The Application of Clinical Lithotripter Shock Waves on RNA Nucleotide Delivery to Cells

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Abstract

The delivery of genes into cells through the transfer of ribonucleic acids (RNAs) has been shown to cause a change in the level of target protein expression. RNA based transfection is conceptually more efficient than commonly delivered plasmid DNA because it does not require division or damage of the nuclear envelope thereby increasing the chances of the cell remaining viable. Shock waves (SWs) have been shown to induce cellular uptake by transiently altering the permeability of the plasma membrane, thereby overcoming a critical step in gene therapy. However, accompanied SW bioeffects include dose dependent irreversible cell injury and cytotoxicity. Here, the effect of SWs generated by a clinical lithotripter on the viability and permeabilisation of three different cell lines *in vitro* was investigated. Comparison of RNA stability before and after SW exposure showed no statistically significant difference. Optimal SW exposure parameters were identified to minimise cell death and maximise permeabilisation, and applied to enhanced green flu-

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orescent protein (eGFP) messenger RNA (mRNA) or anti-eGFP small interfering RNA (siRNA) delivery. This resulted in eGFP mRNA expression levels increasing up to 52-fold in CT26 cells, whilst a 2-fold decrease in GFP expression was achieved following anti-eGFP siRNA delivery to MCF-7/GFP cells. These results demonstrate that SW parameters can be employed to achieve effective nucleotide delivery, laying the foundation for non-invasive and high safety RNA-based gene therapy.

Keywords: Shock waves, high amplitude acoustic waves, ultrasound, mRNA, siRNA, gene therapy, drug delivery

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Introduction

Nucleic acid-based therapies provide a powerful approach to the treatment of genetic diseases, by introducing healthy replacements of mutated or absent genes, or gene-specific inhibitory molecules into target cells; to ultimately reinstate typical cellular function either through the expression of normal protein or the repression of defective protein. However, biological or chemical vectors for delivery are limited by potential viral toxicity and poor targeting, respectively (Mehier-Humbert and Guy, 2005). On the other hand, physical transfection systems for the delivery of nucleic acids have attracted substantial attention in recent years, as they permit accessibility of the target site and entry into the cell's cytosol. Such methods include electroporation, the gene gun, laser irradiation, magnetofection and microinjection. Notably, electroporation has achieved comparably high transfection levels, where up to 1000-fold increase in gene expression has been reported relative to the admittedly highly inefficient level achieved with standard plasmid DNA injection (Wells, 2004). However, one common drawback all such methods share is the inability to access deep-seated tissues without compromising safety. As a result, sonoporation – the process of transiently permeabilising the cell membrane using ultrasound – provides the most practical and least invasive device based option when deep access is needed (Mo et al., 2012). Nonetheless, the efficacy of sonoporation strongly depends on the acoustic parameters of the employed technology, because a trade-off exists between being able to maintain high cell viability and also achieve nucleotide uptake.

Lithotripsy technology has been applied clinically for over 30 years to fragment kidney stones extracorporeally (Chaussy et al., 1980; Cleveland

and McAteer, 2007), with later applications in macromolecule (Gambihler et al., 1994; Delius and Adams, 1999) and plasmid DNA transfer into tumours (Miller et al., 1999; Bao et al., 1998; Song et al., 2002) and chondrocytes *in vitro* (Murata et al., 2007). Lithotripter generated shock waves are characterised as high-amplitude short-pulsed acoustic waves that exert mechanical forces on the focal zone, through two known mechanisms: direct shear stress and the formation, growth and subsequent violent collapse of cavitation bubbles (Cleveland and McAteer, 2007; Madersbacher and Marberger, 2003). Transfer has been attributed to a transient disruption of the plasma membrane taking the shape of defects or pores of at least 50 nm in diameter (Ben-Dor et al., 2000). The short time duration of the shock wave pulse results in a temperature rise $<1\,^{\circ}\mathrm{C}$, producing negligible thermal effects (Huber et al., 1999). This aspect favours shock wave assisted gene therapy over high intensity focused ultrasound as the latter results in tissue heating that may damage the cells, thereby compromising viability which is a prerequisite for gene expression to take place; as well as potentially impacting on the functionality of the delivered nucleotide.

A wide spectrum of shock wave-induced DNA transfection efficiencies has been reported *in vitro*. Lauer et al. (1997) observed poor permeabilisation levels of between 0.1–0.5% which was found to be independent of the cell concentration utilised. Huber et al. (1999) optimised their shock wave exposure to cells and determined a 3-fold stimulation enhancement of reporter gene expression. The introduction of cavitation nuclei was deemed necessary for robust shock wave effects by Miller et al. (1999) and was achieved by intentionally having residual air in their cell samples. More recently, Millán-

Chiu et al. (2014) reported a maximum of 2.9% of cells exposed to shock waves were transfected based on refined but suboptimal parameter settings. In contrast, Bao et al. (1998) showed that at 50% cell viability, cells exposed to 200 shock waves produced a 50-fold increase in reporter gene expression per million cells.

The introduction of nucleotide into the cell as DNA, has seen faster research uptake compared to RNA due to its inherent stability. In contrast, RNA is labile and more difficult to synthesise. Nonetheless, mRNA provides greater reliability of transfection because it does not require nuclear entry for protein expression and thus is not limited to cycling cells (Gilboa and Vieweg, 2004; Bettinger et al., 2001). As such, any host genome integration and risk of insertion-based mutagenesis is averted (Pinel et al., 2014). Moreover, mRNA promotes relatively faster reporter gene production as the initial transcription phase in gene expression is foregone (Ponsaerts et al., 2003). Furthermore, unlike DNA, mRNA is free from immunogenic CpG motifs that may elicit host immune response (Pinel et al., 2014).

