

Microbubbles, nanodroplets and gas-stabilizing solid particles for ultrasound-mediated extravasation of unencapsulated drugs; an exposure parameter optimization study.

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Abstract

Ultrasound-induced cavitation has been proposed as a strategy to tackle the challenge of inadequate extravasation, penetration and distribution of therapeutics into tumours. Here, the ability of microbubbles, droplets and solid gas-trapping particles to facilitate mass transport and extravasation of a model therapeutic agent following ultrasound-induced cavitation is investigated. Significant extravasation and penetration depths on the order of millimetres are achieved with all three agents, including the range of pressures and frequencies achievable with existing clinical ultrasound systems. Deeper but highly directional extravasation was achieved with frequencies of 1.6 and 3.3 MHz compared to 0.5 MHz. Increased extravasation was observed with increasing pulse length and exposure time while an inverse relationship is observed with pulse repetition frequency. No significant cell death or any haemolytic activity in human blood was observed at clinically relevant concentrations for any of the agents. Overall, solid gas-trapping nanoparticles were found to enable the most extensive extravasation for the lowest input acoustic energy, followed by microbubbles then droplets. The ability of these agents to produce sustained inertial cavitation activity whilst being small enough to follow the drug out of the circulation and into diseased tissue, combined with a good safety profile and the possibility of real-time monitoring, offers considerable potential for enhanced drug delivery of unmodified drugs in oncological and other biomedical applications.

Keywords: Drug delivery; extravasation; nanoparticles; cavitation; microstreaming; microbubbles; droplets; focused ultrasound

INTRODUCTION

The efficacy of current and emerging therapeutics is severely limited by inadequate penetration and distribution throughout the tumour (Carlisle and Coussios 2013; Minchinton and Tannock 2006). While the leaky character of tumour vasculature may help in the passive extravasation of anticancer drugs into the perivascular region (Iyer et al. 2006), unregulated extravasation of fluids leads to increased intratumoural and interstitial pressure that hinders penetration and distribution of the drug into the deeper and often less vascularised parts of the tumour (Baxter and Jain 1989). The problem is exacerbated when larger therapeutic agents such as antibodies, drug-loaded liposomes and oncolytic viruses are used, since passive transport is limited due to their size (Jain and Stylianopoulos 2010).

Ultrasound-induced acoustic cavitation has been proposed as a means of promoting extravasation and in situ delivery of anticancer agents (Arvanitis et al. 2011; Prieur et al. 2016). Acoustic cavitation refers to the dynamic response of gas and vapour cavities to an acoustic field (Coussios and Roy 2008). Low acoustic pressure amplitudes lead to stable, low energy bubble oscillations, known as non-inertial cavitation, and the onset of nonlinear scattering, whereas higher acoustic pressure amplitudes lead to rapid expansion and violent collapse of the bubbles, a process known as inertial cavitation (IC) (Flynn et al. 1964; Holland and Apfel 1990).

IC has been shown to induce shockwaves and liquid microjets when in proximity to physical boundaries such as vessel walls or cell membranes (Mitragotri 2005). Microstreaming, associated with sustained bubble oscillations in an absorbing medium, (Eller 1969; Gould 1974), has been shown to promote the penetration and

extravasation of free drugs for a variety of applications (Bhatnagar et al. 2016; Datta et al. 2006; Kooiman et al. 2010; Li et al. 2015; Rifai et al. 2010).

The onset of acoustic cavitation at pressures within the range corresponding to medical applications relies on existing gas pockets or nuclei in a medium. Blood or tissue contain relatively few such (endogenous) nuclei, and consequently cavitation tends to be stochastic and large acoustic pressures are required for the initiation of bubble growth and subsequent collapse (Holland and Apfel 1990). Introducing (exogenous) cavitation nuclei decreases the threshold of cavitation and substantially increases the probability of cavitation inception (Datta et al. 2006; Kwan et al. 2015a).

There are currently three broad classes of cavitation nuclei in clinical use or under development: coated gas bubbles, acoustically vaporizable liquid droplets (Miller et al. 2000), and solid gas-entrapping nanoparticles. The aim of the present work is to compare the ability of selected nuclei from each of these three classes to achieve mass transport and extravasation of a co-administered, unencapsulated therapeutic agent, in response to a range of acoustic parameters. Agents that encapsulate a drug for the purposes of ultrasound-triggered release, or studies that aim to transport therapeutics across the cell membrane or the blood brain barrier, fall beyond the scope of the present work.

Microbubbles are clinically approved for use in diagnostic applications and show great potential as cavitation nuclei in therapeutic applications. Inertial cavitation with microbubbles has been shown to significantly enhance extravasation of an adenovirus in vitro compared to non-inertial cavitation (Arvanitis et al. 2011; Bazan-Peregrino et al. 2013). The underpinning mechanism was shown to be enhanced transport rather than sonoporation (defined as alteration of the permeability of the cell

membrane) because the virus used is unable to infect the cell through anything other than an intact cell membrane.

Drawbacks of microbubble formulations as cavitation nuclei include their relatively large size, short circulation times and rapid destruction upon ultrasound exposure (Kwan et al. 2015b; Mannaris and Averkiou 2012). This prevents the extravasation and accumulation of microbubbles in tumour tissue (Dimcevski et al. 2016), thus limiting their capacity to enhance drug delivery over timescales that are comparable to the circulation of the drug. In their viral delivery study, Carlisle et al. (2013) clearly identified the need to re-administer microbubbles every 120 seconds as a significant impediment to translating these effects in humans.

Haemolysis and platelet aggregation resulting from microbubble interaction with ultrasound has been shown in vitro. Specifically, haemolysis levels increase with pressure, UCA concentration, total exposure time, increasing pulse length and pulse repetition frequency (PRF) (Chen et al. 2003b), but decrease with increasing acoustic frequency (Brayman et al. 1997), with cavitation considered to be the cause of most of these effects (Jakobsen et al. 2005). The safety of microbubble use in conjunction with a therapeutic agent was also evaluated in a phase I clinical study (Dimcevski et al. 2016) where a clinical scanner was used to enhance the delivery of gemcitabine to patients with inoperable pancreatic cancer. The study reported significantly improved median survival (from 8 to 18 months), with no increased toxicity or frequency of side effects. However, the authors felt limited by the fact that microbubbles flow through the vasculature and capillaries, allowing direct contact only with endothelial cells and resulting in enhanced drug uptake only by these cells, and motivated the need for exploring other cavitation nucleation agents to potentially further improve therapeutic efficacy. Building on these findings, there would be significant benefits conferred by

long- circulating, sub-micrometre sized particles that enable accumulation of cavitation nuclei within tumour tissues via passive, or active extravasation by microstreaming.

Phase-change agents composed of perfluorocarbons that remain liquid at body temperature, and then vaporize upon application of ultrasound are one approach that has been developed to overcome this obstacle. This process known as acoustic droplet vaporisation (ADV) was originally introduced by Correias and Quay (1996) as a contrast agent. Droplets which are $< 1\mu\text{m}$ in diameter, and can therefore potentially extravasate passively from the leaky vasculature, have also shown excellent potential as systemic drug carriers (Rapoport et al. 2007) and imaging contrast agents (Sheeran and Dayton 2012). Other applications of ADV include embolotherapy (Kripfgans et al. 2005), HIFU ablation (Sheeran and Dayton 2012) and targeted drug delivery (Wang et al. 2013).

The thresholds for ADV and subsequent IC for droplets depend on the size, composition and materials used, temperature, their aqueous environment, and, the ultrasound excitation frequency and pulse length. There is consequently large variability in reported ADV thresholds found in literature (Sheeran and Dayton 2014).

