Towards an understanding of the mechanisms of therapeutic ultrasound on biomimetic models of cancer

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Towards an understanding of the mechanisms of therapeutic ultrasound on biomimetic models of cancer

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Abstract. Therapeutic ultrasound is transforming the treatment of a range of malignancies in a non-invasive and non-systemic manner. Low-intensity ultrasound (LIUS) has been proposed to selectively eradicate cancer cells but the underlying biological mechanisms remains unknown. To gain an understanding of this phenomenon, 2D breast cancer monocultures were sonicated at varying acoustic intensities (0.1–0.5 $W{\cdot}cm^{-2}$) and excitation times (1–10 minutes). Additionally, 2D monocultures consisting of healthy cell lines were sonicated at varying acoustic intensities (0.1–0.5 $W{\cdot}cm^{-2}$) to determine any distinguishing biological responses. To begin recapitulating *in vivo* conditions, breast cancer cells were also seeded into 3D collagen hydrogels. At a 1 MHz frequency, 20% duty cycle, 100 Hz pulse repetition frequency, a significant drop in cancer cell viability is observed at a sonication intensity of 0.5 $W{\cdot}cm^{-2}$ and over 10 minute excitation time. Healthy counterparts subjected to the same parameters revealed no distinguishing effects. Sonication of breast cancer cells seeded in 3D collagen hydrogels revealed no effect in cell viability compared to non-sonicated controls. The acoustic wave propagation software OptimUS was used to determine the influence culturing plates have on ultrasound propagation, revealing these materials can significantly vary the acoustic field at frequencies relevant to LIUS.

1. Introduction

Breast cancer is the most commonly diagnosed malignancy in the UK, with an average 55,920 new cases and 11,499 deaths each year since 2016 [1]. The advances in the clinical diagnosis and treatment of breast cancer have improved patient survival outcomes, with 80.6% of women aged 14–55 currently surviving for ≥ 10 years, compared with 57.1% at ages 75–99 [1]. The nature of conventional breast cancer treatments can cause physical, mental and economic consequences to patients undergoing these regimes. These situations may be exacerbated further as these problems can persist once cancer patients transition to cancer survivors, significantly impacting quality of life (QoL). The invasive and systemic nature of these treatments can also increase the risk of secondary cancer, with breast cancer survivors possessing a 70% higher risk of developing any cancer relative to the general population [2]. This highlights the need to develop tolerable and effective therapies with the aim of improving QoL alongside patient overall survival.

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Therapeutic ultrasound (US) is a non-invasive, non-ionising and relatively inexpensive technology that has been gaining traction in the field of cancer therapy. US is commonly used as an imaging technique for the diagnosis of various diseases and has expanded towards a therapeutic modality due to its ability to induce a variety of responses in biological media, including tissue. Generally, the biophysical effects of US can be divided as either thermal or non-thermal [3]. Increasing attention has been drawn to the non-thermal biophysical effects US is able to provide, which are typically encountered in low intensity applications. Under these regimes, temperatures rises remain within 1°C of physiological normal, encompassing the effects of acoustic cavitation and acoustic radiation forces (ARF) [4]. To date, there is no accepted definition of what constitutes the threshold for low intensity US, but most research suggests it to be < 3 $W{\cdot}cm^{-2}$, termed LIUS. For the purposes of this paper, this is the definition we will be adopting.

Acoustic cavitation occurs when biological matter is exposed to mechanical energy and forms gas filled voids within tissues or fluids, termed microbubbles. At low acoustic intensity applications this is characterised as stable cavitation, where microbubbles oscillate around an equilibrium point. During these linear oscillations, the flow of the surrounding liquid exposes cells to mechanical shear stress. This microstreaming in close proximity to cell surface membranes can lead to reversible perforations on the cell membrane that increases permeability [5]. This mechanism has allowed the use of US-mediated cavitation to enhance drug delivery. For example, there is significantly poor penetration of antibody therapies to allow the treatment of Alzheimer's Disease (AD). These antibody therapies possess molecular weights of approximately 150 kDa, and the blood-brain barrier (BBB) does not allow the passage of molecules exceeding a molecular weight of 500 Da to enter the brain parenchyma [6]. The potential of LIUS` in neoadjuvant scenarios has been shown in cancer treatments. Low intensity magnetic resonance guided focused US (MRgFUS) has successfully disrupted the BBB via stable cavitation, allowing the delivery of etoposide and doxorubicin to treat glioblastoma [7].

Mechanically sensitive mechanoreceptors present in cell surface membranes can sense ARFs, converting a mechanical stimulus into a biological response, termed mechanotransduction [8]. To sense and enable biological responses to such changes in the cellular environment, this mechanism requires cell-cell/extracellular matrix (ECM) adhesion and cytoskeleton contractility. LIUS has been shown to stimulate integrin mechanoreceptors, activating signalling cascades that converge on downstream signalling pathways such as Rho/ROCK and ERK/MAPK [9]. Additionally, mechanosensitive Piezo ion channels are also associated in transducing LIUS sonication responses in cells, where Piezo-1 has been suggested to enhance $[Ca^{2+}]$ and stimulate Akt/mammalian target of rapamycin (mTOR) signalling [10]. Ultimately, these downstream pathways play a key role in regulating gene expression of various essential cell functions, including cell viability, proliferation, differentiation, migration and adhesion. Accumulating evidence has indicated that LIUS stimulation of these pathways is responsible for the beneficial effects observed in healthy cells. For example, stimulating osteoblasts to promote bone formation has enabled the clinical use of LIUS for bone-fracture healing [11].