For the reduced translation of aberrant cellular protein, small interfering RNA, which also has a cytoplasmic site-of-action, has been reported to achieve effective gene knock-down (Bertrand et al., 2002) through the command of sequence-complementary mRNA degradation (Hall, 2004). As such, siRNA holds promise in the treatment of oncogenes and other disorder-generating gene products. Hence, RNA may be particularly suited to delivery by lithotripsy because, provided the right parameters can be identified, opening of the plasma membrane may be achieved without needing to impart damage to the nucleus.

To date, shock waves have not yet been exploited for mRNA-based transgene expression, and in the context of *in vitro* studies, the application of tissue mimicking materials (TMM) has been scarce. Similarly, there are few reports of shock wave induced siRNA delivery in the literature. In addition, reports detailing shock wave dose dependent bioeffects, were performed using early generation technologies (reviewed in (Brümmer et al., 1990)) with a considerable number of studies using machines such as the Dornier XL1 lithotripter (Brümmer et al., 1989; Gambihler et al., 1994; Delius and Adams, 1999; Lauer et al., 1997) and the Siemens Lithostar (Huber et al., 1999; Oosterhof et al., 1989) which are no longer available. In view of newer clinical lithotripter technology, which provide an enabling pathway to translation, little, if any work has been conducted on shock wave mediated cancer treatment at the cellular level.

In this work, we present studies aimed at achieving and describing delivery of RNA to cells using a state-of-the-art clinical shock wave source. We report cell line based optimal shock wave parameters for the enhancement of RNA transfection of cancer cells.

Materials and Methods

Cell lines & cell culture

Mouse colorectal carcinoma CT26.WT cells (ATCC, CRL-2638; American Type Culture Collection, Rockville, MD, USA), with kind provision from the Department of Oncology (Oxford University, UK), were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher

Scientific, Waltham, MA, USA), in a humidified atmosphere containing 5% CO_2, at 37 °C. Cells were grown to a minimum of 90% confluence and ≥95% cell viability for use in shock wave experiments. Cells were washed with Dulbecco's Phosphate-Buffered Saline (PBS) solution (Thermo Fisher Scientific), harvested by brief trypsinization, and neutralised with medium twice the volume of the trypsin-EDTA (Thermo Fisher Scientific). A cell pellet was formed by centrifugation at 300×g for 5 minutes, and re-suspended in serum-containing medium. To ensure cell density and homogeneity, the prepared cell suspension was agitated using a vortex mixer for a few seconds before counting. Total cell counting was performed using the Trypan blue (Thermo Fisher Scientific) dye exclusion method and a hemocytometer. A cell stock solution was then prepared by extracting the total number of cells required from the cell suspension and diluting with medium to the total required volume. The stock solution was spun once more before dispensing into sample units.

Two other cell lines were similarly cultured: immortalised human kidney (HK-2) cells (ATCC, CRL-2190) and human breast cancer (MCF-7) cells stably expressing GFP (AKR-211, Cell Biolabs Inc., San Diego, CA, USA). These were treated using 10% FBS supplemented Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) in lieu of RPMI 1640.

Shock wave generation & set-up

The shock waves were generated by the Storz Modulith SLX-F2 lithotripter (Storz Medical, Kreuzlingen, Switzerland) with the kind permission of Oxford Stone Group (Churchill Hospital, Oxford, England). The lithotripter consisted of an electromagnetic cylinder coil with dual focal zones. The wide

focus zone was applied to all experiments, with a nominal size of 9 × 50 mm (diameter × length) at peak positive pressures ranging from 5 to 90 MPa. Experiments were carried out in a water-filled tank with a low density polyethylene (LDPE) membrane for shock waves to enter, which was coupled to the shock wave transducer by a thin layer of silicone oil. Cell samples were suspended underwater (Figure 1(a)) and positioned centrally in the focus through fluoroscopic projections at 0 ° and 30 ° relative to the vertical axis.

Ideal experimental conditions for cells were created by heating the tank water to 37 °C using a Grant GD100 water heater (Grant Instruments Ltd, Royston, UK). For experimental reproducibility as well as in view of a lack of cavitation nuclei in non-gas-bearing body tissues, the water was degassed using a pinhole degasser for a minimum of one hour.

Shock wave field characterisation

To determine the pressure at the focal point, the acoustic field was measured using a Müller-Platte polyvinylidene fluoride (PVDF) needle hydrophone (Article-No.: 100-100-1, Müller Instruments, Oberursel, Germany) with a 40 ns rise time. The signals produced by the hydrophone were recorded using a digital oscilloscope (Le Croy waveRunner 44Xi, 400 MHz sampling rate; LeCroy Corporation, Santa Clara, CA, USA). The hydrophone was positioned using a manual three-axis linear stage. Measurements were taken along the lateral axes (X and Y) in the focal region area, in increments of 1 mm for up to 10 mm, and along the propagation axis (Z) in increments of 2 - 5 mm for up to 45 mm. Five waveforms were recorded at each location and converted to pressure using the calibration furnished by the manufacturer.

The procedure was repeated with the hydrophone inside a vial only at the focal point.

