In vitro investigation of the individual contributions of ultrasound-induced stable and inertial cavitation in targeted drug delivery

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Abstract

Ultrasound-mediated targeted drug delivery (UmTDD) is a therapeutic modality under development, with potential to treat cancer. Its ability to produce local hyperthermia and cell poration through cavitation non-invasively makes it a candidate to trigger drug delivery. Hyperthermia offers greater potential for control, particularly with magnetic resonance imaging (MRI) temperature measurement. However, cavitation may offer reduced treatment times, with real-time measurement of ultrasonic spectra indicating drug dose and treatment success. Here, a clinical MRI-guided focused ultrasound surgery (MRgFUS) system was used to study UmTDD *in vitro*. Drug uptake into breast cancer cells in the vicinity of ultrasound contrast agent was correlated with occurrence and quantity of stable and inertial cavitation, classified according to sub-harmonic spectra. During stable cavitation, intracellular drug uptake increased by a factor up to 3.2 compared to the control. This paper demonstrates the value of cavitation monitoring with a clinical system, and its subsequent employment for dose optimisation.

Keywords: Targeted Drug Delivery, Focused Ultrasound, Microbubbles, Cancer cell, Cavitation, Subharmonic, Ultrasound contrast agent.

Introduction

Hyperthermia and cavitation are the ultrasound-related mechanisms that have been reported to cause increased intracellular drug uptake (Böhmer et al. 2009; Liu et al. 2001; Wu and Nyborg 2008; Gourevich et al. 2013). The term 'cavitation' describes two different physical processes, stable and inertial, with the latter sometimes termed 'transient' cavitation, that affect cells in different ways. Whilst evidence exists that both types of cavitation can be utilised in ultrasound-mediated targeted drug delivery (UmTDD) (Sundaram et al. 2003; Schlicher et al. 2006; Karshafian et al. 2009), the literature provides conflicting information on which method may yield the most effective results under a given set of experimental conditions (Fan et al. 2013; Domenici et al. 2013).

Stable and inertial cavitation have been shown to have different acoustic signatures, based on their differing underlying physics and the mechanical index (MI) of the ultrasound from which they originate (Leighton 1997; Brennen 1995). During stable cavitation, the bubbles oscillate, changing size and sometimes shape. These oscillations are associated with the phenomenon of microstreaming (Wu and Nyborg 2008) and can be detected by characteristic scattering of the applied ultrasound. In the frequency domain, the detected ultrasound depends on the type of oscillation (de Jong et al. 2009) and consists of harmonics, subharmonics and ultra-harmonics of the fundamental driving ultrasound frequency. The scattered acoustic signal acquired during sonoporation occurring as a result of inertial cavitation (Frenkel 2008) also has a characteristic signature in the frequency domain, in this case broadband. Passive detection of scattered acoustic emissions acquired during therapeutic ultrasound has been used to predict efficacy thresholds for ablation (Jensen et al. 2013), and explored as a means to investigate exploitable conditions for sonoporation (Fan et al. 2014; Somaglino et al. 2011; Chen et al. 2003; Hallow et al. 2006) e.g. by using the sub-harmonic spectrum amplitude as an indication of sonoporation occurrence (Hensel et al. 2009). Thus further insight into the mechanisms responsible for biological effects related to UmTDD can be gained through defining and correlating the types of cavitation generated with transfection and cell viability results (Bazan-Peregrino et al 2012; Gao et al, 2008; Fan 2031; Hassan 2010).

An additional major concern regarding UmTDD *in vitro* is the ability to differentiate cavitation effects from those correlated with increased temperature and verification that any heating produced unavoidably does not contribute to the observed biological effects. In this context, in the work presented here the experimental environment was based on a clinical magnetic resonance imaging guided focused ultrasound surgery (MRgFUS) system which allowed differentiation between the heating and cavitation mechanisms associated with ultrasound application. This system was characterized to gain understanding of the physical properties affecting the outcomes of cell culture experiments, and hence aid in overcoming difficulties in reproducibility of results in *in vitro* UmTDD experiments as has been raised previously by different authors (ter Haar et al. 2011; Hassan et al. 2010; Hensel et al. 2011; Kinoshita 2007; Leskinen and Hynynen, 2012).