The work focussing on solely using LIUS to directly induce biological responses in different cancer cell types remains limited. For example, LIUS has been used to selectively induce the inhibition of cellular proliferation in human breast ductal carcinoma T47-D cancer cells. When compared to non-tumorigenic mammary epithelial Michigan Cancer Foundation (MCF)-12A cells, LIUS sonication did not result in harm, but rather induced increased cellular proliferation under the same US parameters [12]. Additional studies have also demonstrated increased cancer cell death outcomes when subjected to specific LIUS parameters, including myeloid leukaemia [13], hepatocellular carcinoma [14], prostate cancer [15] and colorectal adenocarcinoma [16]. This ability to induce distinguishing biological responses between cancer and healthy cells in a non-invasive and relatively inexpensive manner, could alleviate the issues for patients underlying breast cancer treatment. Combining this with its potential in a neoadjuvant role would provide a versatile form of cancer treatment. However, the underlying

biological response that represents cancer death, whether it be apoptosis, necrosis or lysis, remains unknown.

Despite promising results being published, it is important to consider that biological responses in US based *in vitro* experiments are significantly susceptible to the design of how samples are sonicated. This is likely to skew results if not addressed appropriately. US based *in vitro* experiments demonstrate a high degree of variation in a number of factors, including the use of different cell lines, biological outcome assaying, US parameters and the geometry of equipment utilised [17]. This highlights the lack of generally accepted guidelines that inform the design of *in vitro* sonication experiments to ensure consistency and reliability in US research. Without this, interpreting and comparing results across published data is difficult, thus limiting scientific progress. Further exacerbating this, *in vitro* sonication research has a reliance on simplistic 2D cell monoculture. Although advantageous in terms of their ease of use, low cost and wide availability, they are inadequate to reflect the complex tumour microenvironment (TME). Taking this approach risks obtaining ineffective results when translating towards clinical studies, which is a time-consuming and expensive process. 3D cancer models aim to bridge the gap between *in vitro* 2D monocultures and *in vivo* cancer models by replicating the conditions found in the TME, such as capturing the complex tumour-surrounding stoma crosstalk, but also providing more realistic cancer proliferative rates and nutrient-oxygen gradients. Culturing cells within a collagen type I hydrogel ensures recapitulation of the geometry of cell-matrix attachment and interaction. Such 3D models will act to replace the need for *in vivo* animal models, which is a goal of the 3R (replacement, reduction and refinements) framework to progress towards more humane research [18].

The goal of this study was to act as a first stage step towards informing the future design of LIUS *in vitro* sonication experiments that will use biomimetic 3D cancer models and consider the potential factors that could influence results. This early iteration includes the use 2D *in vitro* monocultures to observe the effect of using specific LIUS parameters on the human breast adenocarcinoma MCF-7 and breast adenocarcinoma M.D. Anderson-Metastatic Breast Cancer-231 (MDA-MB-231) cells, and use healthy Human Dermal Fibroblasts (HDFs) and MCF-10A cells to determine any potential distinguishing effects. This was followed up by beginning the transitioning into 3D cancer models to observe any difference in comparison to 2D *in vitro* scenarios using collagen hydrogels [19]. Finally, acoustic wave propagation modelling using OptimUS was used to predict the US field when traversing through six-well culturing plates at 500 kHz, a frequency relevant to LIUS applications.

2. Materials and Methods

All chemicals obtained from Gibco™ and Invitrogen™ were provided through ThermoFisher Scientific, Loughborough, UK, unless stated otherwise**.**

2.1. Cell lines

The human breast adenocarcinoma MCF-7 and the non-tumorigenic breast epithelial MCF-10A cell lines were provided by Mr Michael Toeller and Dr Nina Moderau from Imperial College London. The human breast adenocarcinoma MDA-MB-231 cell line was obtained from the European Collection of Authorised Cell Cultures (via Sigma-Aldrich, Dorset, UK). The Human Dermal Fibroblasts (HDFs) were acquired from Invitrogen™. The immortalised MCF-7 and MDA-MB-231 cells were cultured in low glucose (1 $g{\cdot}L^{-1}$) Dulbecco's Modified Eagle Medium (DMEM) and non-immortalised HDFs in high glucose (4.5 $g{\cdot}L^{-1}$) DMEM. Immortalised MCF-10A cells were cultured in DMEM/F-12 HEPES. All culturing media was supplemented with 10% Fetal Bovine Serum (FBS) and 100 units mL^{-1} penicillin and 100 $\mu g{\cdot}mL^{-1}$ streptomycin (all media and supplements mentioned above were obtained from Gibco™ unless stated). The MCF-10A cell line required additional additives, with the appropriate media being supplemented with Epidermal Growth Factor (EGF, PeproTech), Hydrocortisone, Insulin and Cholera-Toxin (all obtained from Gibco™ unless stated). All cell types were regularly washed in sterile conditions with phosphate-buffered saline (PBS, Gibco™), detached using 1× no phenol red

TrypLE™ Express Enzyme (Gibco™) throughout passages and incubated at 37ºC with an atmospheric pressure of 5% CO_2. Human Dermal Fibroblasts were used at <10 passages.

2.2. 2D in vitro experiments

All cell lines were seeded at a cellular density of 1.5×10^5 onto polystyrene plastic six-well multi-well plates (Corning® Costar®) to establish 2D monolayers on Day 1. Cells were cultured up to a period of 3 days, maintained in their respective media as highlighted in *section 2.1*. Ultrasound sonication was performed on Day 2, 24 hours following their seeding onto their respective culturing vessels, according to the parameters outlined in *section 2.4*. Controls consisted of cells not subjected to any ultrasound sonication. Cell viability analysis was carried out on Day 3, 24 hours following sonication, as highlighted on *section 2.5*.