Sample Preparation & Treatments

Polypropylene (PP) vials (T7813, Sigma-Aldrich) with a volume of 2 mL were filled with the suspended cells at a concentration of 500,000/mL without any visible residual air in the vials. Prepared vials were immediately chilled and maintained in an ice-box throughout the duration of the experiment, except during treatment. This prevented the occurrence of the temperature-dependent endocytosis process during sample and experiment preparation (Khalil et al., 2006). For every independent cell viability experiment, shams were prepared alongside the treated samples. Shams captured the effect of the heated water by being placed inside the tank for the duration of the average shock wave treatment, but not being subjected to any shock wave impulses. Sample vials were treated to a combination of shock wave parameter variations: number (125, 250, 500, 1000), energy level (3, 6, 9) and pulse repetition frequency (PRF; 1, 2 Hz). All experiments were performed with the wide focal zone of the lithotripter.

mRNA & siRNA stability

Sterilised vials were filled with 1 μg/mL of eGFP mRNA (StemMACS, Miltenyi Biotec Ltd, Woking, UK) in serum-free RPMI 1640 medium, in the absence of cells, and exposed to shock waves. The rabbit reticulocyte lysate (RRL) cell-free gene expression system (Nuclease Treated L4960, Promega, Madison, WI, USA) was utilised to assess mRNA translation activity after shock wave treatment. The translation reaction was prepared in accor-

dance to the supplier's protocol. Constituent volumes were adapted to the mRNA concentration as well as to permit micro-plate fluorometric reading, while maintaining relative proportions. A control mixture not containing any mRNA was also prepared to measure background due to the RRL. The reactions were incubated at 37 °C for 75 minutes. Fluorometry was then performed at 485 nm excitation and 520 nm emission.

Electrophoresis-based nucleic acid structural integrity post shock waves was tested. eGFP siRNA (Silencer, Thermo Fisher Scientific) and eGFP mRNA cell-free samples were prepared at 15 and 2.5 μg/mL respectively in UltraPure DNAse/RNAse free distilled water (Thermo Fisher Scientific). For siRNA analysis, traditional 1% agarose gel electrophoresis in 1X Tris-Borate-EDTA buffer was performed. For mRNA analysis the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) and RNA 6000 pico kit (due to low concentrated mRNA samples) were employed. The assay was conducted following the Agilent guide for the required kit. RNaseZAP cleaning agent (R2020, Sigma-Aldrich) was applied to all equipment at the start of the procedure.

Cell Survival & Viability Assay

Cell viability of cells was tested with the MTS assay (CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], Promega). After exposure, 200 μL of cell suspension from each vial was plated into a 96-well plate at two wells per vial. The procedure was repeated for a second 96-well plate. 20 μL of MTS solution was added to each well of the first well plate and incubated for 30 minutes, representing the 1-hour-after-exposure assay; the second well

plate was incubated with the MTS following 24 hours of incubation post exposure. Absorbance was read at 490 nm (1.0 s measurement time) using a Wallac 1420 Victor^2 microplate reader (Perkin Elmer, Inc., Beaconsfield, UK).

Cell Permeabilisation analysis

Membrane permeabilisation analyses were carried out using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). 500 μL of cell suspension from each vial was transferred into tubes, maintaining cells in a chilled environment. A solution of 6 μg/mL propidium iodide (PI; Sigma-Aldrich) was prepared. 100 μL of the solution was added to each tube and briefly vortexed immediately before analysis. An argon laser provided excitation at 488 nm. The software programme CellQuest Pro (BD Biosciences) was used to acquire and analyse the data. 10,000 cells per sample were recorded and sorted by gating in two ways: 1. forward scatter/side scatter in order to identify viable cells and 2. PI staining to identify molecule-internalised cells. Data acquisition was initialised with the negative control samples followed by all other samples in the absence of PI. The percentage of PI-positive cells was obtained by setting a gate in the PI fluorescence intensity frequency histogram of sham samples above which circa 0.1% of cells fell.

Transfection procedure

GFP production and knock-down were tested using eGFP mRNA and eGFP siRNA respectively. This reporter gene was selected due to its inherent stability allowing its accumulation and easy detection in living cells (Li et al.,

1998). CT26 cells at a density of 2.5 million/mL were employed for mRNA transfection experiments while MCF-7/GFP cells at 1.5 million/mL were employed for siRNA transfections. Cells were immobilised in 1% agar in a 2 mL custom-made tissue phantom vessel (Figure 1(b)), and supplied with 5 μg mRNA or 10 μg siRNA in UltraPure DNAse/RNAse-free distilled water, prior to shock wave treatment. For the siRNA transfections, both the agar and the cells were prepared in Opti-MEM Reduced Serum Medium (Thermo Fisher Scientific) before mixing, while for mRNA transfections, cells and agar were prepared in RPMI 1640 Medium and PBS respectively. Transfection shams consisted of samples supplied with the nucleotide of interest but not exposed to any shock wave pulses. Afterwards, vessels were maintained at 37 °C in cell culture incubators and removed only for analyses.

Transfections were assayed by fluorometry using the FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany). For mRNA transfection, fluorescence intensity (FI) was read at 24 and 48 hours after shock wave treatment to allow all potential cells to synthesise and express the GFP. Similarly, siRNA mediated knock-down was assayed at 24, 48 and 72 hours – a time frame defined to capture the translational arrest subsequent to target mRNA degradation. Fluorescence was visualised at the respective final assay time point using the Nikon Eclipse TiE2000 inverted microscope (Nikon Corporation, Tokyo, Japan) with a 10× objective lens; microscopic views within the upper agar region of the tissue phantom vessel were generated using NIS-Elements AR software (Nikon Corporation).

Results

Influence of shock wave parameters on CT26 cell viability and permeabilisation

Expression of delivered transgenes is only possible where live cells exist. Cell response to shock waves is dependent on several factors including SW parameters, the physical environment of cells and the cell type. Shock waves can result in enhanced proliferation (Weihs et al., 2014), or marked cytotoxicity (Brümmer et al. (1989); Gambihler et al. (1990); Miller et al. (1999)). Thus, shock wave-induced cytotoxicity was investigated to identify a parameter space that maximised the cell viability.