The perfluorocarbons used in droplet formulations have been utilised in blood substitutes and oxygen carriers (Lickliter et al. 2015) at higher concentrations than are likely to be required for ultrasound mediated therapy based on laboratory studies. Similarly, the surfactants that stabilise the droplets are of the same type as those used in ultrasound contrast agents and other drug delivery systems that have been deemed safe for human use. In animal studies, however, repeated administration of high doses of lipid-coated droplets, resulted adverse effects and sometimes death. The upper dose limit was determined at 3×10^9 droplets/kg or a perfluoropentane dose of 0.2g/kg (Zhang et al. 2010). Another potential limitation of ADV and other sub-micrometre agents

developed to date, is that higher acoustic intensities are required to induce cavitation compared to gas microbubbles (Boissenot et al. 2016). Once the liquid droplet changes to a bubble, the newly formed bubble is exposed to higher acoustic intensities and therefore any safety concerns associated with bubble cavitation such as haemolysis and capillary rupture (Samuel et al. 2012) are amplified.

Even though droplets have yet to be fully approved for use as contrast agents and to date there are no known reports of ADV for therapeutic delivery in humans, their sub-micrometre size, potentially extended circulation half-life and ease of manufacture make droplets a promising theranostic agent.

Recently, gas nanobubbles were successfully stabilised on cup-shaped polymeric shells of submicron diameter that exhibited IC at low acoustic intensities, achievable with existing diagnostic and therapeutic systems. In their introduction of these submicron solid gas-entrapping nanoparticles, Kwan et al. (2015b) have shown that these nanoparticles remain completely unresponsive up to a certain pressure threshold – 0.5 MPa at 0.5 MHz ($MI = 0.7$), after which they start producing broadband emissions, an indication of IC (Kwan et al. 2015a). These sub-micron cups also showed sustained cavitation activity for at least four times longer than existing microbubble constructs (Kwan et al. 2015b) and were successfully used to achieve a 10,000-fold increase in intravenously delivered vaccinia virus activity within target tumours when compared to the application of ultrasound without cups (Myers et al. 2016). This same study demonstrated that, under identical conditions, cavitation mediated by sub-micron cups enhanced the delivery of the virus by more than sixfold compared to microbubbles, and hypothesized that this effect was due to improved intratumoural penetration of sub-micron cavitation nuclei. Furthermore, the structure and activity of small molecule,

antibody and virus based drugs are maintained following exposure to the cavitation events instigated by such cavitation nucleation agents (Myers et al. 2018).

In contrast to microbubbles, the solid shell of sub-micron solid gas-stabilizing particles cannot dissolve in the body and its clearance mechanisms have yet to be fully elucidated. To date, no adverse effects have been reported in any of the *in vivo* studies using gas stabilising polymeric cups.

The novelty of the present work is to compare the ability of three classes of cavitation nucleation agents, namely, microbubbles, polymer-coated droplets and polymeric cups, to achieve mass transport and extravasation of a co-administered, unencapsulated therapeutic agent, over a range of acoustic conditions. At 200 nm, the model agent was chosen to be comparable in size to the largest macromolecular agents in clinical use today, including oncolytic viruses, liposomal drug carriers and antibody-drug conjugates. Extravasation of the agent was quantified by fluorescence microscopy. The toxicity of each cavitation agent was tested in a haemolysis assay and the safety of the optimised exposure regime was further assessed for each class of cavitation agent using cell viability assays following insonation in cell embedded tissue mimicking flow phantoms.

MATERIALS AND METHODS

The aims of this study are addressed experimentally using a protocol that features cavitation nuclei flowing inside a tissue mimicking phantom, wherein extravasation of a fluorescent model drug in suspension with the nuclei is quantified using microscopy techniques following ultrasound exposure. A schematic diagram of the experimental setup used is shown in [Figure 1](#). The main components of the setup

are the focused ultrasound (FUS) transducer, the in vitro tissue mimicking flow phantom model (with an embedded 1-mm channel through which the cavitation nuclei and the model drug can flow) and a passive cavitation detector (PCD) that is used to passively record the acoustic emissions produced from cavitation. The FUS and PCD are fully controlled from custom-made software using graphical programming language (LabVIEW, National Instruments, Austin, TX, USA).

Therapeutic/focused ultrasound

An arbitrary waveform generator (33220A, Agilent, Santa Clara, CA, USA) was used to create the transmit signal which was amplified by a 300W RF power amplifier (A-300, ENI, Mountain View, CA, USA) and sent to the focused transducer via a 50-ohm matching network. Two spherically-focused single-element FUS transducers were used, H107 and H102 (Sonic Concepts, Bothell, WA, USA) with a centre frequency of 0.5 and 1.1 MHz respectively. The third harmonic of the H102 transducer was used for the 3.3 MHz experiments. The aperture and the geometric focus of the transducers are 64 mm and 60 mm respectively. Both FUS transducers used were previously calibrated in water using a 0.4-mm-diameter needle hydrophone (HNA-0400, Onda Corporation, Sunnyvale, CA, USA). All acoustic pressures reported in this study are in MPa peak rarefactional pressures (PRP).

Cavitation nuclei

SonoVue microbubbles

SonoVue (Bracco, Geneva, Switzerland) was reconstituted according to manufacturer's recommendations. To avoid variability due to bubble deterioration, a small sample was drawn from the vial prior to each exposure to prepare a final concentration of approximately 1×10^7 bubbles/mL (10% of original concentration)

(Schneider 1999). Each SonoVue vial was used for a maximum of 2 hours following reconstitution.

Magnetic perfluorocarbon droplets

100 μ L of a solution of oleic acid-coated iron oxide nanoparticles in dichloromethane (25 mg Fe/mL) and 50 μ L of perfluorohexane were added to 1 mL of a solution of poly(ethylene glycol) methyl ether-*block*-poly(D,L-lactide)-*block*-decane (M_n 2 kDa) in water (2.5 mg/mL). The heterogeneous mixture was emulsified with an ultrasonic cell disruptor at 20 kHz, power setting 8, 20 seconds (XL-2000, Qsonica, Newtown, CT, USA), diluted with 1 mL of water and washed three times by pelleting the droplets with a magnet and replacing the supernatant with PBS. The droplets were then further diluted by adding 3 mL of PBS and stored at 4 °C. The final concentration was $\sim 10^8$ droplets/ml as measured by single particle optical sensing (Accusizer, Nicomp, Santa Barbara, USA) with a mean diameter of 700nm as measured by dynamic light scattering (Zetasizer, Malvern, UK).

Submicron polymeric cups

Details of the procedure for polymeric cup preparation can be found in Kwan et al. (2015b). Deionised and degassed water was used to dilute a stock solution of 25 mg/mL (obtained from OxSonics ltd, Oxford, UK) to a final concentration of 1 mg/mL which corresponds to an estimated $1e10$ cups/mL (calculated from weight concentration of cups and density of the polymer) with a mean diameter of 500 nm as measured by transmission electron microscopy (TEM). The effective gas pocket diameter was measured by TEM to be 250 nm (Kwan et al. 2015b).