2.3. Collagen hydrogel 3D in vitro experiments

The MCF-7 and MDA-MB-231 breast cancer cells were seeded at a density of 2.7×10^5 in collagen hydrogels onto polystyrene plastic 24 well multi-well plates (Corning® Costar®) on Day 1. The collagen mixture consisted of 80% Rat-Tail Collagen Type I (First Link, Birmingham, UK), 10% 10x MEM (Sigma Aldrich, Dorset, UK) and 6% neutralizing agent, with the remaining made up of cells suspended in their respective media [20]. The cell containing collagen hydrogels were then settled and allowed to cross-link at 37°C with an atmospheric pressure of 5% CO_2 for 15 min. The neutralising agent was prepared in house, made of 17% 10M NaOH (Sigma Aldrich, Dorset, UK) and 1M HEPES buffer (Gibco™). Cells were cultured up to 7 days in sterile conditions and incubated at 37ºC with an atmospheric pressure of 5% CO_2 in 1 mL of their respective media per well. Ultrasound sonication was then performed on Day 2, 24 hours post-seeding, according to the parameters highlighted in *section 2.4*. Controls consisted of cells containing collagen hydrogels not subjected to any ultrasound sonication. Cell viability analysis was then carried out on days 3, 5 and 7, with wells washed with PBS (Gibco™) prior to being re-supplemented with 1mL of their respective culturing media as highlighted in *section 2.5*.

2.4. Ultrasound device and sonication parameters

A Sonopuls 490 (Enraf Nonius, NL) was used with an operating frequency set to 1 MHz, a duty cycle set to 20% and Pulse Repetition Frequency (PRF) of 100 Hz. Sonication throughout *in vitro* experiments was performed 24 hours following cell seeding. Acoustic intensities were set to either 0.1, 0.25 or 0.5 $W\cdot cm^{-2}$, and excitation periods were altered to 1, 5 or 10 minutes. Aquasonic 100 (Parker Laboratories) ultrasound coupling gel was used to mediate the contact between the ultrasound transducer and the bottom of multi-well culturing plates. It is recognised that in this transducer-culture vessel orientation, cells are sonicated in a spatially non-uniform field, known as the near-field, thus are subjected to variable acoustic pressures. To prevent undesired sonication, samples representing different sonication conditions were kept in isolated culturing vessels.

2.5. Viability analysis

Cell viability was quantified using fluorescence analysis of PrestoBlue™ (Invitrogen™), according to the manufacturer's protocol. The fluorescence analysis of PrestoBlue™ was measured in Relative Fluorescence Units (RFU) with an Infinite M Plex plate reader (Tecan®) at an excitation wavelength of 570 nm and emission wavelength of 610 nm. RFU values represented in figures were established by subtracting the average fluorescence of blank controls from the fluorescence values of experimental wells.

2.6. Acoustic wave propagation modelling/simulations

To determine how the ultrasound field propagates through the six-well culturing plates, 3D meshes were generated and optimised using Meshmixer version 3.5.474 (Autodesk Inc.) and Gmsh (version 4.10.5) [21] to represent these culturing vessels. Acoustic wave propagation was then solved and simulated using OptimUS [22]. A piston source transducer with the dimensions of the Sonopuls 490 (Enraf Nonius, NL) was modelled radiating at a frequency of 500 kHz and with a surface velocity of 0.04 $m\cdot s^{-1}$ in a

surrounding environment of non-attenuating water (density, ρ: 1000 kg·m^{-3}, speed of sound, c: 1500 m·s^{-1}). The transducer was centred in the *y*-*z* Cartesian plane, the propagation taking place along the positive *x*-direction. The well plates were modelled as an acoustic medium, thus neglecting shear waves. Two configurations were investigated whereby the bottom of the well plate was positioned at 5 mm or 75 mm from the source along the *x*-direction, with assigned material properties of polystyrene plastic (density, ρ: 1050 kg·m^{-3}, speed of sound, c: 2400 m·s^{-1} and an attenuation coefficient, α: 1.8 dB·cm^{-1}). It was assumed that the boundary condition at the cell culture surface was perfectly absorbing. The aim of these simulations is not to replicate exactly the conditions under which the experiments were performed, but rather to illustrate the complications that can arise in multi-well plate ultrasonics experiments. All simulations were conducted in 3D. Acoustic wave pressure maps were then generated using MATLAB version R2022a (MathWorks®).

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc.) applying a one-way ANOVA. To evaluate the normality of the data, the Shapiro-Wilk test was used. Data is presented as mean + standard error of the mean (SEM). Statistical significance was considered when P-values are < 0.05.

3. Results

3.1. MCF-7 and MDA-MB-231 breast cancer cell viability is dependent on acoustic intensity

Previous data has demonstrated that LIUS possess the ability to induce cancer cell death. In this study MCF-7 and MDA-MB-231 breast cancer cells were seeded in 2D monolayer and sonicated at acoustic intensities of 0.1, 0.25 or 0.5 W·cm^{-2} for a total time of 10 minutes. PrestoBlue™ analysis, shown on Figure 1, demonstrates that breast cancer cell viability is dependent on acoustic intensity, with reduced viability as the intensity is increased. More specifically, MCF-7 and MDA-MB-231 cell lines show a significant drop in viability compared to controls at an acoustic intensity of 0.5 W·cm^{-2}.