Figure 2 demonstrates a shock wave dose-dependent decrease in the viability of CT26 cells 1 hour after exposure. This effect was greater with increasing energy level and number of shock waves where 500 pulses at the highest attainable energy level (9 E) amounted to a viability of 50–60%. Varying PRF from 1 to 2 Hz did not alter the overall pattern of this loss of viability. At 24 hours, MTS assays were performed again and comparison to 1 hour data allowed the recovery of cells to be characterised. The 24-hour viabilities were comparably elevated in the majority of cases, indicating a population growth phase at or within 24 hours. However, the extent of growth differed between treatments as well as within treatment replicates, with some treatments (e.g. 3 E, 250 pulses, 2-Hz) suggesting metabolic activity within the wells was doubling between the 1 hour and 24 hour readings; while others (e.g. 9 E, 500, 2-Hz) showed minimal sustained depletion of viability implying that in those conditions shock waves instigated damage from which the cells could not recover. The shock wave stimulative effect at low

energy was evidenced at both pulse repetition frequencies in the 24-hour assay; a wider range of energies displayed this effect at 1-Hz, while proliferation peaked at 2-Hz.

Shock wave impact on cell viability was probed further using flow cytometry (*recall* methods). Figure 3 shows example distributions for a sham treated population (a), a minimally exposed population (b, (3 E, 125 shocks, 2-Hz)) and a maximally exposed population (c, (9 E, 500 shocks, 2-Hz)). Based on the untreated (sham) cells, sub-populations such as fragmented cells and cellular debris were identified by side scatter (SSC), which is proportional to granularity. Cell permeabilisation, as characterised by cell staining with PI immediately after shock wave exposure, was quantified by the fluorescence gate separating the SSC/FL2-H plots into left and right halves as shown in Figure 3 (where FL2 is a channel for the detection of emission wavelengths comprising the emission peak of PI). Events in the right half represented the proportion of total PI-positive cells which is shown to increase with increasing shock wave parameter. The minimum SSC of the untreated population provided a baseline for an additional gate to separate intact from damaged cells, where the first quadrant identifies the population of whole and permeabilised cells. It is noted that less than 4% of cells were intact and PI-positive in the sham samples, suggesting that a small proportion of cells became compromised by the removal of culture conditions. Reversible damage (that is, transiently permeabilised cells) was determined by correlating total PI-positive cells with the 24-hours viabilities. In so doing, a parameter space defined by 9 E, 125, 2-Hz and 3 E, 500, 2-Hz was found that permitted temporal membrane permeabilisation to the PI of up to $\sim$15%, without any

associated cell death (Figure 4). For almost all shock wave conditions, results demonstrated an inverse correlation between the percentage of permeabilised cells and viability.

The influence of PRF on cell viability was further investigated, to a maximum of 4-Hz, which could only be realised for energy levels 3 and 9 (data not shown) due to the capacitance of the shock wave source. For a fixed number of shock waves (250) while varying the PRF in increments of 1-Hz, no statistical significance was found between PRFs in both energy level sets ($p = 0.8$ and 0.49 for the 3 E and 6 E sets respectively). Thus, it was thought that for any two treatments where only the PRF was the varying parameter, any difference in apparent viability was due to changes in cell morphology (i.e. cell injury) rather than cell destruction; due to PRF-effectuated cell accelerations and/or collisions.

Optimisation of shock wave parameters

The shock wave settings that imparted the maximum number of permeabilised (as detected by PI staining), but still viable (as detected by MTS) cells was sought. This value may be considered the 'transfectable' population. While all killed cells are permeabilised, not all permeabilised cells are killed. A 2-D interpolation of CT26 cell permeabilisation and 24-hours viability was performed between data points, in increments that related to attainable lithotripter settings, and the proportions of permeabilised cells above the proportions of non-viable cells were derived. Figure 5 demonstrates the resulting contours of high and low transfection power across shock wave energies and number of pulses. The highest value is shown to be produced by 548 shock waves, at 4.5 energy level (*see* white arrow), where 20.19% of

total cells are theoretically capable of being transfected in the presence of a nucleic acid, whilst maintaining 24-hour cell survival rates at 100%. To confirm this effect, the optimum shock wave setting was experimentally tested and a reversible cell permeabilisation of 20.5 $\pm$ 2.7% was found. Karshafian et al. (2009) optimised their ultrasound exposure system by deriving a similar measure that compared the desired and destructive effects of any given shock wave condition.

Notably, Figure 5 also shows that at the highest energies and circa 150 pulses (*see* black arrow), there is a local increase in the percentage of live permeabilised cells, suggesting that a regime consisting of a few shock waves at high peak pressures may exist where appreciable cell permeabilisation is attained.

Shock wave pressure

Figure 6(a) shows pressure waveforms measured at the focus of the lithotripter at the optimal energy level for CT26 cells, in degassed water and with the needle hydrophone in the polypropylene vial. The presence of the vial decreased the peak positive pressure by 57% and increased the duration of the compressive phase from 2.57 μs to 4.63 μs, which was measured from the positive pressure that first exceeds 10% of the peak positive pressure, up to the first time the positive pressure reduces below 10% of the peak positive pressure (IEC61846, 1998). The negative pressure did not change substantially as evidenced by a 1.3 MPa decrease when inside the vial, either as a result of the limiting hydrophone's susceptibility to damage, stemming from cavitation during the negative phase (Smith et al., 2012), or because the negative phase is more sensitive to the low frequency components of the signal

and thus less affected by the vial. The presence of the vial did not appreciably change the shape of the waveform. At the focal point, the peak pressures for energy levels 3, 4.5, 6 and 9 are given in Table 1.