The concentration of each agent was carefully chosen to closely match the molar concentration of gas injected following the rationale that nucleation, growth and inertial collapse of bubbles have been shown to depend upon the dissolved gas concentration

(Plesset and Prosperetti 1977; Rooze et al. 2013). For SonoVue and cups, c – the molar concentration of gas was calculated as

$$c = \frac{n}{RT} \int_0^\infty \left(p_0 + \frac{2\gamma}{r} \right) V(r) P(r) dr \quad (1)$$

where n is the number of bubbles per unit volume, R is the gas constant, T is the temperature, p_0 is the gas pressure at zero surface tension, γ is the surface tension, r is the radius and $V(r)$ is the volume of one bubble and $P(r)$ is the normalised size distribution. A temperature of 300 K and a gas pressure p_0 of 101325 Pa were used for both types of cavitation nuclei. The surface tension of SonoVue was assumed negligible for the purposes of this calculation, while a surface tension of 0.072 N/m was used for cup-stabilized bubbles. The size distribution of the gas pocket reported by Kwan et al. (2015b) was used to calculate $P(r)$ for cup-stabilized bubbles, while the size distribution reported by Greis et al. (2004) was used to calculate $P(r)$ for SonoVue. The volume concentration of gas (μL of gas per mL of solution) can be calculated in a similar way and was found to be 0.93 $\mu\text{L}/\text{mL}$, which is consistent with 10% of the value of 8 $\mu\text{L}/\text{mL}$ stated in the initial scientific discussion for the approval of SonoVue published by the European Medicines Agency (EMA 2004).

For droplets, the molar concentration of gas was calculated as the molar concentration of liquid perfluorohexane (assuming complete vaporisation):

$$c = \frac{n \cdot \rho}{MW} \int_0^\infty V(r) P(r) dr \quad (2)$$

where n is the number of droplets per unit volume, $\rho = 1.63 \text{ g}/\text{cm}^3$ is the density of liquid perfluorohexane, $MW = 338.04 \text{ g}/\text{mol}$ is the molecular weight of perfluorohexane, $V(r)$ is the volume of one droplet and $P(r)$ is the normalised size distribution.

The resulting molar concentrations were calculated at 38, 56 and 96 μM for SonoVue, cups and droplets respectively. Size distribution curves for each agent are shown in Figure A.1

Model Drug

To simulate delivery of large macromolecular therapeutic such as an oncolytic virus or liposomal drug carrier, 200nm carboxylate-modified green fluorescent spheres (ThermoFisher, Waltham, Massachusetts, United States), were added to the cavitation nuclei solutions prior to each experiment. 1 μL of the model drug was added per 1mL of solution. Each solution was checked for possible aggregation prior to each experiment using DLS (ZetaSizerNano, Malvern, Worcestershire, UK), but no aggregation was ever observed.

Tissue mimicking flow phantom

A degassed biocompatible hydrogel composed of 1.25% (w/v) low melting point ultrapure agarose gel (Invitrogen, Carlsbad, CA, USA) with an embedded 1 mm channel was created by casting hot gel in a rectangular tissue-mimicking flow phantom containing three 1 mm metal rods, and withdrawing the rods after cooling and setting of the gel. The gel vessel phantom is a porous gel of approximately 500nm pore diameter (Maaloum et al. 1998), which is comparable to the endothelial gaps in tumour tissue (Hobbs et al. 1998). The phantom contained three 50mm-long channels which allowed multiple conditions to be tested in the same gel, thus minimising variability. A clear and acoustically transparent Mylar film was used to isolate the gel from the surrounding water and allow free propagation of ultrasound and subsequent microscopy. A picture of the flow phantom is displayed in Figure 1. A low-pulsatility peristaltic pump (Minipulse Evolution, Gilson, Middleton, WI, USA) was used to flow

the model drug and cavitation nuclei solution at a constant flow rate of 0.2 mL/min (4.2 mm/sec). The flow rate was chosen to avoid channel rupture and leakage while still in agreement with previously published data of tumour perfusion (Kallinowski et al. 1989). Using the multiple channel pump head flow was achieved by both “drawing” and “infusing” thus reducing any pressure build-up in the channel which would damage the channel.

Passive Cavitation Detection

Acoustic emissions arising from cavitation were sensed by a PCD setup described previously (Hockham et al. 2010; Kyriakou et al. 2011; Mannaris et al. 2018). A 7.5 MHz single element PCD (V320, Panametrics, Olympus, Waltham, MA, USA), of element diameter 12.5 mm and focal length 75 mm, was coaxially and confocally aligned with the FUS transducer via a central circular opening in the FUS. The acquired PCD signal was filtered using a 5 MHz high pass filter (FILT-HP5-A, Allen Avionics, River Grove, IL, USA), amplified by a factor of 5 with a low noise amplifier (SR445A, Stanford Research Systems, Sunnyvale, CA, USA) and recorded with a 14-bit PCI Oscilloscope device (PCI-5122, National Instruments, Austin, TX, USA) at a rate of 100 MHz. The high-pass filter was used to reject strong reflections from the flow phantom at the fundamental FUS frequency (and harmonics due to non-linear propagation).

The pressure threshold to initiate cavitation activity of each type of nuclei was determined by 200-cycle FUS excitation pulses that were ramped at small pressure steps under constant flow. Use of a low PRF (0.5 Hz) assured that the ultrasound focus was refreshed with cavitation nuclei for each exposure. A typical PCD data trace consisted of an initial $\sim 85\mu\text{s}$ segment (‘background’) that was free of any signal content

in the filter pass band, followed by scattering and cavitation emissions ('signal') whose durations varied with drive pulse length. The background and signal segments of each trace were analysed in MATLAB (Mathworks, Natick MA, USA) to determine if inertial cavitation had occurred, and the full ensemble of PCD traces were reviewed to calculate a probability of cavitation at the prescribed ultrasound settings. The identification of inertial cavitation was based upon broadband spectral elevation in the signal relative to the background. For this analysis, FUS harmonic components were removed either with a comb filter or sum of harmonics (Lyka et al. 2016) processing. Based on review of the signal and noise levels across all experiments, harmonic-suppressed traces were deemed to consistently exhibit inertial cavitation when the mean-squared signal to background ratio exceeded e^3 .

Ultrasound imaging

A Philips iU-22 ultrasound scanner (Philips Ultrasound, Bothell, WA, USA) was used to provide real-time imaging of the flow phantom during the experiment. As seen in Figure 1, a linear L12-5 probe was placed at an angle of 60° to the FUS and PCD propagation axis, in order to minimise interference between the imaging beam and the PCD trace. The imaging allowed monitoring of the integrity of the channel and uniformity of the flow, detection of possible leaks, and any air bubbles that would interfere with the results. A low, non-destructive mechanical index ($MI < 0.05$) was used in order to avoid any interference of the imaging with the therapeutic pulse and cavitation nuclei. During SonoVue experiments, contrast mode was used to check the consistency of the bubble concentration.

Cavitation mediated extravasation

All experiments were carried out in a large tank filled with deionised water at 37 °C that was degassed overnight prior to each experiment. With the channel filled with air, both the FUS and PCD were driven in pulse-echo mode using a pulser-receiver (DPR300, JSR Ultrasonics, Pittsford, NY, USA) in order to ensure that the FUS was aligned with the middle of the flow channel and that the PCD focus was aligned with the FUS. The cavitation nuclei and model drug solution was then introduced at a constant flow rate and was kept flowing during FUS exposures. New tubing was used for each type of cavitation nuclei to avoid cross contamination. Control experiments were done using degassed water with the model drug in the absence of cavitation nuclei. The input voltages to the FUS matching network were also recorded and converted to peak negative pressures using previous hydrophone calibrations.

Microscopy

All widefield microscopy was carried out using an inverted microscope (Eclipse Ti; Nikon Inc, Melville, NY, USA). Flow channels were flushed with de-ionised water following ultrasound exposure to remove any remaining fluorescent particles. A rectangular prism containing the flow channel, approximately 40mm long, was then excised and placed on a glass microscope slide for imaging. Side and top-views in both brightfield and fluorescence were acquired around each exposure location. Nikon filter cubes were used to image green fluorescence, FITC (Exciter 465–495, Dichroic 505, Emitter 515–555) or red fluorescence, TRITC (Exciter HQ545/30×, Dichroic Q570LP, Emitter HQ620/60 m). Extravasation, defined here as any fluorescence detected outside the flow channel, was quantified in ImageJ (Schneider et al. 2012). The fluorescent images were first thresholded to highlight any pixels that exhibited higher fluorescent

intensity compared to the background followed by a manual ROI selection (see Figure 1) that was used to quantify total area, length and width of extravasation.