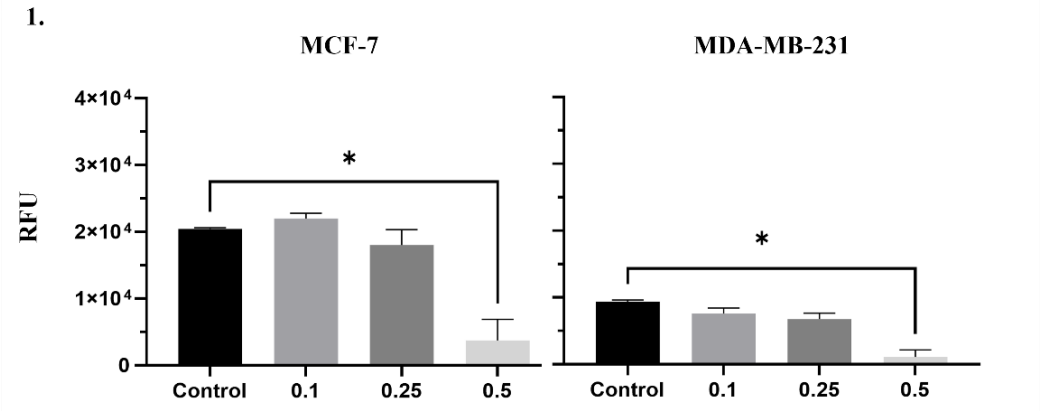

Fig. 1. MCF-7 and MDA-MB-231 cell viability at different acoustic intensities. MCF-7 and MDA-MB-231 breast cancer cells subjected to LIUS sonication 24 hours post-seeding at either an intensity of 0.1, 0.25 or 0.5 W·cm^{-2}. Controls consisted of no LIUS sonication. All sonication wells were subjected to sonication for 10 minutes at an operating frequency of 1 MHz, and a duty cycle of 20% at a pulse repetition frequency of 100 Hz. Cells were analysed 24 hours after treatments with PrestoBlue™ Cell Viability Reagent. Biological repeats n = 3, * $P < 0.001$.

3.2. Healthy HDFs and MCF-10A cell viability also dependent on acoustic intensity

Despite the initial focus of observing the biological responses of LIUS sonication on breast cancer cells, a healthy counterpart comparison would help determine whether LIUS selectively eradicates breast cancer cells. To address this, HDFs and MCF-10A cells were seeded as a 2D monolayer and were sonicated at an acoustic intensity of 0.1, 0.25 or 0.5 W·cm^{-2} for a total time of 10 minutes, shown in Figure 2. MCF-10A were selected as a healthy counterpart for comparison, because they are a commonly used non-cancerous cell line in breast cancer modelling. Additionally, HDFs are the main cell type present in the dermis of the skin, thus would be present during *in vivo* scenarios. PrestoBlue™ analysis revealed that the viability of these healthy cell lines is also dependent on acoustic intensity, but there is no distinguishing bioeffect under these specific parameters compared to the breast cancer cells. More specifically, MCF-10A show a significant drop in viability at an acoustic intensity of 0.5 W·cm^{-2}, while HDFs demonstrate this from 0.1 W·cm^{-2}.

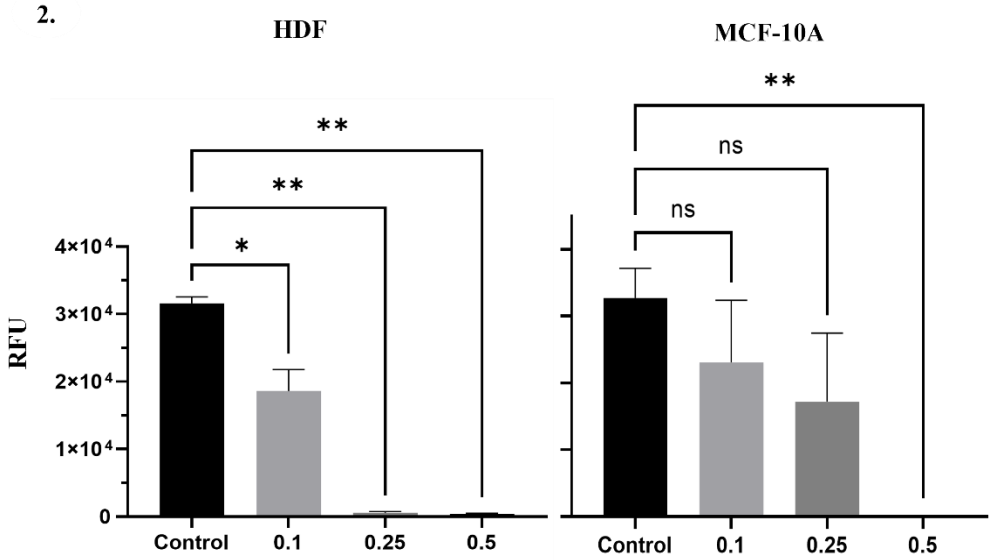

Fig. 2. Human Dermal Fibroblasts and MCF-10A cell viability at different acoustic intensities. Healthy Human Dermal Fibroblasts and MCF-10A cells subjected to LIUS sonication 24 hours post-seeding at either an intensity of 0.1, 0.25 or 0.5 W·cm^{-2}. Controls consisted of no LIUS sonication. All sonication wells were subjected to sonication for 10 minutes at an operating frequency of 1 MHz, and a duty cycle of 20% at a pulse repetition frequency of 100 Hz. Cells were analysed 24 hours after treatments with PrestoBlue™ Cell Viability Reagent. Biological repeats n = 3, * $P < 0.002$ and ** $P < 0.0001$.

