro 3D foam cell spheroid model with therapeutic ultrasound. Once implanted, mcPLGA MPs sustained the release of Dex, resulting in an enhanced therapeutic effect signified by a reduction in oxidized lipid levels and reduced secretion of pro-inflammatory cytokines in the foam cell spheroids. Under diagnostic ultrasound excitation, mcPLGA MPs showed a high contrast enhancement with a CTR value comparable to the commercially available ultrasound contrast agents after HIFU-induced implantation. Broadly, this novel and innovative method opens up a non-invasive route to monitor, improve, and verify treatment for inflamed arterial tissue as a result of atherosclerosis, angioplasty, and other peripheral vascular diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge.

Spectral density curves of mcPLGA MPs; SEM and fluorescent images of hsPLGA MPs; fluorescence microscope image of spheroids embedment; fluorescence images of spheroids (PDF)

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Notes

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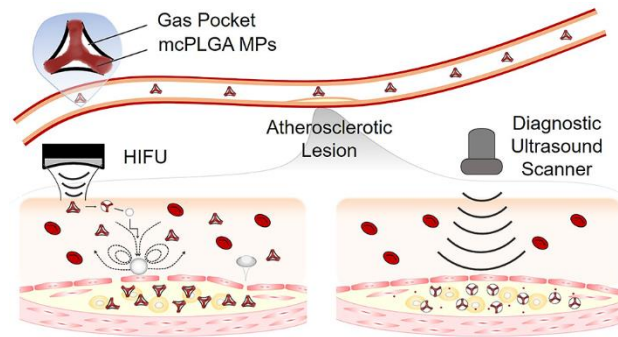
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