The Ultrasound effects on non tumoral cell line at 1 MHz therapeutic frequency.

L Di Giambattista\textsuperscript{1,2,6}, P Grimaldi\textsuperscript{1}, I Udriu\textsuperscript{3}, D Pozzi\textsuperscript{4}, G Cinque\textsuperscript{5}, MD Frogley\textsuperscript{5}, AM Cassarà\textsuperscript{1}, A Bedini\textsuperscript{3}, C Giliberti\textsuperscript{3}, R Palomba\textsuperscript{3}, S Buogo\textsuperscript{6}, A Giansanti\textsuperscript{1} and A Congiu Castellano\textsuperscript{1,6}

\textsuperscript{1}Physics Department, Sapienza, University of Rome (IT).
\textsuperscript{2}CISB, Sapienza, University of Rome (IT).
\textsuperscript{3}DIPIA, ISPESL, via Urbana 167, Rome (IT).
\textsuperscript{4}Experimental Medicine and Pathology Department, Sapienza, University of Rome (IT).
\textsuperscript{5}Diamond Light Source Ltd, Didcot, Oxfordshire (UK).
\textsuperscript{6}CNR-Institute of Acoustics O.M. Corbino, Rome (IT).

l digiambattista@caspur.it

Abstract. The aim of this research is to investigate some bioeffects due to Therapeutic Ultrasound (1 MHz and 50<\textit{P}a<60 W/cm\textsuperscript{2}) which could allow to enhance drugs or genes delivery in non tumoral cells [1]. Ultrasound (US) has been demonstrated to alter the cell membrane permeability due to a biophysical mechanism, Sonoporation, and exploited as a promising non-invasive gene transfer method. We have used the NIH-3T3 cell line as a model system and exposed it to US medical equipment for 15, 30, 45, 60 minutes at distances of 10 and 15 cm from the source transducer, corresponding to the far field region where \( z > \frac{a^2}{4d} = 4.0\pm0.4 \) cm. We have worked with the maximum power in pulsed system with 75\% duty cycle. Characterization of the unfocused, planar and with a circular geometry 1 MHz source transducer, was performed and the acoustics pressure was measured by a calibrated 0.5 mm needle hydrophone; moreover, the pressure field generated by the source transducer was simulated. The US effects on cells were assessed by Fourier transform infrared (FTIR) Imaging with focal plane array (FPA) detector. By the IR analysis, the US exposure on non tumoral cells has induced a change of the intensity for CH\textsubscript{2} asymmetric stretching (2924 cm\textsuperscript{-1}) band in the lipid region (3000-2800 cm\textsuperscript{-1}) that it could detect an energy-dependent process. It has already shown that cells invest energy to catalyze lipid movement in order to maintain a specific transmembrane phospholipid distribution. Although asymmetry is the rule for control cells, the loss of asymmetry could be associated with the permeability change of plasma membrane inducing temporary pores.

1. Introduction
In the last years, the Ultrasound has been employed in gene delivery for the advantages over other systems as virus or nonvirus-mediated systems; the advantages are a lower invasive-method, a higher delivery efficiency, more applications and minimal cell death. Ultrasound systems are available for both in vitro and in vivo therapies [1]. The biological (thermal, cavitation and microstreaming) effects of ultrasound can result from physical and biological factors, such as frequency, intensity, time.
exposure, duty cycle, temporal and spatial structure of sound field, the physiological state and the size-volume of a sonicated sample, and external conditions like temperature, pressure, microstreaming, shear stress. Such a great number of variables complicates the analysis of the phenomena [2]. We have introduced the FTIR microspectroscopy based on the FPA detector to study the bioeffects of ultrasound on a cellular system in the range of Therapeutic Ultrasound (1 MHz in pulsed system with 75% of duty cycle).

According to a previous test [3], we have characterized the source transducer and we have established the conditions of experimental set up, such as the size of plexiglass tank or the distances from sample to US source. Finally, we have simulated the distribution of pressure with this experimental set up through multimodules software.

2. Materials and Method

2.1. Cell culture and sample preparation

In this research, we have used a fibroblast cell line (NIH-3T3); the NIH-3T3 were grown with a solution of Dulbecco’s Modified Eagle’s minimum essential Medium (DMEM) without calcium with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine at 37°C in humidified atmosphere containing 95% air and 5% CO₂. Before the US exposure, the NIH-3T3 were cultured as monolayer in 35-mm petri dish on CaF₂ windows, pre-treated with polylysine. The viability of both the untreated (control) and the US treated (sonicated) cells has been determined by Trypan blue (TB) exclusion test (over 90% of viability). After the treatment with ultrasound, the control and sonicated cells were fixed in paraformaldehyde (2% for 15 min), washed in Dulbecco's Phosphate Buffer Saline (PBS) and in distilled water to remove PBS residues, and dried in a desiccator.

2.2. Experimental set up

As shown in figure 1, we have built a plexiglass tank filled with partially degassed water and the source transducer was placed on the bottom.

![Figure 1. The general scheme of experimental setup.](image)

A vertical directivity test of source transducer was realized and the maximum signal by transducer has shown a shift of θ=0.8° (figure 2).