3.3. Breast cancer cell viability is also dependent on the excitation period

The effect of altering the excitation period on cell viability when using an optimum acoustic intensity responsible for reducing cell viability was tested. Focussing on the breast cancer cell lines, MCF-7 and MDA-MB-231 cells were seeded as a 2D monolayer and sonicated at an acoustic intensity of 0.5 W·cm^{-2} for a total time of 1, 5 or 10 minutes. Here, PrestoBlue™ analysis demonstrated that breast cancer cell viability in both cell lines is also dependent on the excitation period, shown in Figure 3. As the sonication time increases, the cell viability decreases, with the optimum excitation time determined to be 10 minutes at 0.5 W·cm^{-2}.

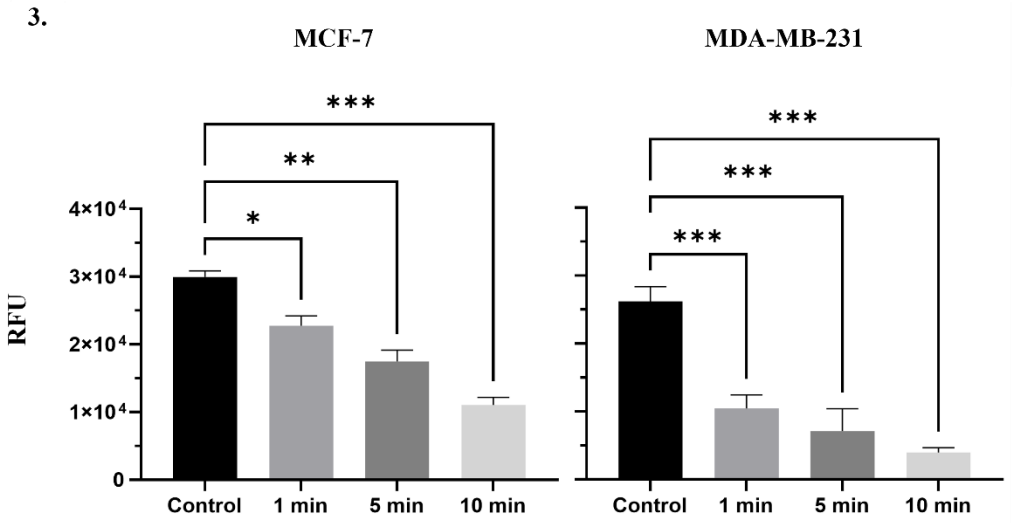

Fig. 3. MCF-7 and MDA-MB-231 cell viability at different excitation periods at 0.5 W·cm^{-2}. MCF-7 and MDA-MB-231 cells subjected to LIUS sonication 24 hours post-seeding for either 1, 5, or 10 minutes at an acoustic intensity of 0.5 W·cm^{-2}. Controls consisted of no LIUS sonication. All sonication wells were subjected to sonication at an operating frequency of 1 MHz, and a duty cycle of 20% at a pulse repetition frequency of 100 Hz. Cells were analysed 24 hours after treatments with PrestoBlue™ Cell Viability Reagent. Biological repeats n = 3, * $P < 0.015$, ** $P < 0.0005$ and *** $P < 0.0001$.

3.4. Translating 2D monolayers to 3D collagen hydrogels prevents biological responses in MCF-7 and MDA-MB-231 breast cancer cells

To bring in the element of biomimicry and moving away from the basic and relatively unrepresentative 2D monolayer cell cultures, MCF-7 and MDA-MB-231 breast cancer cell lines were seeded onto collagen hydrogels, and sonicated at an acoustic intensity of 0.5 W·cm^{-2} for a total time of 10 minutes. Here, PrestoBlue™ analysis demonstrated that there is no change in breast cancer cell viability at days 3, 5 or 7 compared to the controls, shown in Figure 4. This suggests that the transition from a 2D monolayer to a more biomimetic environment eliminates the ability for LIUS to impact breast cancer viability under these specific parameters. The culture of these cells in a 3D collagen hydrogel confers a protective effect on the cells from US.