Using the side view images, the extent of extravasation along the axial and lateral planes (z and x) of the FUS beam was quantified whereas the top view images were used to quantify extravasation along the elevation plane (y-axis). The lateral and elevation planes were similar, which was as expected given that the FUS beams of our circular transducers are axisymmetric. Results are therefore shown only for the axial and lateral planes.

Rationale for the Choice of Ultrasound Exposure Conditions

Table 1 shows the matrix of exposure parameters investigated in this work. Three excitation frequencies were tested: 0.5 MHz, deemed the lowest frequency to minimize tissue cavitation in the absence of cavitation nuclei, and a commonly used frequency in tumour extravasation studies; 1.6 MHz, a commonly used frequency for therapeutic applications in the clinic as well as in imaging of obese patients; and 3.3 MHz, a frequency in the range commonly used by conventional clinical imaging scanners. The maximum pressure at 0.5, 1.6 and 3.3 MHz was 2, 3.5 and 5.5 MPa respectively. All these pressures correspond to a $MI \approx 3$, but it should be noted that the MI is not strictly applicable to pulses longer than a single cycle. With the exception of investigating PRF, the duty cycle (DC) was limited to <5% in order to minimise any thermal effects.

The total acoustic energy density delivered between each experiment was kept constant whenever possible. For example, when investigating the effect of pulse length, 300 bursts of 2500 cycles were compared against 600 bursts of 1250 cycles, 1500 bursts of 500 cycles and so on.

Safety

Thermometry

To rule out any ultrasound induced hyperthermia and possible implications in extravasation, a fine wire thermocouple was embedded in the agar phantom as close to the channel as possible without puncturing the channel. The FUS was aligned with the thermocouple both acoustically (pulse-echo) and thermally (using single bursts and measuring temperature elevation). Temperature elevation was recorded for the maximum energy settings in our parameter space (2500 cycles, 2 MPa at 0.5 MHz, 8000 cycles, 3.5 MPa at 1.6 MHz and 16500 cycles, 5.5 MPa at 3.3 MHz) for each individual type of cavitation nuclei.

Haemolysis

A haemolysis assay was used to assess the death of red blood cells (RBC) in whole blood incubated for 3 hours with serial dilutions of the 3 types of cavitation nuclei. Four-times concentrated stock solutions were prepared for each final concentration of cavitation nuclei to account for the dilution with whole blood. Stock solutions were prepared by diluting the cavitation nuclei with their native solvent i.e. PBS (droplets), 0.9% NaCl (SonoVue) or a glucose solution (cups). The range of concentrations tested was $1e7$ to $1e10$ cups/mL (0.001-1.0 mg/mL), $5e5$ to $1e8$ droplets/mL (D1000-D5) and $1e5$ to $2e7$ bubbles/mL. Informed consent was received from the donor and the study approved was by the university's ethics committee.

For each concentration tested, three samples were prepared by adding 150 μ L of whole blood to 50 μ L of the corresponding stock solution of cavitation nuclei. A positive control was prepared by adding 150 μ L of whole blood to 50 μ L of a 0.4% solution of triton X-100. A negative control was also prepared for each type of cavitation nuclei by adding 150 μ L of whole blood to 50 μ L of the native solvent of

each nuclei. The samples were incubated at 37 °C for 3 hours. 32 µL of each sample was then diluted to 600 µL and centrifuged at 5000 g for 5 minutes. The absorbance of the supernatants was measured in triplicate in a plate reader (Omega POLARStar, BMG Labtech, Ortenberg, Germany) at 541 nm and normalised to the absorbance of the positive control.

Cell viability

The safety of the proposed drug delivery method was further evaluated for each cavitation agent by measuring cell viability following ultrasound induced cavitation in a cell-embedded tissue mimicking flow phantom.

A549 cells were obtained from the European Collection of Cell Cultures, and were cultured in high-glucose DMEM (11965-092, Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; 10270-106, Life Technologies, Carlsbad, CA, USA) to give 'growth media'. Two stains from the LIVE/DEAD Viability/Cytotoxicity Kit (L3224, ThermoFisher, Waltham, MA USA) containing ethidium homodimer ("dead stain" indicating loss of membrane integrity) and calcein ("live stain" indicating intracellular esterase activity) were added to cells in growth media, which were then combined with low-melting point agarose dissolved in PBS at 37 °C. The resulting mixture had an agarose concentration of 1.5% (w/v), a cell concentration of 4e5 cells/mL, 1.6 µM of calcein, and 2.5 µM of ethidium homodimer. The cell-gel mixture was allowed to set in the tissue-mimicking flow phantom (described above) at 4 °C for approximately 30 minutes.

Prior to the experiment, the feasibility of using the LIVE/DEAD kit in a cell-embedded gel matrix and the ability of ethidium homodimer to stain "freshly killed" cells was verified by imaging cells in agarose (same conditions as above) before and

after exposure to UV light for 1 hour, followed by incubation at 60 °C for 30 minutes. Figure A.2 in the Appendix shows fluorescent images of the cells before treatment (mostly green) and after UV/Heat-treatment (mostly red) indicating that ethidium homodimer successfully stained the freshly killed cells.

Following the same protocol described above, each cavitation agent was flowed through the cell embedded agarose phantom and exposed to FUS in the absence of the model drug. Two frequencies (0.5 and 1.6 MHz) were selected for the cell viability study and the “extreme” conditions at each frequency i.e. 2500 cycles at 0.5 MHz and 8000 cycles at 1.6 MHz were evaluated. The duty cycle was fixed at 5%. Approximately 1 hour after completion of ultrasound exposures, rectangular prism sections of the cell-embedded gels were excised around each channel and imaged under the microscope. Z-stack scans of each channel were acquired around the middle of the channels in brightfield, green and red fluorescence. Using ImageJ, analysis of the fluorescence images was achieved as follows: *i.* background subtraction and ‘despeckle’, to remove any background noise; *ii.* automatic thresholding (Otsu method), *iii.* segmentation into “exposed” and “unexposed” sections and *iv.* cell counting using Analyze Particles tool. This procedure was repeated for each of the 5 different stacks and summed. To account for cell density variability between samples, the percentage of dead cells (% dead) is calculated, by dividing the number of red cells by the total number of cells (red + green). The % dead for each condition is normalized by dividing by the % dead in the exposed segments by the % dead in adjacent “unexposed segments”, thus eliminating any differences in the cell population due to other factors (e.g. location, non-uniform dye concentrations etc.)

RESULTS

Passive cavitation detection

The probability of inertial cavitation (PIC) for each type of nuclei is shown in Figure 2. Across frequencies and pressures tested, the PIC of SonoVue microbubbles is 100% with the exception of 0.5MHz, pressure < 0.4 MPa where the PCD signal primarily consists of narrowband emissions associated with non-inertial cavitation. In agreement with previous work (Kwan et al. 2015b), a clear threshold is seen with cups which seem unresponsive up to a certain pressure before exhibiting high PIC. At 0.5 MHz the threshold lies between 0.5 and 1 MPa. At 1.6 MHz, the threshold is between 0.6 and 1.2 MPa while at 3.3 MHz the threshold is between 1.4 and 2.8 MPa. As seen in Figure 2(a), no cavitation beyond that already produced in water alone was detected with droplets at 0.5 MHz. At 1.6 and 3.3 MHz, cavitation from droplets becomes detectable at pressures >3 and >4 MPa respectively. It must be noted that the results presented correspond to this particular formulation of droplets whose cavitation threshold was significantly higher compared to previous formulations and experiments carried out in our laboratory (Lee et al. 2015). As discussed above, there is a plethora of droplet formulations, with a range of substantially different properties and exhibiting significantly different dependencies upon ultrasound exposure conditions. Such variation makes it impossible to cover all such formulations in this work and also highlights the need for further study and standardisation of this type of agent.