![Figure 2. Polar diagram (log scale) for unfocused, planar and circular source transducer.](image)

The excitation pressure waveform was measured at different distances from transmitting transducer by 0.5 mm needle hydrophone (Precision Acoustics LTD, Interchangeable Probe) with a sensitivity of -272.7dB re 1V/µPa at frequency of 1 MHz (table 1).
Table 1. The values of voltage and pressure at different distances.

| Distance (cm) ± 0.05 | Voltage\textsubscript{rms} (mV) | Pressure (MPa) |
|---------------------|-------------------------------|----------------|
| 0.8                 | 53.4 ± 2.8                    | 1.62 ± 0.08    |
| 3.0                 | 54.3 ± 2.7                    | 1.70 ± 0.08    |
| 10.0                | 33.7 ± 1.7                    | 1.03 ± 0.05    |
| 15.0                | 49.3 ± 2.5                    | 1.53 ± 0.08    |
| 20.0                | 44.0 ± 2.2                    | 1.34 ± 0.07    |

The US medical equipment has been set to 1 MHz with 100% of maximum power in pulsed system with 75% duty cycle. According to the preliminary analysis, the distances \((h0)\) between the cellular sample and the transducer were chosen at 10 and 15 cm (figure 2) and the US exposed times were fixed at 15, 30, 45, 60 minutes. At both distances, we have established the values of pulse average intensity \((I_{PA})\): \(I_{PA} = (52 ± 6)\) W cm\(^{-2}\) at 10 cm and \(I_{PA} = (58 ± 6)\) W cm\(^{-2}\) at 15 cm. The temperature inside the petri dish and water of tank was monitored with a thermocouple; a maximum increase of 2°C or less was revealed during US exposed time.

2.3. FTIR microspectroscopy and data pre-processing for image analysis

An FTIR microscope (Hyperion 3000) coupled to an Vertex 80v FTIR spectrometer (both from Bruker Optics, Germany) was used for IR measurements: the Hyperion 3000 microscope was equipped with a computer-controlled x,y stage and was sealed using a specially box purged in 99.8% pure nitrogen. This microscope was equipped with a mercury cadmium telluride (MCT) based focal plane array detector (FPA) of 64x64 pixels where each pixel corresponded to an area of 40x40µm, so that the visible field corresponded to an area 170x170µm\(^2\) with 15x magnification objective. Each pixel corresponds to a single acquired FT-IR spectrum. The FTIR images were acquired continuously with a spectral resolution of 8 cm\(^{-1}\) and by co-adding 512 scans of absorbance spectra were recorded in the range from 3000 to 900 cm\(^{-1}\) (lipid, proteins and nucleic acids regions). Every cellular sample was measured on CaF\(_2\) window in Transmission mode and a background spectrum/image of CaF\(_2\) slide was recorded before each cellular sample measurement in order to account for variations in water vapour and CO\(_2\) levels. Three images were acquired on the CaF\(_2\) slide, resulting 30 images with 4096 spectra for the whole experiment. The resulting FT-IR images were pre-processed by different softwares (OPUS 6.5, OriginPro 8.0, Mathematica 7.0); to remove poor quality spectra, the data sets were subjected to a quality test such as Signal-to-Noise ratio (S/N) [3]. In the (S/N) test, the 4096 spectra were evaluated using a criterion of 100 as a threshold of the ratio in accordance with the FTIR spectroscopy; the signal with the maximum absorbance was evaluated in the frequency region of the Amide I band (1595-1800 cm\(^{-1}\)) and the noise was calculated as the standard deviation in the spectral range 1800-1900 cm\(^{-1}\). The spectra with a S/N ratio smaller than 100 were labeled as bad spectra and the corresponding pixels, bad pixels. The number of bad spectra inside a map is denoted with \(N_{bs}\); the maps having more than 1000 bad pixels have been discarded. After this pre-processing analysis, the spectral data were processed by a baseline correction with a rubberband method using 64 baseline points; then, through the vector normalization, we have normalized the whole spectral region (3000-900 cm\(^{-1}\)) at CH\(_2\) asymmetric stretching (2924 cm\(^{-1}\)) band for the analysis of the lipid region. The chemical maps were created from these spectra by plotting different spectral parameters, such as intensity peak or intensities peaks ratio, as a function of x-y pixel position.

2.4. Simulation details
In this study, 2-D simplified model has been built using multimodules software. Finite element analysis has been used to model the ultrasonic waves propagation in the plexiglass tank at distances of 10 and 15 cm from US source. The boundary condition was sound hard boundary (wall) for the whole tank; at interface between the water and air, the boundary condition was set to continuity. The mesh size has been adapted to every acoustics subdomain in order to resolve the wavelength. The maximum dimension of the mesh has been set on a value corresponding to 2th of the acoustic wavelength of water (IEC 61102). A frequency-dominion analysis was selected for solving the problem and a linear system solver adopted for high frequency was selected.