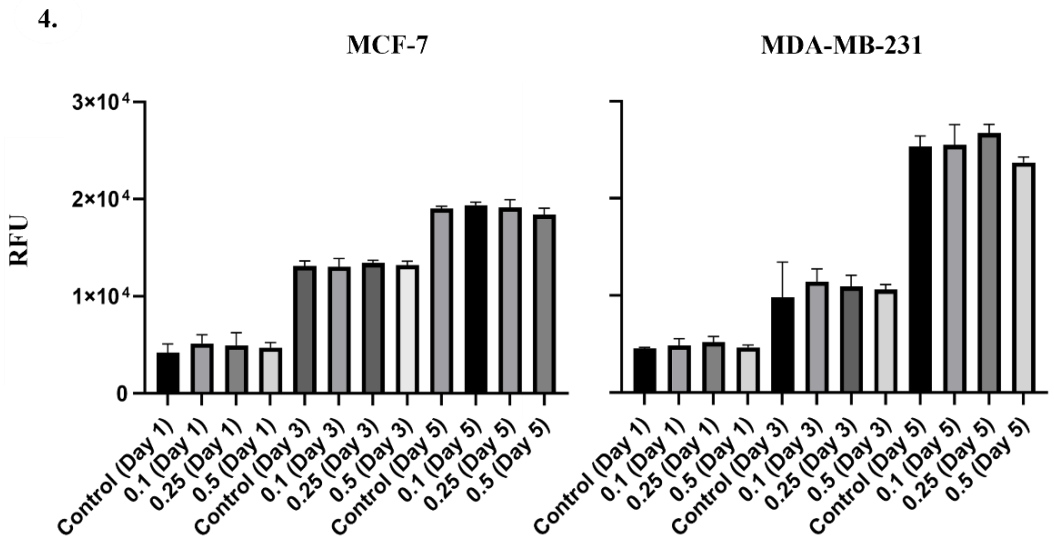

Fig. 4. MCF-7 and MDA-MB-231 cell viability at different acoustic intensities in 3D cell culture. MCF-7 and MDA-MB-231 breast cancer cells seeded onto collagen hydrogels were subjected to LIUS sonication 24 hours post-seeding at either an ultrasound intensity of 0.1, 0.25 or 0.5 $W \cdot cm^{-2}$. Controls consisted of no LIUS sonication. All sonication wells were subjected to sonication for 10 minutes, an operating frequency of 1 MHz, and a duty cycle of 20% at a pulse repetition frequency of 100 Hz. Cells were analysed on 1, 3 and 5 days after cell sonication with PrestoBlue™ Cell Viability Reagent. Biological repeats n = 3. NS.

3.5. Acoustic wave propagation through six-well culturing plates

To gain an understanding of the impact of the six-well plates used throughout *in vitro* experiments on US propagation, the acoustic field was calculated by modelling a piston source transducer radiating at 500 kHz at surface velocity of 0.04 m/s, with dimensions representing the Sonopuls 490 transducer. Six-well plate meshes were then positioned at 5 mm or 75 mm above the transducer to capture how the field interacts with the culturing vessels in these configurations, shown in Figure 5. In comparison to an undisturbed field, where the six-well plate is not present, the culture vessel significantly alters the acoustic field at 5 mm from the source, with less drastic changes when positioned at 75 mm. The 5 mm configuration represents the *in vitro* scenario, where our culture vessels were placed directly on top of the transducer with coupling media in between. This demonstrates cells being in the region of the US beam with variable acoustic pressures, termed the near-field. In comparison to the 75 mm configuration, acoustic pressures are more homogenous upon propagation through the six-well plate at this distance, where the US beam is more uniform. Additionally, overhead views of the acoustic pressure magnitudes on the surface of the well plates demonstrate the lateral spread of US into adjacent wells, with significant spread being observed when the culturing vessel is placed 5 mm compared to 75 mm.