Extravasation

Representative fluorescent images of extravasation are shown in Figure 3, arranged by type cavitation nuclei (columns) and frequencies (rows). Ultrasound direction is shown by the arrow (bottom-to-top of page) and this applies to all

microscope images in this paper. A qualitative difference is observed in the extravasation patterns, particularly at the higher frequencies, with droplets typically producing a single ‘filament’ of extravasation, whilst cups and microbubbles produce a broader ‘fireworks’-like pattern. Tunnelling, which is indicative of bubble burrowing (Caskey et al. 2007), is observed mostly with microbubbles (right column) and droplets (left column) and less so with cups. The penetration distance in the direction of sound propagation is comparable across all three agents, but transport transversely to the acoustic axis differs considerably.

No extravasation was detected during control exposures without cavitation nuclei regardless of the ultrasound parameters used.

Effect of frequency

Representative fluorescent images of the flow channel following exposures with polymeric cups at 0.5, 1.6 and 3.3 MHz are shown in Figure 4. Top view images of the flow channel at 0.5, 1.6 and 3.3 MHz are shown in Figures 4 (a), (b) and (c) respectively, and the corresponding side view images in (d), (e) and (f). A more confined but omnidirectional extravasation was observed at 0.5 MHz while higher frequencies lead to extravasation which was always in the direction of ultrasound propagation. This was valid for all types of nuclei with the exception of droplets that produced no cavitation and/or extravasation at 0.5 MHz. Maximum extravasation distance was achieved at 1.6 MHz (Figure 4 b,e), followed by 3.3 MHz (Figure 4 c,f). The width of extravasation decreases with increasing frequency which matches well the beam width of the FUS.

Figures 5-7 show a numerical summary of the results from all the experiments performed. All the results are averages of a minimum of four independent experiments at each setting and error bars represent one standard deviation. The total area of

extravasation is plotted against interval between pulses (rather than PRF), pulse length in cycles and exposure time. Shaded elliptical areas near each marker represent the area of extravasation at the given setting and are drawn to scale. In the case where backwards extravasation was also detected then a second ellipse is added below the marker. At the reader's disposal, individual graphs for extravasation length and width are supplied in the Appendix (Figures A.3 and A.4).

Effect of PRF

PRF was varied from 1-100 Hz (10-1000ms interval between pulses). For this series of experiments, 300 bursts of 2500, 8000 and 16500 cycles were used for 0.5, 1.6 and 3.3 MHz respectively and the results are shown in Figure 5. The reader should note that different axis scales have been used for better clarity of the graphs.

A more omnidirectional extravasation is seen with SonoVue microbubbles at 0.5 MHz throughout the range of pulse intervals investigated. With cups, backwards extravasation is observed only with short pulse intervals (Figure 5, 0.5 MHz or Figure A.3). Higher frequencies produce narrower but deeper penetration which is always in the direction of ultrasound propagation.

Across all frequencies, cavitation mediated extravasation with microbubbles and cups increases with pulse interval until a plateau is reached. This is not true for droplets especially at 1.6 MHz (Figure A.3) where an inverse relationship between extravasation and pulse interval is observed. This might be attributed to dissolution of the vapor bubbles.

Effect of Pulse Length

Figure 6 plots extravasation as a function of pulse length. The total energy delivered was kept constant by adjusting the number of bursts while varying the pulse

length. As expected, across frequencies and cavitation nuclei, longer bursts facilitate more extravasation. Interestingly, a threshold exists for all agents, where longer bursts have no added effect, especially at the higher frequencies.

Overall, shorter bursts are required for extravasation to be achieved with cups compared to microbubbles and droplets. This would be favourable in a clinical scenario where less energy would be needed in order to achieve the desired delivery effect. In addition, significant extravasation levels are achieved with cups at the lower frequencies, which is important when trying to achieve delivery in deep tissues.

Effect of Exposure time

Extravasation as a function of exposure time is shown in Figure 7. Longer exposure times resulted in enhanced extravasation and would thus be beneficial in terms of cavitation mediated extravasation in a clinical setting, something that can be favoured with long-circulating cavitation nuclei.

Real time monitoring

Low MI harmonic imaging of the experimental procedure is shown in Figure 8. While SonoVue microbubbles can be clearly seen entering the channel from the left (Figure 8(a)), the FUS pulse rapidly destroys the microbubbles around the target area indicated by the arrow. The opposite is true with submicron agents however that are invisible to ultrasound as shown in Figure 8(b), until activated by the FUS pulse. The hyperechoic signal detected outside the channel in Figure 8(c) is an indication of cups cavitating even after they have extravasated from the channel. This capacity for sustained cavitation may allow mapping of the extent of the therapeutic process in real time.

In Figure 8(d) the hyperechoic signal before the target region indicates the presence of bubbles along with droplets, possibly due to some of the droplets vaporising during the experimental procedure (handling, flowing etc.) as previously described (Rapoport 2016). Similar to SonoVue, these bubbles are rapidly destroyed by the FUS around the target region. Nevertheless, at the focus, we have ADV and a prolonged cavitation activity that, as described above, would allow monitoring of the therapeutic process.

Safety

Thermometry

In all the conditions tested, maximum temperature increase detected was 2°C above ambient. This suggests that thermal effects are negligible in the context of the present study.

Haemolysis

Haemolysis results are shown in Figure A.5. No significant RBC lysis was observed with any agent for the concentrations tested.

Cell Viability

The results of the investigation of cell viability in the ultrasound exposed tissue-mimicking phantom are shown in Figure A.6. No statistically significant difference ($P>0.05$) in cell viability was found between any of the cavitation agents tested (and the control of glucose alone), at both ultrasound exposure conditions tested. It should be noted that while this contradicts some of the literature, where safety studies of blood brain barrier opening with microbubbles and ultrasound showed endothelial cell damage (Sheikov et al. 2004), it is not surprising since there is no reason for the safety

threshold of an endothelial cell to be similar to that of a cell firmly embedded within a viscoelastic medium.

Discussion

The ability of microbubbles, droplets and polymeric cups to facilitate mass transport and extravasation of a model drug was investigated here. Significant extravasation and penetration depths on the order of millimetres were observed with all three agents, while no safety concerns were raised for any of the three agents, following cell viability and haemolysis assays.

Low frequencies led to more omnidirectional extravasation whereas higher frequencies facilitated deeper but highly directional extravasation. A combination of the two seems ideal in efficiently covering the entire tumour volume. The reason for this observation is unclear and more work is required to elucidate the underlying mechanisms.

Across frequencies, we observe that extravasation with cups and microbubbles increases with increasing pulse interval (decreasing PRF) until a plateau is reached. This effect is linked to the flow rate used, which dictates the rate of replenishment of the target region with cavitation nucleation agents. In a 2003 study, Chen et al. (2003a) investigated the effect of ultrasound parameters in cardiac delivery of plasmid DNA incorporated into liposome microbubbles in vivo. Their reported increase in expression with triggered compared to continuous ultrasound can be attributed to the lack of replenishment of the myocardium with microbubbles when continuous ultrasound was used.

As expected, longer pulses achieve enhanced extravasation. Longer pulses lead to stronger radiation forces and better established microstreaming which has been closely associated with enhanced extravasation and tumour penetration (Li et al. 2015; Myers et al. 2016).