Following arduous and lengthy experimental work *in vitro* and *in vivo* (Chen et al. 2010), therapeutic ultrasound is becoming an established clinical modality used mainly for tissue ablation, hyperthermia, and lithotripsy (Crouzet al. 2010; Lafon et al. 2002; ter Haar 1995; Barnett et al. 1994; Jolesz 2009). However, notwithstanding the work of others (Bazan-Peregrino et al 2012; Razavi et al. 2014), cavitation-based UmTDD is not yet sufficiently developed for clinical application and development is still under way, as required for tumour treatment, of techniques to monitor cavitation and to determine how cavitation may be used to predict resultant drug dose. This paper aims to contribute new knowledge on optimized values and conditions relating to use of cavitation in UmTDD to assist in development of this important therapeutic modality.

Materials and Methods

Experimental Apparatus and Setup

An ExAblate 2000 system (InSightec, Tirat Carmel, Israel) was used as the sonication platform in the described *in vitro* study. The source of ultrasound in this system is a concave phased array with 26 annuli each divided into eight sectors to give a total of 208 transmitting ultrasonic array elements organised in 26 transducer rings (Tr), The radius of curvature is 160 mm, aperture 120 mm, and the operating frequencies can be set in the range of 0.95 - 1.35 MHz. Quasi-continuous excitation is used, making the bandwidth extremely small for any given frequency. The array elements can be turned on and off individually and also allow phase adjustment with 45° quantisation. An additional complete concave element with radius 11.5 mm acts as a receiver, located coaxially with the transmitting array to allow spectral responses to be recorded. Signals are acquired from the receiving element for 150 ms every 200 ms, using a low-pass filter with a cut-off frequency of approximately 700 kHz to reduce the amplitude of the transmitted signal. After each acquisition, the signal is subjected to a fast Fourier transform (FFT) and the frequency domain result is recorded.

The ExAblate 2000 system complies with international HITU standards - IEC62555 (HITU radiation force) and IEC62556 (HITU field characterization) as well as IEC60601 ("Medical electrical equipment: Particular requirements for the basic safety and essential performance of high intensity therapeutic ultrasound (HITU) equipment"), and is calibrated accordingly. In addition, it was tested prior to each set of sonications, using the same method as for its clinical application, i.e. by sonication of a specially manufactured daily quality assurance (DQA) phantom which verified the dimensions of the focal zone as well as its delivery to a specified location.

Ninety six-well polystyrene µ-clear plates with a 0.2 mm thick base (Greiner Bio-One, Stonehouse, UK) were found to be the best cell culture environment, with manageable heat effects and space to fit the focal spot within the 7 mm diameter and 10 mm depth of each well. Titer-Tops sealing film (EMS, Hatfield, PA, USA) with a thickness of 0.1 mm was used to seal the open tops of the wells to maintain the sterility of the cells' microenvironment and to reduce acoustic boundary interface effects by allowing complete plate immersion in water.

To ensure that the wells did not behave as ultrasonic waveguides during sonication (Sommer et al. 1997, Redwood 1960), which would shift focal point toward the distal end of the well, we reduced the focal zone diameter which excited different propagation modes, allowing ultrasound propagation in the well not as a plane wave but with some interference between the modes (Figure

1A). This indicated there is a trade-off between the spot size and waveguide effects when sonicating an inverted 96-well plate using focused ultrasound.

In order to validate the optimal parameters for sonication inside a well, acoustic field measurements were performed as described below. The full experimental setup is depicted in Figure 1B and Figure 1C shows the order in which the wells were sonicated. To reduce secondary effects caused by accumulated dissipation of acoustic power in neighbouring wells, the sonicated wells were separated by wells with various contents (e.g. with microbubbles (MBs), without MBs and empty). The sonication order was also set in such a way as to avoid sonicating two neighbouring wells one after another and to minimize time-related effects on MBs by sonicating wells containing MBs first, as shown in Figure 1C. The 96-well plate was placed 92 mm above the transducer.