Table 1: Shock wave Focal Pressure

Energy Level	Peak Positive (MPa)	Peak Negative (MPa)
3 E	8.6 ± 0.3	4.7 ± 0.3
4.5 E	14.9 ± 0.2	5.0 ± 0.1
6 E	20.7 ± 0.8	6.4 ± 0.4
9 E	37.0 ± 0.5	7.18 ± 0.54

The variation in peak positive pressure in the focal zone of the lithotripter at energy level 4.5 was also measured. Figure 6(b) shows peak pressure in the lateral plane (X,Y) and along the propagation path (Z). The maximum error in hydrophone positioning was a standard deviation of 0.86 MPa. In the lateral direction the pressure amplitude dropped to 50% as demarcated by the -6 dB threshold line, at a radial distance of 3.9 mm, while in the axial direction the -6 dB focal zone was asymmetric about the focus, and spanned 42.4 mm. Therefore the incident pressure field was relatively uniform within the exposure vessels (vial & TMM were 9 or 10 mm wide and 40 mm long), although the vessel material is likely to have resulted in some variation.

Permeabilisation and viability of HK-2 & MCF-7/GFP cells

The impact of shock waves on other cell lines was investigated using HK-2 and MCF-7 cells to gauge variability across different cell types. Cell permeabilisation and viability data were processed similarly to data from CT26 cells. Appropriate settings for the flow cytometer's detectors and amplifiers were adjusted accordingly. However, for MCF-7/GFP cells, PI positivity was

detected using the FL3 channel due to the GFP signal bleeding into the FL2 channel. The overlap between FL2 and FL3 detectors at 620 nm, enabled PI detection in either channel.

The cell viabilities of HK-2 cells were shock wave dose dependent, with values ranging from ∼70% to ∼129% relative to sham controls. In contrast, the permeabilisation of HK-2 cells (Figure 7) showed little correlation with shock wave parameters. However, a measurable level of shock wave induced permeabilisation was demonstrated, highlighting the sensitivity of the cell line to ultrasound. As HK-2 cells are transformed rather than of cancer origin, this provides evidence of shock wave applicability in non-cancerous gene therapy applications.

Results of MCF-7/GFP permeabilisation, shown in Figure 7, confirmed the differential effect of shock waves on cancer cells, previously observed with CT26 cells, where both shock wave energy and number of pulses were effective discriminants. However, the cell line revealed reduced amenability to be efficiently transfected as the highest proportion of permeabilised cells above that of killed cells was found to be 4.1% by exposure to 134 shock waves, energy level 5.0, at 2-Hz. Although up to 23.5% of cells could be permeabilised (500 shocks, 9 E), these parameters were associated with a 25% loss in viability. This is consistent with reports that have described MCF-7 cells as 'hard-to-transfect' in the literature (Fire et al., 2005).

RNA stability

Having identified shock wave parameters that would allow transfection whilst maintaining cell viability, the effect of shock waves on the stability of nucleotide was tested. The structural stabilities of mRNA and siRNA

were determined using the Bioanalyzer and traditional gel electrophoresis respectively. Figure 8(A-1) depicts representative electropherograms of sham and optimum-shock wave treated mRNA. The distinct 18S and 28S ribosomal peaks of typical RNA were present in both treatments. No shift in nucleotide [nt] size of both peaks was observed with optimal shock wave exposure. In addition, the absence of smaller peaks between the two ribosomal peaks (typically observed in partially digested RNA) suggested that the structure-based functionality of the exposed mRNA had not been impaired. However, a 22% decrease in the 28S ribosomal peak intensity was detected with shock wave exposure (1,765 $\pm$ 183 pg/μL for the shams versus 1,363 $\pm$ 115 pg/μL for the shock wave treated, based on n=3). On the stability of siRNA, Figure 8(B) demonstrated that optimal shock waves for MCF-7/GFP cells did not substantially impact the structure or concentration of siRNA, with comparably equal migration and similar fluorescence intensity of the bands with respect to the non-shocked siRNA.

Maintenance of mRNA biological stability was assayed by performing cell-free protein translations of the GFP mRNA transcript, allowing for absolute measurements of shock wave-induced damage to the activity of the mRNA as there was no cell interference during exposure. Figure 8(A-2) demonstrates the translational activity of sham controls and optimum-shock wave treated samples, in terms of fluorescence intensities. An 18% drop in mean fluorescence intensity between sham and optimum shock wave was observed (167,570 versus 137,125 fluorescence units, respectively) which was not found to be statistically significant. Thus, results from both stability methods were in agreement on the mRNA effects impacted by shock

waves. Though direct investigations on the (in)stability of mRNA in acoustic fields have not been previously presented, Forbrich et al. (2013) reported ultrasound-enhanced cellular liberation of endogenous mRNA. They determined significant numbers of liberated mRNA molecules as assayed by reverse transcription and quantitative polymerase chain reaction (qPCR), thus entailing functional post-exposure mRNA. Furthermore, while ultrasound-assisted delivery of complexed mRNA has been reported (De Temmerman et al., 2011), no comparison can be made between free and complexed mRNA.

Ultimately, the reported reduction in RNA quality due to shock waves was shown to be slight and non-significant compared to the potential of enhanced nucleotide delivery.