Our results indicate longer bursts, higher frequencies and lower pulse intervals favour extravasation with droplets. This is in agreement to the findings of Kripfgans et al. (2000) who reported a decrease in the IC threshold from 4.5 MPa at 1.5 MHz to 0.75 MPa at 8 MHz and those of Williams et al. (2013) who found inverse relationships between IC threshold and frequency and IC threshold and pulse length. This is an indication that there is a thermal component involved with ADV and IC with the current droplet formulation. We further tested this hypothesis by determining the threshold for IC with droplets at two different intervals: 200 and 50 ms (see Figure A.7). A significant level of IC is seen at pressures as low as 4 MPa when 50 ms interval is used whereas an excess of 7 MPa is required for IC with a 200 ms interval. This may potentially be related to destruction of the bubbles during long pulses as observed by Sheeran et al. (2013). The complexity of the agents is such however that other phenomena such as superharmonic focussing (Miles et al. 2016; Shpak et al. 2014) cannot be dismissed and further work is required to elucidate the mechanisms involved.

Despite the fact that microbubbles require lower pressures to initiate IC, we observe that cups require less energy in order to achieve extravasation. Shorter bursts (Figure 6) and shorter exposure times (Figure 7) are needed to produce notable extravasation with cups. Due to their submicron size, cups can passively extravasate or self-propel through the agar pores during a cavitation event, as observed by Bhatnagar et al. (2016) who used the same cups to enhance transdermal delivery and penetration of a model vaccine both in-vitro and in-vivo. Not only were higher doses and

penetration depths achieved with ultrasound and cups compared to a chemical enhancer but surprisingly, the cups were able to penetrate even further than the model drug, an indication of self-propulsion. Droplets have a similar advantage in terms of their size but require higher energies to achieve significant extravasation compared to both microbubbles and cups.

Real time monitoring of the therapeutic process is an exciting possibility. Sustained cavitation activity from submicron agents like cups and droplets, which have the capacity to extravasate and follow the drug in the tissue, makes it possible to use passive or active detection methods to map the extent of a successfully treated region. This is not possible with microbubbles however, which stay in circulation, and any signal emanating from microbubbles is therefore limited to the vasculature.

Limitations of the study

The authors acknowledge the fact that the tumour mimicking flow phantom setup used in this study is clearly a marked simplification of the complex in vivo tumour environment. Increased interstitial pressure, the complex capillary structure and variable flow rates found in the tumour will introduce complexities that are impossible to reproduce in vitro. The material properties of the gel are also quite different to tissue. Even though not seen in microscopy images of sectioned gels (figure A.8), possible channel formation in the current setup would probably not occur in vivo. We do believe however that the main mechanisms behind cavitation enhanced extravasation, namely microstreaming and radiation force, remain the same and will produce similar outcomes in vivo. In order to verify the results, experiments are already in progress to test the optimal conditions determined in this study in a murine cancer model. In practice, only a subset of the droplets is likely to vaporise. In the absence of a better alternative, we

have assumed complete droplet vaporisation in order to match the molar gas concentration.

The minimal heating observed in the non-perfused lossless phantom may be quite different from the situation in either a perfused phantom or in real tissues. The question of thermal effect/safety should be an item for further study. Nevertheless, preliminary experiments with thermocouples implanted in a tumour during similar exposures, also showed negligible heating (data not shown).

CONCLUSION

The ability of three main classes of cavitation agents, microbubbles, droplets and polymeric cups, to facilitate extravasation of a large model therapeutic has been investigated and the optimal acoustic parameters identified. Significant extravasation has been achieved with all three agents with cups requiring less overall energy followed by microbubbles and droplets, while no cellular safety concerns have been identified for any of the agents and the conditions tested. Real time monitoring of the therapeutic process and mapping of the extent of the treated area is made possible by sustained inertial cavitation.

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from the donor for the haemolysis study which was approved by the university's ethics committee.

REFERENCES

- Arvanitis CD, Bazan-Peregrino M, Rifai B, Seymour LW, Coussios CC. Cavitation-enhanced extravasation for drug delivery. *Ultrasound Med Biol* 2011;37:1838–1852.
- Baxter LT, Jain RK. Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection. *Microvasc Res* 1989;37:77–104.
- Bazan-Peregrino M, Rifai B, Carlisle RC, Choi J, Arvanitis CD, Seymour LW, Coussios CC. Cavitation-enhanced delivery of a replicating oncolytic adenovirus to tumors using focused ultrasound. *J Control Release* 2013;169:40–47.
- Bhatnagar S, Kwan JJ, Shah AR, Coussios CC, Carlisle RC. Exploitation of sub-micron cavitation nuclei to enhance ultrasound-mediated transdermal transport and penetration of vaccines. *J Control Release* 2016;238:22–30.
- Boissenot T, Bordat A, Fattal E, Tsapis N. Ultrasound-triggered drug delivery for cancer treatment using drug delivery systems: From theoretical considerations to practical applications. *J Control Release* 2016;241:144–163.
- Brayman AA, Strickler PL, Luan H, Bared SL, Raeman CH, Cox C, Miller MW. Hemolysis of 40% hematocrit, Albunex-supplemented human erythrocytes by pulsed ultrasound: frequency, acoustic pressure and pulse length dependence. *Ultrasound Med Biol* 1997;23:1237–1250.
- Carlisle R, Choi J, Bazan-Peregrino M, Laga R, Subr V, Kostka L, Ulbrich K, Coussios CC, Seymour LW. Enhanced tumor uptake and penetration of virotherapy using polymer stealthing and focused ultrasound. *J Natl Cancer Inst* 2013;105:1701–1710.

- Carlisle R, Coussios C-C. Mechanical approaches to oncological drug delivery. *Ther Deliv Future Science*, 2013;4:1213–1215.
- Caskey CF, Stieger SM, Qin S, Dayton PA, Ferrara KW. Direct observations of ultrasound microbubble contrast agent interaction with the microvessel wall. *J Acoust Soc Am* 2007;122:1191–1200.
- Chen S, Shohet R V, Bekerredjian R, Frenkel P, Grayburn PA. Optimization of ultrasound parameters for cardiac gene delivery of adenoviral or plasmid deoxyribonucleic acid by ultrasound-targeted microbubble destruction. *J Am Coll Cardiol* 2003a;42:301–308.
- Chen WS, Brayman AA, Matula TJ, Crum LA, Miller MW. The pulse length-dependence of inertial cavitation dose and hemolysis. *Ultrasound Med Biol* 2003b;29:739–748.
- Correas JM, Quay SD. EchoGen(TM) emulsion: A new ultrasound contrast agent based on phase shift colloids. *Clin Radiol* 1996;51:11–14.
- Coussios CC, Roy RA. Applications of acoustics and cavitation to noninvasive therapy and drug delivery. *Annu Rev Fluid Mech* 2008;40:395–420.
- Datta S, Coussios CC, McAdory LE, Tan J, Porter T, De Courten-Myers G, Holland CK. Correlation of cavitation with ultrasound enhancement of thrombolysis. *Ultrasound Med Biol* 2006;32:1257–1267.
- Dimcevski G, Kotopoulis S, Bjanes T, Hoem D, Schjott J, Gjertsen BT, Biermann M, Molven A, Sorbye H, McCormack E, Postema M, Gilja OH. A human clinical trial using ultrasound and microbubbles to enhance gemcitabine treatment of inoperable pancreatic cancer. *J Control Release* 2016;243:172–181.
- Eller AI. Growth of Bubbles by Rectified Diffusion. *J Acoust Soc Am* 1969;46:1246–

1250.