3. Results and discussion

3.1. Spectra pre-processing

By the results with S/N quality test, we have established the number of bad spectra in each data set and have evaluated their weight in the 4096 spectra. In figure 3, we have reported as an example, the dispersion of (S/N RMS) values around average value vs. pixels # (4096 spectra) for the control cells and the sonicated cells at 15 cm for 45 minutes (SON\textsubscript{45\_15}) that has shown a larger number of bad spectra (data no shown for all samples).

![Figure 3](image)

**Figure 3.** On the left, RMS of S/N ratio vs. pixels for control cells with 4051 good spectra and 44 bad spectra; on the right, RMS of S/N ratio vs. pixels for sonicated cells at 15 cm for 45 minutes with 3344 good spectra and 752 bad spectra.

We have not discarded the FT-IR maps with \(N_{bs}<1000\) to monitor the presence of major number of empty spaces due to the US that perturbed the spatial cellular distribution on the slide forming the islands of cellular monolayers. We have compared the number of bad spectra vs. time of FTIR maps between the control and sonicated cells at both distances from the US source; in figure 4, we have reported the results only for the distance of 15 cm.

![Figure 4](image)

**Figure 4.** The number of bad spectra (\(N_{bs}\)) vs. time within the maps reported at the 15 cm from the US source.
3.2. The simulation of pressure field

We have presented a simplified 2-D geometry to study the acoustics wave propagation inside the plexiglass tank (LxH=20x25cm). The results have been validated by comparison with experimental data (figure 5).

Figure 5. The distribution of pressure at distances of 10 (left) and 15 (right) cm from US source.

3.3 FTIR spectral imaging

FTIR spectral imaging enables the determination of the distribution of several molecules of interest; we have studied the chemical map due to the FTIR- imaging through the analysis of $L_{2I}$ that indicates the intensity of the $\text{CH}_2$ asymmetric stretching (2924 cm$^{-1}$) band. The intensities of IR peaks provide quantitative analysis about sample contents, depending on the nature of molecular structure, their bonds, and their environment. As reported in figure 6, we have compared the values of this parameter vs. time obtained from the chemical map at both distances.

Figure 6. $L_{2I}$ parameter vs. time at 10 cm (black point) and 15 cm (white point) due to chemical map is reported, showing differences between the values of chemical map at two distances. The error bars correspond to $\pm 5\%$ of the measured value.

From spectral data, the changes of $L_{2I}$ parameter obtained from the map were correlated with the applied ultrasonic (US) energy (the ultrasonic energy per unit of surface). In figure 7, this correlation has a R-value of about 94% with a probability of about 99% (Fisher test) at the distance of 15 cm. At
10 cm from the source, the degree of correlation decreases to a R-value of about 75\% with a probability of about 98\% (Fisher test) (data not shown).

![Graph showing correlation between L$_{21}$ intensity and US energy](image)

**Figure 7.** Correlation between the L$_{21}$ intensity and the US energy (the ultrasonic energy per unit surface) with a R-value of about 0.94 and a probability of about 99\% (Fisher test). The error bars correspond to ± 5 \% of the measured value.

4. Conclusion

In this paper, we have analyzed the effects due to US medical equipment at 1 MHz frequency in pulsed system on non tumoral cell line. The unfocused, planar and with a circular geometry source transducer was characterized through a vertical directivity test where the maximum signal has shown a shift of of $\theta=0.8^\circ$. At the chosen distances of 10 and 15 cm, we have established the values of pulse average intensity ($I_{PA}$): $I_{PA} = (52 \pm 6) \text{ Wcm}^{-2}$ at 10 cm and $I_{PA} = (58 \pm 6) \text{ Wcm}^{-2}$ at 15 cm. The experimental setup did not cause a thermal effect on cells; the temperature was registrated inside the petri dish and the water by thermocouple and it was increased of 2°C or less during US exposed time. The simulation of acoustics wave propagation has been validated by comparison with experimental data. The results of FTIR microspectroscopy have shown that the US experimental conditions have not induced a DNA mutations [4] whereas in the lipid region (3000-2800 cm$^{-1}$) there was an effect on L$_{21}$ parameter vs. time that could be useful in the study of the ultrasound-mediated gene transfection in vitro. The change of L$_{21}$ parameter has proved as membrane phospholipid asymmetry was perturbed; this movement of transbilayer lipid distributions can be associated to the formation of temporary pores on plasma membrane, ruled out the formation of permanent pores that can induced the cell death.

References

[1] Newman CM, Lawrie A, Brisken AF, Cumberland DC 2001 *Echocardiog.* 18 339-347
[2] Lewin PA, Lypacewicz G, Bautista R, Devaraju V 2000 *Ultrasonics* 38 135-139
[3] Conti L, Grimaldi P, Udroui I, Bedini A, Giliberti C, Palomba R, Congiu Castellano A 2010 *Vibrat. Spectrosc.* 52 79-84
[4] Di Giambattista L, Grimaldi P, Udroui I, Pozzi D, Cinque G, Frogley MD, Giansanti A, Congiu Castellano A 2009 *Biophys. and Bioengin. Letters*, 2, 2.

Acknowledgments

The authors are grateful Dr. M. Lattanzi for his assistance with Mathematica software and Dr. S. Belardinelli for his assistance in the laboratory experiments.