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Structural and permeability sensitivity of cells to low intensity ultrasound: Infrared and fluorescence evidence *in vitro*

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ABSTRACT

This work is focused on the *in vitro* study of the effects induced by medical ultrasound (US) in murine fibroblast cells (NIH-3T3) at a low-intensity of exposure (spatial peak temporal average intensity $I_{ta} < 0.1$ W cm^{-2}). Conventional 1 MHz and 3 MHz US devices of therapeutic relevance were employed with varying intensity and exposure time parameters. In this framework, upon cells exposure to US, structural changes at the molecular level were evaluated by infrared spectroscopy; alterations in plasma membrane permeability were monitored in terms of uptake efficiency of small cell-impermeable model drug molecules, as measured by fluorescence microscopy and flow cytometry. The results were related to the cell viability and combined with the statistical PCA analysis, confirming that NIH-3T3 cells are sensitive to therapeutic US, mainly at 1 MHz, with time-dependent increases in both efficiency of uptake, recovery of wild-type membrane permeability, and the size of molecules entering 3T3. On the contrary, the exposures from US equipment at 3 MHz show uptakes comparable with untreated samples.

1. Introduction

US is beginning to emerge as a powerful stimulus in numerous medical applications [1], including new methods for localised treatments as gene therapy and drug delivery [2–4]. The challenge is to drive the biological properties of cellular membranes as an efficient way to non-invasively transfer DNA, drugs or other molecules into target cells. These applications take advantage of the pivotal US bioeffect investigated by numerous *in vitro* experiments, which is commonly referred to as the sonoporation phenomenon [5,6].

In the absence of ultrasonic heating, US-induced bioeffects are commonly assumed to be caused by acoustic cavitation [7,8]. Cavitation is typically generated through the activation of small dissolved gas nuclei in the presence of an acoustic pressure field [9–11]. According to sonoporation studies [5,6], cavitation-promoted plasma membrane fractures can allow the uptake of poorly membrane-permeable exogenous vectors in viable cells, although the lack of reversibility of the process results in cell death [12]. Some applications may benefit from killing cells [13], however drug delivery scenarios seek to maximise intracellular uptake while maintaining constant cell viability [14,15].

Although the ability of US to deliver fluorescent molecules into viable cells has been demonstrated in numerous *in vitro* studies, the mechanism and the quantitative dependence of bioeffects on acoustic parameters remains poorly understood [15–21].

In particular, low-intensities of US exposure ($I_{ta} < 0.1$ W cm^{-2}) have been shown to induce bioeffects in cells without any evidence of inertial or stable cavitation being present [17].

Based on these findings, an intramembrane cavitation model, named bilayer sonophore (BLS), has recently been proposed [18] as a unique hypothesis to explain US-induced bioeffects at both non-cavitation ($I_{ta} < 0.1$ W cm^{-2}) and cavitation ($I_{ta} > 0.1$ W cm^{-2}) regimes. According to this model, the bilayer membrane is capable of absorbing mechanical energy from the US field and transforming it into expansions and contractions of the intramembrane space, overcoming the molecular attraction forces between the bilayer leaflets. This mechanism would induce different effects on the cell membrane, such as an increase of membrane permeability,

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potentially facilitating the uptake of drugs and genes, and the enhancement of tissue permeability [18].

In this framework, microscopic and spectroscopic techniques offer the sensitivity and capability to detect morphological, structural and functional fine alterations of sonicated cells, as they are not yet significantly advanced [22–27]. In previous papers [28,29], we have shown how the effects of US on biological samples can be effectively revealed by means of Fourier Transform Infrared (FTIR) spectroscopy, a non-destructive technique which is able to monitor conformational and functional changes exhibited by specific subsets of macromolecules inside a cell population.

Proceeding from this approach, here we combine FTIR spectroscopy, flow cytometry and fluorescent microscopy to investigate the sensitivity of biological cells undergoing therapeutic US in regimes of sub-cavitation. To this aim, we focus on the murine fibroblasts cell culture NIH-3T3 in representing a well characterised *in vitro* biological model to analyse alterations which may occur at both the structural and plasma membrane permeability level, in the presence of 1 and 3 MHz US at varying field intensities and times of exposure.

Within these working conditions, we analyse the intracellular uptake of the fluoroprobe calcein, which is extensively considered a small model drug molecule (radius = 0.6 nm) also in relation to sonoporation, and use these results to better understand the FTIR analysis of lipid, protein and nucleic acids composing the cell. Thus, the question of whether the presence of disruptions in the cell membrane (pore formation) is consistent with the uptake of cell-impermeable molecules has been addressed.

2. Materials and methods

2.1. Cell and culture conditions

The experiments were carried out using murine fibroblasts NIH-3T3. The cells were cultured as a monolayer in a humidified atmosphere with 95% air and 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich, St. Louis, MO) with 10% foetal bovine serum, 1% penicillin and 1% glutamine/streptomycin. Samples were prepared in well plates (Falcon® Easy Grip™ tissue culture dish, 35 × 10 mm) at concentrations varying between 7×10^5 cells/ml and 9×10^5 cells/ml. Three millilitres of Phosphate Buffered Saline (PBS) Dulbecco's Formula without calcium and magnesium were used in each experiment.