Shock wave-mediated dissemination of 250-kDa FITC-dextran in agar is a function of macromolecule availability

The application of shock waves has the added potential to improve the transfer of nucleotide from blood vessels and into target tissue. Having identified shock wave exposure conditions which were conducive to cell viability and maintenance of nucleotide structure, the impact of these conditions on mass transfer out of a model channel and into a TMM was investigated. Commercially available FITC-dextran (FITC-D) was employed to simulate as closely as possible the size of eGFP mRNA (1000 nt = $\sim$ 320 kDa) in order to determine RNA-like penetration in 1% agar achieved using selected shock waves. Using the custom-made tissue phantom vessels (*see* Figure 1(b)), the sample could be loaded into the vessel (0 mm), exposed from beneath and transfer toward the shock wave source (up to -20 mm) or away from the shock wave source (up to +20 mm) could be measured. A set of FITC-D amounts

were chosen to encompass the average *in vitro* 20 - 30 μg transferred DNA amounts reported in the literature (Miller et al. (1999), Lauer et al. (1997)). Figure 9 depicts results of scaled fluorescence against distance below (-2.5 to -20 mm) and above (2.5 to 20 mm) the channel. The channel is illustratively demarcated by the dash-dot lines. At all three dextran amounts (red line), an elevated fluorescence signal in the upper agar region (between +2.5 mm and +20 mm) in the presence of shock waves was demonstrated compared to the sham treated samples (blue line). At the farthest distance (20 mm), scaled intensities were $\sim$ 6, 8 and 25 -fold higher than respective sham intensities at the three FITC-D amounts. Contrast between shams revealed some degree of passive dissemination into the agar from the channel, where increasing dextran mass increased the fluorescence in the vicinity of the channel.

GFP mRNA transfection

Experiments were performed to investigate if cells embedded in TMM could produce transgene from nucleotide delivered through a channel compartment within the TMM. Cells were transfected with eGFP mRNA in the absence and presence of the optimal shock waves and incubated for periods of 24 and 48 hours. For fluorescence reading, the 40 x 10 x 5 mm mylar window was discretised into 1 x 1 x 5 mm volumes and fluorescence values recorded for each. Due to cell growth and/or migration, the calculated mean FIs included the channel regions as well. Figure 10a reports the levels of GFP expression at the two time points for both sample treatments. The fluorescence of no-treatment samples comprising just cells was subtracted away from the recorded FIs of the shock wave and sham treated samples. For a given independently conducted experiment, the FI variance between repli-

cates was generally higher at 24 hours than at 48 hours, which was thought to be due to time differences in the onset of protein synthesis. Low numbers of successfully transferred nucleic acids have been previously attributed to the often observed stochasticity in gene expression (Schwake et al., 2010).

At 24 hours after shock wave exposure or sham treatment, the FI of shock wave treated cells, compared to sham was substantially $\sim$ 6-fold higher. When cells were incubated for a further 24 hours, a $\sim$ 52-fold increase ($p < 0.05$) in FI was evident for the shock wave treated cells. FI levels could not be correlated with the number of GFP-positive cells, due to the multiplanar presence of cells in deep tissue phantom samples as well as the GFP spatial heterogeneity observed microscopically (*see* Figure 10b). The images represent 835.2 $\times$ 624 μm areas (0.6 μm/px @ 1392 $\times$ 1040), depicting intensely green fluorescent cells when exposed to the optimum shock waves. In contrast, little GFP signal could be detected microscopically in the sham samples. RNA transfection promotes transient gene expression, hence, strongly expressing cells are desirable to produce sufficient therapeutic benefit.

GFP siRNA transfection

In addition to providing production of therapeutic protein, the tissue mimicking phantom developed here was tested to see if the delivery of siRNA could be achieved to provide knock-down of a GFP reporter gene. An initial assessment was performed on fluorescence quantification for four MCF-7/GFP concentrations over a 4 log range and a linear relationship was found between FI and cell concentration indicating the ability of the quantification method to detect small changes in the number of fluorescent cells.

Results of shock wave mediated GFP knock-down are shown in Figure

11(A). For siRNA delivery, the optimal shock waves for MCF-7/GFP cells were delivered to samples. Three forms of negative control were tested: 1) gene knock-down specificity (using scrambled siRNA whose sequence is intentionally non-complementary to that of the mRNA encoding GFP), 2) sham treatment and 3) no treatment (absence of both GFP siRNA and shock wave exposure). An additional no-treatment control based on non-GFP MCF-7 cells was tested to distinguish between GFP and autofluorescence detection. For all samples, the region of analysis was restricted to the agar area 5 mm above the channel, in which a differential effect between treatments was shown.

Comparisons of relative fluorescence between treatment groups using one-way ANOVA, revealed statistical significance at all three time points ($p < 0.05$). The maximum depletion of GFP signal occurred at 48 hours in cells exposed to siRNA and shock waves. This result was 17% higher than sham controls. The largest difference in GFP fluorescence ($\sim$23%) between sham and siRNA + shock waves was recorded at 72 hours. Figure 11(B) compares the spatial fluorescence across the TMM phantom between siRNA/+ SW and sham siRNA. GFP reduction was also observed in all three negative controls with a peak loss of $\sim$5% relative to sham, occurring in the scrambled siRNA + shock waves samples, implying a small percentage of shock wave induced cell death at 72 hours. The FI decrease over time was generally invariable between negative controls, suggesting a degree of cytotoxicity to affect MCF-7 cells exposed to the TMM *in vitro* system for over 24 hours. Furthermore, at 72 hours an increase in fluorescence in all groups, but particularly in the nucleotide containing treatments was observed. Two possible reasons for the

slight fluorescence recovery are: 1) non-GFP MCF-7 cells (data not shown) revealed an upward trend in cellular fluorescence with increasing time, to a maximum of 12% relative to the initial (background) fluorescence read at zero hours. Such autofluorescence, which is indicative of cell necrosis and increases with decreasing metabolic activity, may have explained the upturn of fluorescence at 72 hours. 2) Cell proliferation up to 72 hours was likely to have produced the increased GFP signal due to an increase in the number of cells. In the case of the siRNA + SW treatment, the net increase in fluorescence was due to a concurrent loss of siRNA function due to its degradation (of which 72 hours defines a time well beyond its onset, considering siRNA's half-life of 24 hours (Bartlett and Davis, 2006)).