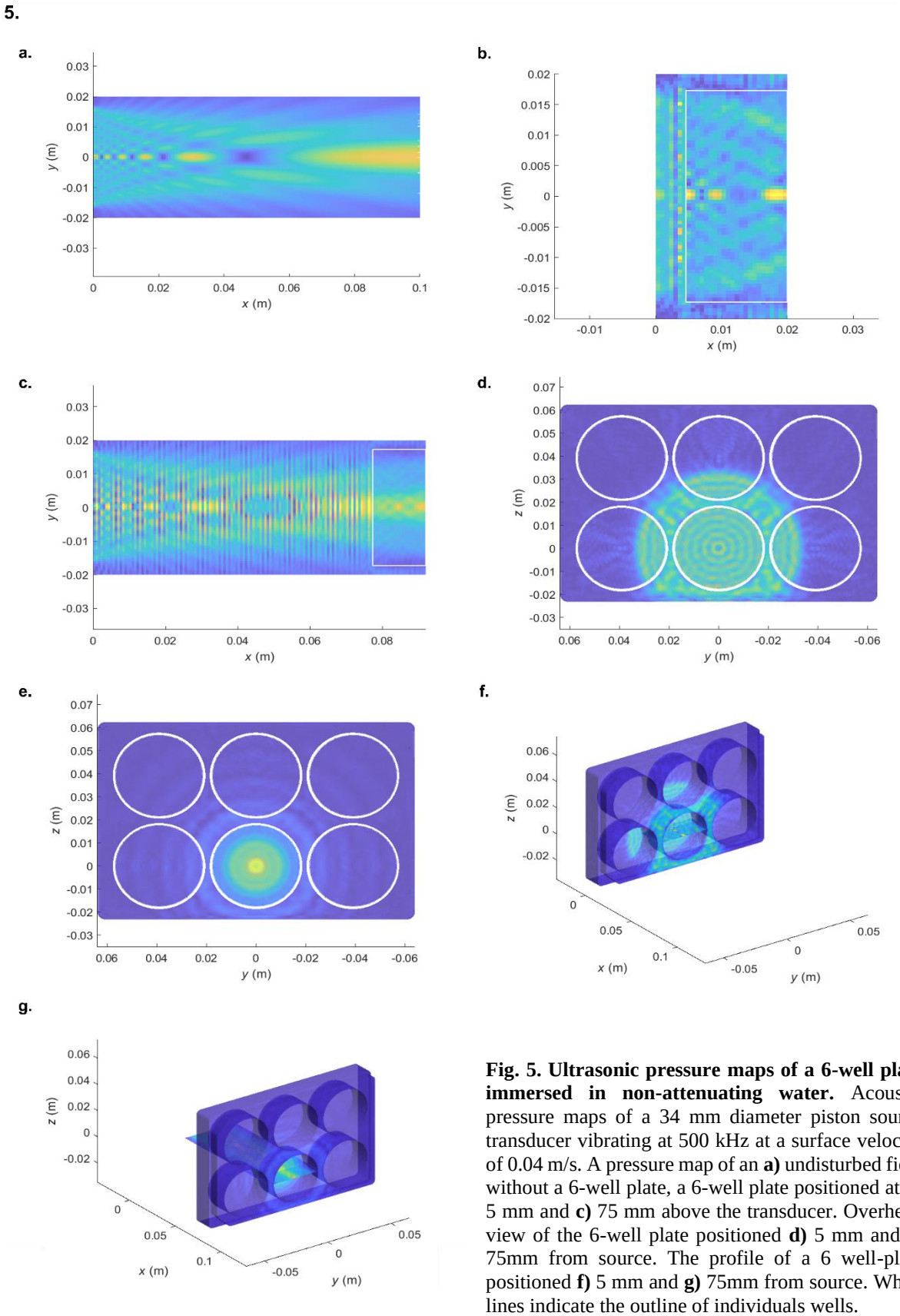

Fig. 5. Ultrasonic pressure maps of a 6-well plate immersed in non-attenuating water. Acoustic pressure maps of a 34 mm diameter piston source transducer vibrating at 500 kHz at a surface velocity of 0.04 m/s. A pressure map of an **a)** undisturbed field without a 6-well plate, a 6-well plate positioned at **b)** 5 mm and **c)** 75 mm above the transducer. Overhead view of the 6-well plate positioned **d)** 5 mm and **e)** 75mm from source. The profile of a 6 well-plate positioned **f)** 5 mm and **g)** 75mm from source. White lines indicate the outline of individuals wells.

4. Discussion

The sonication of our cells was performed in the near-field, therefore they are being subjected to a spatially non-uniform US beam with variable acoustic pressures, which in turn could result in the inaccurate interpretation of results. Despite MCF-7 and MDA-MB-231 breast cancer cell results being in agreement with previously published data, the healthy HDF and MCF-10A cells did not produce distinguishing biological outcomes. As mentioned in the introduction, it has been reported that healthy MCF-12A cells did not respond when subjected to near-field LIUS sonication and in fact responded to US with increased cellular proliferation compared to T47-D breast cancer cells [12]. This study used similar US parameters to our own, using a frequency of 1.5 MHz and intensities ranging from 10-100 $mW{\cdot}cm^{-2}$. However, the configuration of our US set-up did not possess the advantage of positioning the transducer inside the culturing media. In turn this avoids the need for using a dedicated acoustic coupling medium and prevents the possible influence of the culture medium-air interface. Nevertheless, in a clinical scenario it would ideally require moving towards a far-field configuration where cells can be sonicated at uniform pressures in a homogenous field. Alternatively, the use of a bowl-shaped transducer would utilise the benefits of focussed US (FUS) to achieve precise treatments. FUS has been an area of increasing interest and development to precisely sonicate target areas. For example, there are multiple ongoing low intensity FUS clinical trials trying to access its ability to induce neuro-modulatory effects in areas of epileptogenic activity to treat epilepsy, such as within the temporal lobe [23].

Despite the disadvantages of using near-field sonication, another issue encountered during the design of *in vitro* sonication experiments that will influence biological responses is the type of culturing vessel used. Typically, vessels consist of plastic multi-well plates that possess highly reflective surfaces. This will vary the characteristics of the US field and the subsequent exposure to attached cell layers. Additionally, the presence of culture media-air interfaces will establish standing wave (SW) patterns. This is problematic because SWs have been demonstrated to modify the US field by as much as 700% [24]. Aberrating this further is that SWs are extremely susceptible to geometrical alterations, such as changing the height of culturing and acoustic coupling media, which has been shown to either increase or decrease the local acoustic pressure amplitudes at the site of interest, indicating their unpredictive nature [25]. *In vitro* experiments in this study were performed using a manual process with no automation of any kind. Therefore, small and sufficient geometrical changes were likely introduced during these procedures to influence variations in the US field, thus impacting the outcome of cell sonication. This includes the generation of SW patterns. In order to achieve reproducibility during US based *in vitro* experiments, SWs needs to be minimised or eliminated. Experimenting with US absorbers and reflectors are a common direction taken to tackle this [26]. However, the possible design of acoustically ‘friendly’ materials to make up culturing vessels of which cell cultures are established or maintained in, could limit the extensive variations in the US field and minimise SW generation. Regardless, the ideal US configuration would be to sonicate cells with minimal alterations to the US field, such as preventing the establishment of SWs and minimising the variation of acoustic pressures exposed to biological samples.

The biophysical mechanisms of LIUS that induces the biological responses observed in this study still remain unknown. In this study, it was assumed temperatures did not exceed physiological normal and that cavitation thresholds were not reached due to the low acoustic intensities used, thus were not monitored in this early iteration. Rather, it was assumed that no thermal or cavitation-related biological responses were being induced, but rather by ARF. Ideally, the use of low intensity applications is to avoid thermal related biological responses as those observed in ‘high intensity’ focussed ultrasound (HIFU) applications, reducing the possibility any collateral thermal ablation that could impact patient QOL. Uncontrollable thermal changes can still occur due to factors in the experimental environment. For example, heat generation can result from the prolonged usage of an US transducer, especially in close orientations as those in near-field sonication and in scenarios where multiple biological sample replicates are essential. Regardless, the degree of heating is dependent on the US frequency, acoustic

intensity, attenuation coefficients and the distribution of an US beam, thus should be monitored using thermocouples going forward [27].

Cavitation thresholds are also important to consider, defined as the peak negative pressure of US that initiates cavitation. The application to monitor this during *in vitro* experiments is more complicated to implement, with many methods available such as bubble observation, broadband noise, sonoluminescence and aluminium foil attrition [28]. The importance of understanding cavitation thresholds has been shown when targeting specific events leading up to this mechanism of action and has allowed to produce desired biological outcomes. For example, cavitation mediated BBB disruption to treat a variety of disorders is achieved by actively using intensities and relative pressures that do not result in the collapse of microbubbles [6,7]. This suggests that rather than focussing on acoustic intensities provided by ultrasonic equipment, it is important to determine the accompanied acoustic pressures. These may be different to what is reported by US equipment manufactures. In turn, determining pressure thresholds that induce or lead up to cavitation events could be calculated. For instance, thresholds leading up to microbubble collapse have been measured in tissue mimicking hydrogels such as PVA, cellulose, agar, agarose and gelatine, but are yet to be established in collagen derived hydrogels [29].