Acoustic and Thermal Evaluation

To estimate acoustic power loss and unwanted heat distribution, a separate base portion of a 96-well plate, supplied by the manufacturer, and the Titer-Tops sealing film were mounted in a special frame and scanned acoustically. To determine how much of the acoustic power was dissipated during the sonication, we utilised externally gated, pulsed sonications, each only a few seconds long, using the ExAblate transducer as the ultrasound source. A 0.5 mm polyvinylidene difluoride (PVDF) needle hydrophone (Precision Acoustics, Dorchester, UK) was placed in the focal plane of the transducer to record the waves. Acoustic scanning equipment described previously (Gourevich et al. 2013) was used to scan a 2D plane and the amplitude of the ultrasonic wave-front through a focal plane was recorded in water and with the base and sealing parts of the 96-well plate immersed between the transducer and the hydrophone.

The relatively small well diameter of 96-well plates means that care must be taken when they are used in ultrasound-related *in vitro* experiments. To determine suitability, detect possible limitations, and identify an appropriate sonication pattern, i.e. the focal distance (FD) and the form of the beam, two sets of experiments were done. First, successively increasing proportions (20%, 50% then 100%) of the array elements from the centre outwards were activated coherently to narrow the focal width increasingly, while leaving the remaining elements inactive. Second, increasing proportions (0%, 50% then 100%) of the array elements were excited with random phases to determine the effect of coherence. As detailed in the Results, the most appropriate activation of those investigated was determined to be of the central 50% of the array elements (13 annuli) with coherent excitation.

An additional issue that may influence the focal behaviour is the FD inside a well. Because of the plate geometry, as stated above, various disruptions can occur during the passage of the beam through the well. To establish the optimal height for sonication inside a single well within a plate, which will yield minimal distribution of power to the neighbouring wells, and to evaluate the influence of a sonication on neighbouring wells, acoustic field measurements were performed over an inverted plate at different focal points. The hydrophone in these measurements was less than 1 mm from the surface of the inverted plate, bringing it into close proximity with the theoretical cell location.

To differentiate between the thermal and mechanical effects that are associated with application of ultrasound, thermal measurements were made with an infrared (IR) thermal imaging camera (FLIR Systems CeDIP JADE camera, Kent, UK). A 96-well plate was only partly immersed in water, to overcome the opacity of water to IR wavelengths. The plate was sonicated at room temperature (RT) using different acoustic power levels for 90 s each, allowing cooling time between the sonications.

Cavitation Spectrum Measurements

The definition of cavitation dose, CD, used here is based on an approach in which, for each time acquisition, the magnitude of the signal in the frequency domain is integrated around the subharmonic frequency, f = 0.475 ±0.1 MHz, and these values are further integrated for the whole period of the sonication, as defined in Equation 1 (Hallow et al. 2006).

$$CD = \sum_t \int_{0.375}^{0.575} a(f)df \quad (1)$$

This definition allows a distinction between experiments in which there is no cavitation and those with stable and inertial cavitation, where lower values of CD indicate no cavitation and larger values indicate stable and then inertial cavitation. The fundamental unit of CD in this scheme is mV.

Figure 2 shows three examples of possible cavitation spectrum readings, as recorded by the ExAblate 2000 during ultrasonic exposure of cells within treatment groups containing MB, (A) showing no cavitation; (B) showing a single subharmonic peak from non-linear stable cavitation; and (C) showing broadband noise from inertial cavitation. Linear cavitation was not studied during this research.

Cell Maintenance

Cultures of MCF-7 human breast cancer cells (American Type Culture Collection (ATCC), Manassas, Virginia, USA) were grown as monolayers on 75 cm^2 cell culture flasks (TPP Techno Plastic Products AG, Trasadingen, Switzerland) in Complete Medium (CM) i.e. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (5000 I.U./ml, 5000 lg/ml; Gibco Invitrogen, Paisley, UK). They were kept in humidified air with 5% CO_2 at 37ºC. Re-seeding of the cells was performed twice each week using Trypsin-ethylenediaminetetraacetic acid (EDTA) 0.05% (Gibco Invitrogen, Paisley, UK).

For UmTDD studies, 15,000 cells/well were seeded in 100 µL CM, in µ-clear 96-well plates (Greiner Bio-One, Stonehouse, UK) three days in advance, in order to reach confluence on the day of the experiment. Prior to the application of ultrasound, the CM in the wells was replaced with the following solutions:

- *Uptake studies* - Treatment group: 25 µM doxorubicin (Dox) (Discovery, Wimborne, UK) in CM with 0.025% dimethyl sulfoxide (DMSO) and 4.7% of ultrasound contrast agent (USCA) Sonovue solution, i.e. 4 x 10^6 MBs per well (Bracco Research SA, Milan, Italy); Control: Dox in CM with 0.025% DMSO.
- *Viability assessment* - Treatment group: CM with MB at the same concentration, i.e. 4 x 10^6 MBs per well; Control: CM only.