Using the Tukey Range test, post-hoc pairwise comparisons did not prove statistical significance for scrambled siRNA or no-treatment results, compared to sham siRNA, at any analysed time point. However, significant difference was detected between siRNA + shock waves and sham siRNA [q = 7.08 >q(crit = 6.35), q = 17.12 >q(crit = 13.67), q = 23.2 >q(crit = 19.8) at 24, 48 and 72 hours respectively]. At 48 hours, significance was found between the two shock waves (+ SW) groups, signifying treatment specificity as well as enabling separation of the proportion of siRNA silenced GFP cells, from those collaterally silenced due to shock wave induced damage.

Discussion

Externally applied ultrasound offers an increasingly popular approach to tackling the challenge of gene delivery (Carlisle et al., 2013). Shock wave exposure may be particularly attractive in certain situations because it is

comparatively low cost, clinically available and has a safety track-record through lithotripsy. The presented studies showed that cancer cell lines exposed *in vitro* to lithotripter shock waves, using clinically available parameter settings, resulted in differential cell viability and reversible membrane permeabilisation. In the mouse colorectal carcinoma cell line, the acquired cell viability data revealed high statistical power ($p<0.01$) between 125, 250 and 500 pulse number sets (encompassing the various tested energy levels and PRFs). In fact, microscopic examination demonstrated that doubling the number of pulses produced progressively shrunken cells in the presence of some cellular debris. In the human breast cancer cells, comparably more irreversible permeabilisation was noted as demonstrated by the poor recovery of viability at 24 hours (Figure 7b). A study by Guck et al. (2005) on the elasticity of MCF-7 cells, based on an optical stretching technique, revealed an approximate 10% increase in peak deformability compared to other malignant cells. We speculate that the lower elasticity of MCF-7 cells resulted in greater deformation when subjected to shock waves hence producing greater damage and permeabilisation. On the other hand, cell viability data of the transformed non-cancerous human kidney cells, demonstrated a sensitivity to shock waves comparable to CT26 cells. Brümmer et al. (1990) made a similar conclusion regarding malignant and normal cells as they found no significant difference in their median lethal dose (LD50) values. While HK-2 manifests both normal and cancerous cell characteristics, these non-malignant cells further agreed with the non-distinguishing dose effect of normal cells observed by Brümmer et al. (1990) because uniquely high cell permeabilisation levels could not be established.

Shock wave parametric studies were conducted to determine the settings that maximised cell permeabilisation with no effect on the viability. The optimal number of pulses for MCF-7 cells was four times less than the number required for CT26 cells, whereas the similarity in optimal shock wave energy indicated the presence of an energy threshold below which reversible permeabilisation is negligible.

Sonoporation is a process of temporary benefit, as the cell membrane naturally reforms afterwards if the cell remains viable. The time taken for such repair has not been defined for most cell lines and there is little data concerning the differences between cancer cell lines and primary cells. The ability of transiently defected cells to permit PI entry was characterised as a measure of permeabilisation. PI is a nuclear stain that is detected upon binding to cellular DNA. Therefore, PI is a late marker in the context of plasma membrane permeability, and excludes the population of cells whose nuclear membrane is intact but whose plasma membrane is compromised. It follows that our reversible plasma membrane permeabilisation result of 20.5% and 4.1% for CT26 and MCF-7 cells respectively is an underestimation of the proportion of potentially RNA transfectable cells, as these only require perturbation of the plasma membrane. Comparable results to CT26 transfectability have been obtained using therapeutic ultrasound (centre frequencies of $1 - 5$ MHz) and variable intensity levels, where 28% (Duvshani-Eshet et al., 2005) and 32% (Karshafian et al., 2009) transfected or transfectable cells respectively have been reported per total number of cells. However, the latter was attained in the presence of ultrasound contrast agents to function as nuclei for cavitation. Our shock wave mediated transfection required no nucleation

agents.

Experiments were designed by considering a number of aspects that would affect shock wave propagation to the cells and gene delivery efficiency. The shock wave-induced cell streaming in fluid at low cell concentrations and subsequent reduced bioeffects inspired the development of TMM exposure vessels (Figure 1b) that permitted the analysis of immobilised cells without needing to dislodge them. Cells embedded in agar allowed shock wave forces to act directly on the cells, thus more likely reflecting the transfection levels that may be observed in tissue, than experiments using isolated cells in solution. Secondly, preliminary transfection tests revealed that the cell or nucleotide concentrations were not as important as the cell-to-RNA ratio (data not shown). A supporting study by Bao et al. (1998) scaled up *in vitro* mRNA dose for *in vivo* use and obtained lower transfection efficiencies *in vivo* than they had *in vitro* probably because the assumed cell-to-mRNA ratio may not have applied to both conditions. In our transfections, the lowest nucleotide concentrations which produced a measurable and distinguishable transfection effect between treatments, were employed.

In this work *in vitro* transfections were performed in a new tissue mimicking model, i.e. not suspended in cell culture or growth medium, and having compartmentalised RNA and cells in an effort to simulate nucleic acid administration via the bloodstream. As such, the efficiency of gene delivery could not be assessed in a single cell fashion and thus the number of transfected cells is not given. Notably, this SW exposure system allowed us to report considerable mediation in gene augmentation and gene knock-down with optimal lithotripter shock wave treatment, compared to sham treatment. In

comparison with similarly low administered nucleotide (plasmid DNA) concentrations (1 – 5 μg in 10^6 cells), Huber et al. (1999) reported just 800 cells out of one million cells were successfully transfected while Murata et al. (2007) showed less than a two-fold increase in luciferase expression, relative to control with shock wave application. In this first demonstration, to our knowledge, of shock wave assisted mRNA delivery, over 50-fold increases were attained. Furthermore, siRNA transfection was enhanced with optimal shock wave exposure despite being challenged by the cell line's relatively low amenability to delivery; with a two-fold increase in knock-down relative to sham siRNA at 72 hours being achieved. Similarly, Ha et al. (2015) transfected CT26 cells with anti-GAPDH siRNA through low energy shock wave exposure, and found a ∼three-fold decrease in the relative GAPDH expression when compared to controls in an *in vitro* set-up comprising a shock wave probe immersed in cell suspensions. In the presence of microbubble contrast agents and unfocused ultrasound in lieu of shock waves, Juffermans et al. (2014) demonstrated approximately a four-fold decrease in GAPDH expression.