LIUS sonication of cells embedded in 3D collagen hydrogels resulted in no change in cell viability. The 3D culture environment was thus deemed to have conferred some protective effect against the treatment regimen. This 3D protective effect has been previously demonstrated with the use of cytotoxic drugs [30]. By using a 3D system, the geometry of cell-matrix attachment likely reduced the susceptibility of cell detachment during near-field sonication protocols. This may go some way to explain why the cell viability is significantly reduced when using 2D monocultures, whereas the biological responses induced from LIUS sonication may not be captured. Nonetheless, these results could suggest that the 3D orientation provides a ‘protective’ environment to US exposure. These 3D environments are also more viscous in comparison to cells present in culturing media during 2D monolayer culture. This in turn would increase cavitation thresholds by stabilising microbubbles, thus acoustic microstreaming is reduced and in turn limiting biological related responses. Furthermore, research is limited in establishing US based 3D cancer models, but rather focused on using 2D monocultures. Therefore, there isn’t a representative study assessing the biological responses that LIUS could induce outside of a neoadjuvant role. However, results in this study could advise the possibility to focus on the amount of US energy absorbed rather than the acoustic pressures an incident wave places on biological samples. In other words, the dosage is responsible for a biological response and not the exposure. For example, a study using an US frequency of 1 MHz and an acoustic intensity of 0.2 $W \cdot cm^{-2}$ has shown that 3D cultures of HDFs sonicated for 5 minutes every day for a total of 10 days induced increased HDF cellular proliferation [31]. This could indicate that our MCF-7 and MDA-MB-231 breast cancer cells in a 3D orientation possess a higher dosage threshold required to induce a biological response, thus multiple sonication attempts are required.

As previously mentioned, culturing vessels have an influence on the variation of the US field, and thus on the sonication conditions to biological samples. One of the goals of this study was to use the acoustic wave propagation software OptimUS to calculate US propagation through six-well plates. Limited attempts to calculate US propagation in such culturing vessels have been carried out to date. For example, the finite-element method (FEM) was used to calculate the acoustic pressures and shear wave vibration displacements on the surfaces of six-well plates when a transducer is inserted into an individual well [32]. Although these calculations were limited to a frequency of 45 kHz, FEM was also able to demonstrate the establishment of SW patterns, which is a known occurrence in these culturing vessel types. By comparison, OptimUS calculated the pressure fields of US propagation through the bottom of a six-well plate at a frequency of 500 kHz, but does not model shear waves, and vibration displacements at individual surfaces were not calculated. Regardless, OptimUS and FEM reveal that calculating US propagation using benchmarks of culturing vessels is possible. Additionally, both show

that US can propagate into adjacent wells, which is undesirable due to subjecting cells to a higher US dosage than is intended. Investigations that have focussed on using direct measurements of the US fields have also reveal the lateral spread of US into adjacent wells that are not being subjected to sonication, which in turn correlated with biological responses in these non-sonicated wells [33]. In this study adjacent wells were not used to try and avoid this.

It is acknowledged that the use of OptimUS in this paper does not address the full complexities of the experimental configuration, in terms of the excitation frequency used (500 kHz vs. 1 MHz) as well as the boundary conditions at the cell culture / air interfaces and neglecting shear waves. Nevertheless, these simulations have the merit of illustrating the complexities which can arise at LIUS frequencies in ultrasound experiments in multi-well plates, adding weight to studies carried out within other frequency ranges, using an extensively validated software library [34]**.**

Regardless, how the US sonication set-up is configured, biological samples will be susceptible to variations in acoustic exposure, thus will influence the biological responses. Therefore, steps need to be taken to characterise US equipment before conducting *in vitro* experiments and this is often not appropriately addressed or reported in publications. This highlights the missing gap between acoustics and *in vitro* based research, where the lack of a generalised guideline is limiting scientific progression. In this early iteration, the characterisation of US equipment was not performed, therefore variations in the acoustic field are unknown. To ensure sufficient characterisation of our equipment before conducting experiments, free-field measurements of the transducers and in sites of interest such as cell layers attached to our culturing vessels need to be performed.

5. Conclusion

This preliminary study demonstrated that LIUS sonication induced identical biological responses between MCF-7 and MDA-MB-231 breast cancer and healthy HDFs and MCF-10A cells lines under specific US parameters. The responses to LIUS sonication are shown to be intensity and excitation time dependent, with the highest reduction in cell viability being observed at 0.5 $W \cdot cm^{-2}$ for a 10 minute excitation time. Translating this to 3D culture, MCF-7 and MDA-MB-231 breast cancer cells seeded onto collagen hydrogels resulted in no biological response. This suggests that when cells are in this more *in vivo* representative orientation, it provides a protective environment that increases thresholds required to induce biological responses. OptimUS calculated the pressure fields of US propagation through the bottom of six-well plate model at a frequency of 500 kHz, highlighting the issues of near-filed sonication at these orientations and inform future investigations. The biophysical US mechanism and the underlying bioeffect responsible for inducing biological responses still remains unknown. Going forward, the information obtained from this informative first stage study will help advise the design a platform suitable to carry out LIUS *in vitro* experiments. Ideally, this platform needs to be able to position the transducer precisely at a desired distance and orientation to the culturing vessel, sonicate cells at uniform pressures in a homogenous field, reduce the occurrence of SWs and monitor the potential biophysical mechanisms of US.

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