- *Membrane Permeability assessment* - Treatment group: CM with MB at the same concentration, i.e. 4 x 10^6 MBs per well; Control: CM only.

In all the experiments, the Sonovue MBs were prepared according to the manufacturer's instructions and kept in an ice bath throughout the experiment, except when in use. The MBs were added to the final Dox/CM solution in individual portions per plate and the final solutions were applied immediately to the cell monolayers.

The uptake of Dox into the MCF-7 cell line was investigated using fluorescence intensity measurement (Infinite M200, Tecan Group, Mannedorf, Germany) with excitation and emission wavelengths of 485 nm and 592 nm, respectively. Following ultrasound treatment, it was observed that some cells detached from the mono-layer. This detachment was attributed to ultrasound application and extensive cell washing to remove excess Dox left between the cells. As the detached cells were discarded during the washes, they did not contribute to the total fluorescence reading. Therefore we correlated the uptake, as indicated by the fluorescence measurements, with the number of viable cells. This was achieved by normalization of the fluorescence readings by the total protein amount present within each well after ultrasound exposure, using the bicinchoninic acid (BCA) assay (Smith et al. 1985). The recorded protein amounts following sonication were also compared to the control groups to estimate the level of cell loss due to the different treatments.

The viability of cells after ultrasound exposure was quantified by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Dorset, UK), a colorimetric assay which correlates with cells' viability via their enzymatic activity (Cole 1986; Twentyman and Luscombe 1987). Additionally, a membrane integrity test (CytoTox-ONE, Promega, UK) was conducted to quantify the level of membrane permeability due to ultrasound application (Cho et al. 2002). In the presence of MBs, ultrasound is known to increase cell membrane permeability. Therefore, measurement of lactate dehydrogenase (LDH) levels released from leaking membrane into the media can indicate the level of membrane permeability achieved by sonication. The level of resofurin, which is proportional to the amount of LDH, was measured by fluorescence intensity measurements (Kaur et al 2007).

Experimental Procedure and Ultrasound Application

The solutions described above were added to each well, to a total volume of 420 µL, according to the defined treatment groups (Figure 1C) and the plates were sealed using Titer-Tops (EMS, Hatfield, PA, USA). The cells were sonicated continuously for 10 s with the following parameters; acoustic power level, $P = 1$ W, as the defined output of the ExAblate 2000 system which was focused at the centre of each well in all axes (FD = 97 mm); peak negative pressure, PNP = 0.53 MPa; intensity, I = 18.77 W/cm^2; mechanical index, MI = 0.54; and frequency, $f = 0.95$ MHz. The plate was placed inverted to ensure that the MBs were in proximity to the cell monolayer, owing to MB buoyancy. The sonications were performed in the order shown in Figure 1C, such that the wells containing the MBs were sonicated first.

Following the sonication process, the cells were washed twice with phosphate-buffered saline (PBS; Oxoid, Basingstoke, UK). For the uptake studies, the cells were lysed with 0.5% sodium dodecyl sulphate (SDS; Sigma-Aldrich, Dorset, UK) and the total fluorescence intensity of each well was recorded and normalized by total protein count using the BCA method (Smith et al. 1985). The cellular uptake of the sonicated samples was calculated as a percentage of the unsonicated samples, i.e. the control group, which was seeded in the same 96-well plate:

$$Drug\ Uptake = \frac{\left(\frac{Dox\ Fluorescence\ [RFU]_{sample}}{Total\ Protein\ Amount\ [\mu g]_{sample}}\right)}{\left(\frac{Dox\ Fluorescence\ [RFU]_{control}}{Total\ Protein\ Amount\ [\mu g]_{control}}\right)} \qquad (2)$$

The cell loss in each well after sonication was established according to the BCA absorbance readings relative to the control group, by:

$$Cell\ Loss = \left(\frac{Total\ Protein\ Amount\ [\mu g]_{sample}}{Total\ Protein\ Amount\ [\mu g]_{control}}\right) \qquad (3)$$