Conclusively we have shown that shock wave exposure can successfully induce RNA transfer into cells without imparting cellular or nucleic acid damage, and that this may be possible in a broad range of tissue types by tuning the shock wave exposure parameters. These effects were accomplished using a clinical electromagnetic lithotripter which provides a pathway for clinical translation. Variability in the optimum parameters is expected where different types of shock wave generated lithotripters are utilised having a dissimilar focal volume and shock wave form, from those shown in this work.

Further research is to be undertaken to determine how these findings may impact therapy and its effectiveness.

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Figure Captions

Figure 1: (a) Shock wave permeabilisation equipment and set-up. Shock waves were generated through a 43 L water tank, fitted above the shock wave source. Cell samples were supported by a sample holder, and maintained at 37 °C with a thermostatic heater. The flow degasser reduced O_2 content to 1 – 3 mg/L; (b) custom-made tissue phantom vessel for RNA transfection. The dimensions of the agar + cells containment were 10 × 5 × 40 mm (L × W × H).

Figure 2: Influence of shock wave exposure on cell viability. CT26 cells were assayed at 1 or 24 hours after exposure using the MTS assay. The influence of number of shock waves, energy and pulse repetition frequency was investigated. Data points represent the mean values of three same-day replicates per treatment, for at least three separate day experiments (i.e. n=9). Error bars are the standard errors.

Figure 3: Flow cytometric measurements of CT26 cell permeabilisation and viability. Cell permeabilisation was assessed through propidium iodide (PI) fluorescence-assisted cell sorting (using the FL2-H channel for PI fluorochrome detection) while viability was analysed through forward (FSC) & side (SSC) scatter. Density-plots demonstrate representative cell populations for three treatments: sham (a), 3 E, 125 pulses, 2-Hz (b), 9 E, 500 pulses, 2-Hz (c). The polygon gate (pink) was set to enclose the normal population of intact cells based on the negative control. The fluorescence-based quadrant gates (pink lines) were set to identify the percentage of PI-positive intact events. The data is

representative of 9 repeats.

Figure 4: CT26 cell permeabilisation combined with results of 24-hours viability. Data points represent the means of three same-day replicates, for at least three separate day experiments (i.e. n=9). Error bars are the standard errors.

Figure 5: Transfectability of CT26 cells as a function of the number of shock pulses (0 – 1000) and energy level (3 – 9) at 2-Hz. Transfectability is defined as the percentage of live permeabilised cells. Three independently conducted experiments at 1000 pulses, with three-same day replicates at each tested energy level were included.

Figure 6: (a) Representative shock waveform at energy level 4.5 when measured at the focal point in degassed water and inside the polypropylene (PP) vial; (b) Measurements of peak positive pressure at energy level 4.5, in the direction perpendicular to the shock wave propagation path (X-Y) and along the path (Z). Error bars are the standard deviations (range: 0.07 - 0.86 MPa). The focal zone is marked by the dashed line representing the pressure being 6 dB less than the maximum peak positive pressure.

Figure 7: Influence of shock wave exposure on HK-2 and MCF-7/GFP cell permeabilisation (bars) and 24-hour-after cell viability (lines). Data points represent the mean values of three same-day replicates per treatment, for three separate day experiments (i.e. n=9). Error bars are the standard deviations.

Figure 8: Results of stability for (A) mRNA and B) siRNA post shock wave exposure or sham (control) treatment. Structural stability data depicts (A-1) representative mRNA electrophoretic profiles and (B) agarose gel electrophoresis for siRNA analysis (based on n=3). (A-2) mRNA biological activity data consists of one sample per treatment, for three independently conducted experiments (n=3); error bars represent the standard deviations.

Figure 9: Representative disseminations of 250-kDa FITC-dextran in the custom-made 1% agar tissue phantoms, along the direction of shock wave propagation (i.e. from - 20 to 20 mm) after shock wave or sham treatment. The areas in-between the dash-dot lines represent the channel width. The black arrow indicates the direction of shock wave propagation.

Figure 10: Results of eGFP mRNA delivery to CT26 cells for shock wave and sham treated samples. a) Data is expressed as the means of intensities of six replicates per sample across two independently conducted experiments. The error bars are the standard deviations. Significance was tested using the two-tailed unpaired parametric t-test where * = $p < 0.05$ ($p = 0.04$). b) Representative images of GFP expression at 48 hours after sham or shock wave treatment. Images were converted to binary by thresholding.

Figure 11: Results of anti-eGFP siRNA delivery to MCF-7/GFP cells. A) GFP intensities of shock wave treated (+ SW) and non-treated (- SW) samples consisting of three replicates per sample for one independently

conducted experiment, up to three days after sample preparation or treatment. scR refers to the scrambled siRNA. Data is expressed as the percentage fluorescence relative to its initial fluorescence. Error bars are the standard deviations. Significance was tested using one-way ANOVA and post-hoc Tukey Range tests. The latter revealed statistical significance and is denoted by the asterisks: * = between siRNA + SW and sham; ** = between siRNA + SW and scR + SW, in addition to (*). B) Representative spatial fluorescence intensities across the tissue phantom region of interest for SW and sham siRNA treatments; the dashed lines demarcate the siRNA-incorporated channel. The black arrow represents the direction of SW propagation.