EMA. Initial scientific discussion for the approval of Sonovue. 2004 [cited 2017 Jan 15].

Flynn HG, Papadakis EP, Pierce AD, Mason WP, Thurston RN. Physics of Acoustic Cavitation in Liquids Physical Acoustics. 1964.

Gould RK. Rectified diffusion in the presence of, and absence of, acoustic streaming. J Acoust Soc Am 1974;56:1740–1746.

Greis C. Technology overview: SonoVue (Bracco, Milan). Eur Radiol Suppl 2004;

Hobbs SK, Monsky WL, Yuan F, Roberts WG, Griffith L, Torchilin VP, Jain RK. Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. Proc Natl Acad Sci U S A 1998/05/16. 1998;95:4607–4612.

Hockham N, Coussios CC, Arora M. A real-time controller for sustaining thermally relevant acoustic cavitation during ultrasound therapy. IEEE Trans Ultrason Ferroelectr Freq Control 2010;57:2685–2694.

Holland CK, Apfel RE. Thresholds for transient cavitation produced by pulsed ultrasound in a controlled nuclei environment. J Acoust Soc Am 1990;88:2059–2069.

Iyer AK, Khaled G, Fang J, Maeda H. Exploiting the enhanced permeability and retention effect for tumor targeting. Drug Discov Today 2006;11:812–818.

Jain RK, Stylianopoulos T. Delivering nanomedicine to solid tumors. Nat Rev Clin Oncol 2010;7:653–664.

Jakobsen JA, Oyen R, Thomsen HS, Morcos SK, Members of Contrast Media Safety Committee of European Society of Urogenital R. Safety of ultrasound contrast

- agents. *Eur Radiol* 2005;15:941–945.
- Kallinowski F, Schlenger KH, Runkel S, Kloes M, Stohrer M, Okunieff P, Vaupel P. Blood flow, metabolism, cellular microenvironment, and growth rate of human tumor xenografts. *Cancer Res* 1989;49:3759–3764.
- Kooiman K, Emmer M, Foppen-Harteveld M, van Wamel A, de Jong N. Increasing the endothelial layer permeability through ultrasound-activated microbubbles. *IEEE Trans Biomed Eng* 2010;57:29–32.
- Kripfgans OD, Fowlkes JB, Miller DL, Eldevik OP, Carson PL. Acoustic droplet vaporization for therapeutic and diagnostic applications. *Ultrasound Med Biol* 2000;26:1177–1189.
- Kripfgans OD, Orifici CM, Carson PL, Ives KA, Eldevik OP, Fowlkes JB. Acoustic droplet vaporization for temporal and spatial control of tissue occlusion: a kidney study. *IEEE Trans Ultrason Ferroelectr Freq Control* 2005;52:1101–1110.
- Kwan JJ, Graham S, Myers R, Carlisle R, Stride E, Coussios CC. Ultrasound-induced inertial cavitation from gas-stabilizing nanoparticles. *Phys Rev E Stat Nonlin Soft Matter Phys* 2015a;92:23019.
- Kwan JJ, Myers R, Coviello CM, Graham SM, Shah AR, Stride E, Carlisle RC, Coussios CC. Ultrasound-Propelled Nanocups for Drug Delivery. *Small* 2015b;11:5305–5314.
- Kyriakou Z, Corral-Baques MI, Amat A, Coussios CC. HIFU-induced cavitation and heating in ex vivo porcine subcutaneous fat. *Ultrasound Med Biol* 2011;37:568–579.
- Lee JY, Carugo D, Crake C, Owen J, de Saint Victor M, Seth A, Coussios C, Stride E. Nanoparticle-Loaded Protein-Polymer Nanodroplets for Improved Stability and

- Conversion Efficiency in Ultrasound Imaging and Drug Delivery. *Adv Mater* 2015;27:5484–5492.
- Li T, Wang YN, Khokhlova TD, D’Andrea S, Starr F, Chen H, McCune JS, Risler LJ, Mashadi-Hosseini A, Hwang JH. Pulsed High-Intensity Focused Ultrasound Enhances Delivery of Doxorubicin in a Preclinical Model of Pancreatic Cancer. *Cancer Res* 2015;75:3738–3746.
- Lickliter JD, Wilson DB, Hendrickson H, Raghunand N, Khor S-P, Longacre O, Unger EC. An oxygenation agent and radiation sensitizer, dodecafluoropentane, for the treatment of glioblastoma multiforme. *J Clin Oncol* 2015;33:TPS2078-TPS2078.
- Lyka E, Coviello C, Kozick R, Coussios CC. Sum-of-harmonics method for improved narrowband and broadband signal quantification during passive monitoring of ultrasound therapies. *J Acoust Soc Am* 2016;140:741.
- Maaloum M, Pernodet N, Tinland B. Agarose gel structure using atomic force microscopy: Gel concentration and ionic strength effects. *Electrophoresis* 1998;19:1606–1610.
- Mannaris C, Averkiou MA. Investigation of microbubble response to long pulses used in ultrasound-enhanced drug delivery. *Ultrasound Med Biol* 2012;38:681–691.
- Mannaris C, Boon M. T, Seth A, Bau L, Coussios C-C, Stride EP. Gas-Stabilizing Gold Nanocones for Acoustically Mediated Drug Delivery. *Adv Healthc Mater Wiley-Blackwell*, 2018;0:1800184.
- Miles CJ, Doering CR, Kripfgans OD. Nucleation pressure threshold in acoustic droplet vaporization. *J Appl Phys* 2016;
- Miller DL, Kripfgans OD, Carson PL. Cavitation nucleation agents for nonthermal ultrasound therapy. *J Acoust Soc Am* 2000;

- Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat Rev Cancer Nature Publishing Group*, 2006;6:583–592.
- Mitragotri S. Healing sound: the use of ultrasound in drug delivery and other therapeutic applications. *Nat Rev Drug Discov* 2005;4:255–260.
- Myers R, Coviello C, Erbs P, Foloppe J, Rowe C, Kwan J, Crake C, Finn S, Jackson E, Balloul JM, Story C, Coussios C, Carlisle R. Polymeric Cups for Cavitation-mediated Delivery of Oncolytic Vaccinia Virus. *Mol Ther* 2016;24:1627–1633.
- Myers R, Grundy M, Rowe C, Coviello C, Bau L, Erbs P, Foloppe J, Balloul J-M, Story C, Coussios C, Carlisle R. Ultrasound-mediated cavitation does not decrease the activity of small molecule, antibody or viral-based medicines. *Int J Nanomedicine Dove Press*, 2018 [cited 2018 Feb 15];Volume 13:337–349.
- Plesset MS, Prosperetti A. Bubble Dynamics and Cavitation. *Annu Rev Fluid Mech* 1977;
- Prieur F, Pillon A, Mestas JL, Cartron V, Cebe P, Chansard N, Lafond M, Lafon C. Enhancement of Fluorescent Probe Penetration into Tumors In Vivo Using Unseeded Inertial Cavitation. *Ultrasound Med Biol* 2016;42:1706–1713.
- Rapoport N. Drug-loaded perfluorocarbon nanodroplets for ultrasound-mediated drug delivery. *Adv Exp Med Biol* 2016;
- Rapoport N, Gao Z, Kennedy A. Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy. *J Natl Cancer Inst* 2007;99:1095–1106.
- Rifai B, Arvanitis CD, Bazan-Peregrino M, Coussios CC. Cavitation-enhanced delivery of macromolecules into an obstructed vessel. *J Acoust Soc Am* 2010/11/30. 2010;128:EL310-15.