The viability studies were performed to determine if the sonication on its own, with and without MBs, caused cell necrosis, regardless of the presence of Dox. For assessment of cell viability, the same procedure was performed as in the uptake studies, where the indicated solutions were added to the different groups and the cells were sonicated at the specified parameters. Following the sonication process, 100 μL of fresh CM was added after two washes with PBS, then the cell viability was either assessed immediately or cell recovery studies were performed. The latter included additional 24 h incubation at 37 ºC in 5% CO_2. The cell viability was assessed by addition of 20 μL of MTT and 3 h incubation at 37 ºC in 5% CO_2. The created formazan was dissolved in 100 μL DMSO (Gibco Invitrogen, Paisley, UK), the plate was shaken for 50 s and the absorption signal at 550 nm was recorded with a plate reader (Infinite M200, Tecan Group Mannedorf, Germany). The level of cell viability was calculated relative to the control group of unsonicated cells:

$$Cell\,Viability = \left(\frac{MTT\ Absorbance_{sample} - Background}{MTT\ Absorbance_{control} - Background}\right) \tag{4}$$

In the membrane permeability studies, the plate was centrifuged at 2000 rpm for 10 min to spin down any cells present in the medium following sonication. 50 μL of the medium in each well were transferred into a new black 96-well plate (Greiner Bio-One, Stonehouse, UK) and an equivalent amount of resazurin reagent was added. After an incubation period of 10 min at RT, the stop solution was added and the fluorescent signal was measured with an excitation wavelength of 560 nm and emission wavelength of 590 nm. The fluorescence readings were normalized by the total protein amount using the BCA method as described above. The membrane permeability was calculated as follows:

$$Membrane\ Permeability = \frac{\left(\frac{LDH\ [RFU]_{sample} - Background}{Total\ Protein\ Amount\ [\mu g]_{sample}}\right)}{\left(\frac{LDH\ [RFU]_{control} - Background}{Total\ Protein\ Amount\ [\mu g]_{control}}\right)} \tag{5}$$

All the ultrasound cell culture studies were performed in a water environment at 30ºC, the upper temperature limit of the experimental setup.

For each plate, nine replicates were done for each treatment group and an additional six replicates for each type of control group. Throughout the various uptake, viability and membrane permeability experiments, each set of applied ultrasound parameters was replicated at least three times to ensure statistically significant data collection and correlation with spectral recording. All the values in the results section are presented with their standard deviations.

Results

Characterisation of Setup

In our experimental set up we found that the base of the µ-clear 96-well plate and the Titer-Tops sealing caused minimal attenuation of the acoustic signals, with, on average, 95.7% and 99.8% of the transmitted power passed through the base and the sealing, respectively (Figure 3A). Figure 3B represents the acoustic measurements at different focal points performed at 50% Tr with an acoustic power of 1 W at 0.95 MHz. The acoustic field measurements above the 96-well plate, showed that I in neighbouring wells is less than 4% of the peak intensity of the targeted well, with the minimal I distribution to the neighbouring wells being when the focus is in the middle of the well, i.e. FD = 97 mm. The acoustic field measurements showed that changing the aperture of the transducer by deactivating outer elements increased the focal length and diameter as expected, as well as distorting the focal shape, especially at 20% Tr (Figure 3C). Based on these measurements, the aperture of the transducer to be applied was established as using half the transmitting elements (50% Tr) and chosen such that the focal zone diameter entering the well was 2.6 mm. According to the results of the thermal camera measurements presented in Figure 4 for the chosen ultrasound parameters, with $P = 1$ W for 10 s, the overall temperature rise was only 0.44°C (±0.2°C).

Biological Studies and Cavitation Spectrum Measurement

Analysis of the relationship between the cell results and the cavitation spectrum measurements was applied to two groups. The first group, represented by Figure 5, contains all the measurement points from all the intracellular drug uptake and membrane permeability experiments. The second group, represented by Table 1 and Figures 6 and 7, contains results averaged ±SD in accordance with the sonication order, i.e. time of sonication, presented in Figure 2B.

Figure 5A shows the dependence of intracellular drug uptake on the CD. There is a distinct separation in outcome depending on the presence or absence of MBs. Without MBs, no significant cavitation occurs, as is also evident in the uptake levels which are below a factor of 1.5 relative to the control group. On the contrary, with MBs, the drug uptake varies by factors from 1.5 up to 3.2 with varying CD. Figure 5B depicts the correlation between the permeability of the cells' membranes as a result of ultrasound application.