Cell viability of both the untreated (control) and the US treated (sonicated) cell samples has been determined by the Trypan blue exclusion test, for all of the transducers-samples distances and at all times of exposures. Sample viability, before US exposure, was better than 95% for each trial.

2.2. Ultrasound exposure system and experimental protocol

For the US exposures, we used two conventional medical devices (Nuova Elettronica, Italy), consisting of two submersible piezoceramic circular transducers (6 cm diameter) tuned at 1 and 3 MHz, and the corresponding waveform generators. Such systems can work in continuous and pulsed modes; the duty cycle of both 1 MHz and 3 MHz US generators has been selected in *High* pulsed mode, in which the signal is delivered for 750 ms, followed by a pause of 250 ms. The power can vary in the range from 10% to 100% of the maximum power (maximum nominal intensity I_{max} = 2.5 W cm^{-2}), allowing for selection of the maximum value maintains the integrity of the monolayer after sonication: 100% of I_{max} for 3 MHz, 75% of I_{max} for 1 MHz. Ultrasonic transducers were placed at the bottom of a tank filled with degassed water; the temperature of the waterbath was kept constant at ~25 °C as monitored by thermocouple system (Lutron electronic enterprise co., LTD.) both inside and outside the Petri dish.

A cell culture-treated Petri dish (9.6 cm^2) containing three millilitres of PBS solution hermetically lidded was positioned at the water surface and inserted to half of its thickness in the waterbath, in line with the transducer, according to the scheme reported in Fig. 1. Taking into account therapeutic applications in which the position of the US source is fixed and the time of exposure changed at a selected nominal power and duty cycle, the biological samples were sonicated at three sample-transducer distances, called Source-dish Surface Distance (SSD) (see Fig. 1): 5, 10, 15 cm, and at each distance for 5, 15, 30, 45 and 60 min. The SSD distance was varied by decreasing/increasing the water level of the tank and in turn the position of the Petri dish.

The characterisation of the acoustic field produced by the US sources at the two frequencies was performed using a needle hydrophone (Precision Acoustics) of 1 mm diameter (S.N. 1470) with a sensitivity of 1670.4 mV/MPa at 1 MHz and 958.2 mV/MPa at 3 MHz (±14%).

In this work, the intensity of the acoustic field is provided in terms of *Spatial Peak Temporal Peak* (I_{tp}), which represents the higher intensity value measured when the pulse is on and therefore it is particularly significant to correlate mechanical bioeffects induced by pulsed US; on the other hand, I_{ta} represents the maximum spatial intensity measured when the pulse is on, mediated for the period of pulse repetition.

I_{tp} values transmitted through the cell monolayer as measured nearby the cell culture plate for 1 MHz US exposures are 0.11 W cm^{-2} (SSD = 5 cm), 0.12 (SSD = 10 cm) and 0.09 W cm^{-2} (SSD = 15 cm); for 3 MHz they are 0.06 W cm^{-2} (SSD = 5 cm), 0.04 W cm^{-2} (SSD = 10 cm) and 0.01 W cm^{-2} (SSD = 15 cm). Because of the frequency dependence of US transmission coefficient, the measured values I_{tp} at 3 MHz fall below those corresponding to 1 MHz, although the nominal intensity of the US generators at 3 MHz and 1 MHz was set at 100% and 75% of I_{max}, respectively.

In terms of I_{ta}, the measurements provide values I_{ta} < 0.026 W cm^{-2}, which fall significantly below the recommended threshold of 0.1 W cm^{-2} in the sub-cavitation regime.

2.3. FTIR spectroscopy

The spectroscopic measurements were performed with a Jasco spectrophotometer FT-IR 410 in transmission mode; for each SSD and exposure time, cells were grown to confluence on a window

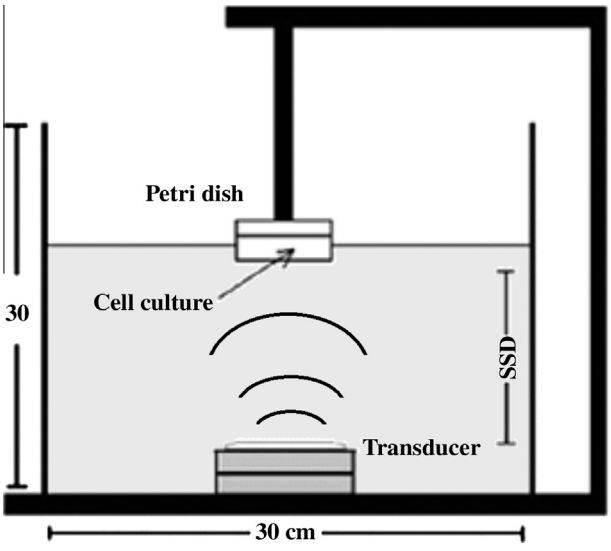

Fig. 1. Experimental set up for US exposures.