- Rooze J, Rebrov E V., Schouten JC, Keurentjes JTF. Dissolved gas and ultrasonic cavitation - A review. *Ultrason. Sonochem.* 2013.
- Samuel S, Duprey A, Fabiilli ML, Bull JL, Fowlkes JB. In vivo microscopy of targeted vessel occlusion employing acoustic droplet vaporization. *Microcirculation* 2012;19:501–509.
- Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;9:671–675.
- Schneider M. Characteristics of SonoVue trade mark. *Echocardiography* 1999;
- Sheeran PS, Dayton PA. Phase-change contrast agents for imaging and therapy. *Curr Pharm Des* 2012;18:2152–2165.
- Sheeran PS, Dayton PA. Improving the performance of phase-change perfluorocarbon droplets for medical ultrasonography: current progress, challenges, and prospects. *Sci* 2014;2014:579684.
- Sheeran PS, Matsunaga TO, Dayton PA. Phase-transition thresholds and vaporization phenomena for ultrasound phase-change nanoemulsions assessed via high-speed optical microscopy. *Phys Med Biol* 2013;
- Sheikov N, McDannold N, Vykhodtseva N, Jolesz F, Hynynen K. Cellular mechanisms of the blood-brain barrier opening induced by ultrasound in presence of microbubbles. *Ultrasound Med Biol* 2004;
- Shpak O, Verweij M, Vos HJ, de Jong N, Lohse D, Versluis M. Acoustic droplet vaporization is initiated by superharmonic focusing. *Proc Natl Acad Sci* 2014;
- Wang CH, Kang ST, Yeh CK. Superparamagnetic iron oxide and drug complex-embedded acoustic droplets for ultrasound targeted theranosis. *Biomaterials*

2013;34:1852–1861.

Williams R, Wright C, Cherin E, Reznik N, Lee M, Gorelikov I, Foster FS, Matsuura N, Burns PN. Characterization of submicron phase-change perfluorocarbon droplets for extravascular ultrasound imaging of cancer. *Ultrasound Med Biol* 2013;39:475–489.

Zhang M, Fabiilli ML, Haworth KJ, Fowlkes JB, Kripfgans OD, Roberts WW, Ives KA, Carson PL. Initial Investigation of Acoustic Droplet Vaporization for Occlusion in Canine Kidney. *Ultrasound Med Biol* 2010;

Figure Captions

Figure 1: Schematic diagram of the experimental setup, illustrating the FUS generation and passive cavitation detection loops. On the top right, a picture of the flow-through tissue-mimicking phantom and an example of the ROI selection following fluorescent microscopy.

Figure 2: Probability of Inertial Cavitation as a function of pressure at 0.5, 1.6 and 3.3 MHz. 200-cycle pulses at 200 ms interval under constant flow were used to ensure complete replenishment of ROI. Harmonic-suppressed traces were deemed to consistently exhibit IC when the mean-squared signal to background ratio exceeded e^3 .

Figure 3: Representative fluorescent images of agarose flow channel following cavitation mediated extravasation with droplets (left column), submicron polymeric cups (middle column) and SonoVue microbubbles (right column) at 0.5, 1.5 and 3.3 MHz at 2, 3.5 and 5.5 MPa respectively. Different nuclei result in different extravasation profiles. Direction of ultrasound propagation indicated by arrow.

Figure 4: Effect of frequency on cavitation mediated extravasation. Top view images of agarose channel following exposure with cups at (a) 0.5MHz, 2 MPa (b) 1.6 MHz, 3.5 MPa and (c) 3.3 MHz, 5.5 MPa and corresponding side view images at (d-f). Omnidirectional extravasation was observed at 0.5 MHz while deeper directional extravasation was observed at 1.6 and 3.3 MHz.

Figure 5: Extravasation as a function of PRF (interval between pulses). Pulse length fixed at 2500, 8000 and 16500 cycles for 0.5, 1.6 and 3.3MHz at 2, 3.5 and 5.5 MPa respectively. 300 bursts for each exposure. Shaded elliptical areas represent the axial (length of ellipse) and lateral (width of ellipse) extravasation at the given setting. Scale

bars are 2mm and act as a reference for the size of the ellipses. Backwards or omnidirectional extravasation indicated by a second ellipse below the marker.

Figure 6: Extravasation as a function of pulse length for 0.5, 1.6 and 3.3 MHz at 2, 3.5 and 5.5 MPa respectively. Pulse interval was kept constant at 100ms. The total energy density delivered between exposures was kept constant by adjusting the number of bursts as the pulse length was varied. Scale bars are 2mm and act as a reference for the size of the ellipses

Figure 7: Extravasation as a function of exposure time. 2500, 8000 and 16500-cycle bursts were used for 0.5 MHz, 2.5 MPa, 1.6 MHz, 3.5 MPa and 3.3 MHz, 5.5 MPa respectively. Pulse interval was kept constant at 100 ms. Scale bars are 2mm and act as a reference for the size of the ellipses.

Figure 8: B-Mode images of agarose flow channel during cavitation enhanced extravasation using (a) SonoVue microbubbles, (b) polymeric cups and (d) droplets. Real time extravasation imaged with polymeric cups at (c). Low non-destructive harmonic imaging was used.

Figure A.1: Size distribution curves for, droplets, SonoVue and cup-stabilised gas pockets. For reference purposes, the size distribution of the entire cup population (solid cup + gas pocket) is also given.

Figure A.2: Positive control demonstrating the ability of the Live/Dead kit to successfully stain freshly killed cells. Green/live cells before UV-treatment, turn Red/dead following UV radiation for 30 minutes.

Figure A.3: Penetration distance (length of extravasation) as a function of PRF (interval between pulses), pulse length and exposure time for 0.5, 1.6 and 3.3 MHz at 2, 3.5 and

5.5 MPa respectively. Negative values represent backwards (towards the FUS source) or omnidirectional extravasation.

Figure A.4: Penetration width as a function of PRF (interval between pulses), pulse length and exposure time for 0.5, 1.6 and 3.3 MHz at 2, 3.5 and 5.5 MPa respectively. Negative values represent backwards (towards the FUS source) or omnidirectional extravasation.

Figure A.5: Haemolysis assay in whole blood with serial dilutions of the cavitation nuclei. Values are shown as a percentage of the positive control (Blood + Triton X-100)

Figure A.6: Cell viability following cavitation mediated extravasation in a cell embedded agarose gel. The ratio of dead cells to the total number of cells is used to calculate the percentage of dead cells in the exposed segment and compared to an un-exposed segment of the same size.

Figure A.7: Probability of inertial cavitation with droplets at 1.6 MHz, 8000 cycles at 200ms (5Hz) and 50 ms (20 Hz) pulse intervals. Harmonic-suppressed PCD traces were deemed to exhibit IC when the mean-squared signal to background ratio exceeded e^3 .

Figure A.8: Brightfield (BF) and fluorescent images of entire channel following extravasation at 1.6 MHz with (a)-(b) SonoVue, 3.5 MPa, 100ms interval, 8000 cycles, and with (c)-(d) cups, 3.5 MPa, 100ms interval, 8000 (d-left) and 4000 (d-right) cycles. No obvious channel formation is seen in any of the images. No clear observation of channel formation in any of the images. Nothing detectable In the SonoVue BF image (a) suggesting that any remaining bubbles have probably been destroyed. In (c), cups can be seen in the BF.

List of tables

Table 1: Parameter matrix of the acoustic exposure conditions investigated

Frequency (MHz)	0.5	1.6	3.3
Pressure (MPa)	0.5-2.0	0.5-3.5	0.5-5.5
Duty cycle (%)	0.5-50	0.5-50	0.5-50
PRF (Hz)	1-100	1-100	1-100
Cycles/burst	125-2500	200-8000	165-16500
Exposure time (s)	1-30	1-30	1-30