As can be seen in Figure 5B, there is a significant increase in the membrane permeability of the cells, by a factor up to 3.5, in the presence of MBs, with an increasing trend with decreasing CD values. Figure 5C shows the dependence of drug uptake on the cell loss, where a linear fitting represents the relation between the uptake values and the number of cells missing from each well after sonication and washing. According to this trend, higher levels of cell loss correspond to higher uptake values, which can be explained by the fact that larger effects on the cell membrane cause greater penetration of the cytotoxic drug into the cells. This is also evident from the linear correlation between the uptake and the cell membrane permeability (Figure 5D).

Table 1 differentiates between stable and inertial cavitation for the different wells containing MB, according to the order of sonication. The classification in Table 1 was performed according to the maximum CD spectrum reading for each sonication; thus, if the sonication is classified as stable, it is assumed that no inertial cavitation has taken place. From Table 1, it can be seen that the occurrence of inertial cavitation decreases over time, corresponding to the order of sonication, whilst stable cavitation occurs instead. Our results show that the recorded CD = 0.22 ± 0.06mV and higher correspond to the occurrence of inertial cavitation, whereas CD = 0.15 ± 0.02 mV and lower correlate with stable cavitation.

Figure 6 presents the dependence of the drug uptake, membrane permeability and CD on the time passing after the first sonication. As previously mentioned, the treatment groups without MB were sonicated last. It can be seen that sonications without the MBs produce consistent results both for the intracellular drug uptake and membrane permeability, with up to 20% variation from the average value: these results are not time dependent. On the other hand, sonications performed on the wells with MBs manifest a bigger span of results and are time-dependent.

The dependence of immediate viability and cell recovery studies on the order of the sonications is presented in Figure 7A and B, respectively. The trend-lines shown in Figures 6 and 7 are the linear fitting representations of the dependence of the groups containing MBs on the order of sonication.

Discussion

This study was designed to define the individual contributions of stable and inertial cavitation to intracellular uptake of a chemotherapic agent within cancer cells. The initial step was the characterisation of the experimental arrangements including 96-well plates sonicated with a clinically approved system. This has confirmed that the use of the standard cell culture environment does not compromise the properties of the ultrasound propagation. Moreover, the acoustic energy levels that were used do not cause significant thermal effects, allowing differentiation between hyperthermia and cavitation.

Our Dox uptake results indicate a threshold between drug uptake and cavitation activity with and without MBs. While there was no effect on either the uptake or the membrane permeability in the absence of MBs, the intracellular drug uptake was, on average, increased by a factor of 2.14 ± 0.46 relative to the control group in the presence of MBs.

Membrane integrity tests were conducted to gain better understanding of the mechanism of uptake enhancement by MBs in acoustic environment. The membrane integrity assay quantified the level of LDH present in the media due to leaking cell membranes. Accordingly, it was possible to evaluate the membrane permeability achieved by sonication. In this study we utilised a simple fluorescence-based measurement technique to validate the mechanism of drug uptake into the cells. The close correlation between the increases in cellular uptake and the membrane permeability (i.e. 3.2 and 3.5 fold, respectively) indicate that membrane permeability studies involving quantification of LDH levels can be used as a tool to establish the levels of membrane permeability caused by the application of ultrasound.

The average decrease in viability of cells immediately after sonication in the wells containing MBs was recorded as 31% ± 8% relative to the control group. Taken together with the 24 h recovery studies, where the average decrease in the same group was 19% ± 5%, this indicates that some of the cell damage caused by sonication was reversible and did not lead directly to cell death. Therefore we conclude that there is an acoustic intensity window inside which the permeability of the cell membranes increases due to the presence of ultrasound-driven MBs which allows greater intracellular drug uptake, while permeabilisation is not permanent as it does not cause excessively high levels of cell death.

The drug uptake dependence on cell loss along with the cell viability studies indicated that greater cell loss is caused by the increased uptake of toxic drugs into the cells, rather than for any other reason. The average results of the cell culture studies are in agreement with previously described work by other authors using different experimental configurations (Pitt et al. 2004).

The dependence of drug uptake on CD indicates that greater uptake is achieved for smaller values of CD, i.e. $0.12\ \text{mV} < \text{CD} < 0.19\ \text{mV}$, suggesting that stable, non-linear cavitation with a peak at ½ the transmitting frequency is responsible for greater uptake and membrane permeability. Indeed, sorting the results according to the order of the sonicated wells, we have found that inertial cavitation occurred mainly within the first four sonicated wells, whereas stable cavitation was detected mainly in the last three sonicated wells that contained MBs. From the representation of the results as a function of time starting at the first sonication, there is a trend of increased drug uptake and reduced CD. This suggests that the increased drug uptake into the cell monolayers in our experiments was caused by stable, rather than inertial, cavitation. The dependence of the uptake on cell loss shows that stable cavitation also caused a greater cell loss. This is also evident in the immediate viability studies where there is a reduction in viability as a function of time similar to the CD. Nonetheless, this trend is decreased in the 24 h recovery studies as shown by the fact that the slope of the linear trend in the 24 h viability is half that generated from immediate viability studies.

We have calculated that the initial MB concentration was enough to cover the surface of the cells in a single monolayer, yet not high enough to attenuate the ultrasound beam. Therefore, we hypothesize that the initial inertial cavitation in our experiments, during the first three sonications, was caused by destruction of MBs in their original form, being unstable due to their relatively large diameters (e.g. > 9 µm). As, initially, the fraction of MBs in this range of diameters is significantly smaller than for other diameters (Gorce et al. 2000), the impact of the inertial cavitation on cell loss, viability and drug uptake is lower. After 100 s, the time between the first and the seventh sonications, larger quantities of the MBs retained diameters of 8 - 9 µm due to their spontaneous increase in diameter in a time dependent manner at temperature above RT (Guiot et al. 2006; Mulvana et al. 2010, 2011; Vos et al. 2008). These MBs are more attuned to resonate at $f = 0.95$ MHz (Gorce et al. 2000), the ultrasonic frequency in use, and hence a greater effect was achieved due to the irregular oscillations associated with stable non-linear cavitation.

Conclusions

In this study, a clinically approved MRgFUS system was used for cell studies *in vitro*. Adaptation of a clinical system in this way carries the potential for use of a single system from *in vitro* studies through the pre-clinical stage to clinical trials. Moreover, a well-defined experimental setup that can be used in a clinical environment promotes a reproducible baseline of *in vitro* and *in vivo* results, which can promote related research, e.g. in conjugation with novel drug carriers (Wang and Thanoua 2010), in multiple centres. The ability offered by this system to quantify cavitation dose has the potential to explain previously obscure results. The method used for cavitation detection in this study has advantages of robustness and simplicity. More precise cavitation detection methods have been suggested by Gyongy and Coussios, 2010. The use of the passive cavitation detection method they describe, along with the method described in our work, will allow better localization of cavitation and correlation between the sub-harmonics and harmonics and the intracellular drug uptake.

The absolute values of the drug uptake results presented here are in line with previously reported work by other authors using different experimental configurations. Moreover, the spectral recording that has been performed has allowed us to determine that the highest drug uptake was achieved in the presence of stable, non-linear cavitation. According to our findings, there is higher uptake at lower CD values. This is contradictory to the common assumption that UmTDD is governed by inertial cavitation (Somaglino et al. 2011; Razavi et al. 2014). Here we have provided evidence that stable cavitation with a lower CD, in the presence of MB, produces a greater impact on the intracellular uptake. This should be taken into account in future cavitation related TDD studies. Moreover, the hypothesis driven from our studies with Sonovue MB suggests that the time for ultrasound application to the MB is crucial and should be carefully controlled and reported in the literature.

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List of Tables

Table 1- Distribution of the stable and inertial cavitation during the uptake studies, between the ten repetitions in each well with MBs in the plate. For example out of ten repetitions of the third sonication, inertial cavitation occurred nine times and stable cavitation once. The order of the sonications in the plate is shown in Figure 1C.

Sonication Order	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Time (s)	*10*	*28*	*44*	*60*	*76*	*92*	*110*	*126*	*142*
Inertial Cavitation	9	8	9	9	4	5	1	2	1
Stable Cavitation	1	2	1	2	6	5	9	8	9