# Acoustic characterization of ultrasound fields able to induce sonodynamic activity in an in vitro cancer model

# Acoustic Characterization of Ultrasound Fields Able to Induce Sonodynamic Activity in an *in Vitro* Cancer Model

## *Ultrasound metrics for Sonodynamic Therapy*

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***Abstract*—***The corner stone of cancer management is by far chemotherapy, unfortunately toxicity and undesired side-effects of these antineoplastic approach often limit its usefulness. Recently it has been found that certain dye compounds non cytopathogenic per sé, in particular porphyrins, can achieve a cytopathogenic effect when the neoplastic site is subjected to ultrasounds irradiation, this technique is referred to as sonodynamic therapy (SDT). Despite the promising results, the poor reproducibility of the treatment, due to the poor investigation between the ultrasounds field parameters and the SDT activity, hampered the development of robust treatment protocol. Therefore the aim of this work has been the characterization of high intensity ultrasound fields needed to reach the SDT activity. First, by an ultrasound generator system, an in vitro sonodynamic treatment has been performed on HT-29 cell line, previously pre-incubated with 50 μg/mL of aminilevulinic acid, therefore a complete characterization of the ultrasound field in measurement conditions has been carried out. An ONDA type AIMS III scanning tank system with needle hydrophone ONDA type has been used and the real energy that hit the cellular culture in the burette has been evaluated. During insonation process the energy supplied to the cells has been about 5 $10^{-6}$ J introducing, for the first time in the sonodynamic field, a clear parameter of energy supplied to the cells.*

***Keywords—ultrasounds; sonodynamic therapy; porphyrin***

## I. INTRODUCTION

Although ultrasounds affect tissue through a variety of mechanisms, the thermal and non-thermal mechanisms are the most prominent. The thermal anti-cancer applications of ultrasounds, such as high intensity focused ultrasounds (HIFU) that produces coagulative necrosis at a precise focal point, have been more extensively studied than has the therapeutic use of the non-thermal effects of ultrasounds [1]. The tissue interactions associated with the non-thermal effects of ultrasounds include the direct interaction between ultrasounds and the biological membrane, which is able to modify cell permeability and to enhance drug bioavailability (sonoporation) [2], and the ability to modify the chemical properties of particular compounds, known as sonosensitisers (sonosensitisation) [3]. Recently, sonodynamic therapy (SDT) has been proposed as an innovative non-invasive anticancer approach to selectively promote the generation of cytotoxic species via the excitation of particular chemical compounds, known as sonosensitizers, through ultrasound irradiations [4]. Although neither component is individually toxic, together they initiate a sonodynamic process culminating in the generation of highly reactive cytotoxic species such as radicals (hydrogen atoms, hydroxyl, alkoxyl and peroxyl radicals) and singlet molecular oxygen, which can rapidly cause intracellular damages leading to cell death via apoptosis and/or necrosis and/or autophagy [5]. Sonosensitisation is a consequence of acoustic cavitation, i.e., the formation of gas/vapour-filled nuclei that grow into microbubbles in ultrasound-irradiated fluids: under the appropriate irradiation conditions (i.e.: when acoustic pressure amplitude is sufficiently high), the cavitation microbubbles implode violently, causing the temperature and pressure within the cavity to increase drastically [6]. Such extreme conditions are confined to the vicinity of the microbubble, and they are able to trigger cascades of chemical modifications similar to the ones occurring during the photodynamic process. Sonosensitizers are able to absorb acoustic cavitation energy and translate it into the production of reactive species. Collapsing microbubbles are known to promote light emission (sonoluminescence) [7], and, intriguingly, the majority of the compounds showing sonodynamic efficiency also display photodynamic activity: taken together, these two evidences led to the hypothesis that sonoluminescence plays a key role in the transfer of cavitation energy to the sensitizer, and in the sonosensitised generation of reactive species. Despite these promising characteristics, the scarce reproducibility of the treatment parameters and the poor investigation of the correlation between the ultrasound field parameters and the induced sonodynamic activity hampered the development of robust treatment protocol, preventing this approach from reaching clinical applications. Introducing a precise acoustic characterization of high intensity ultrasound fields needed to

reach the sonodynamic activity will have a strong impact on the development of SDT and applications of power ultrasound in medicine.

## II. Materials and Methods

### A. Ultrasound generator system

The ultrasound field is generated by a plane wave 2.54 cm transducer in CW mode at $f$ =1.866 MHz connected to an Amplified Research type AR 100A250A power amplifier and an AGILENT 33250 function generator. A mechanical adaptor has been built to connect the 1 cm diameter burette that contains the cellular culture. When filled with ultrapure water, the adaptor realizes a high reproducibility of measurement conditions and distance (17 mm),from transducer to burette [8], as shown in Fig. 1.

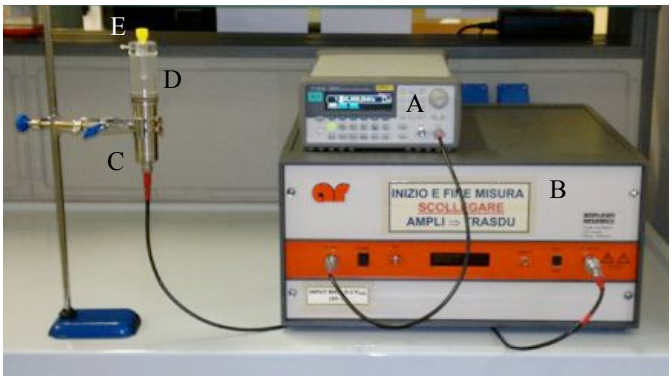


Fig. 1. Ultrasound generation System: A) Function generator; B) Amplifier; C) Piston like ultrasound transducer; D) Mechanical adaptor; E) Burette

### B. Cell culture

The HT-29 human colorectal adenocarcinoma cell line (ICLC, Interlab Cell Line Collection, Genova, Italy) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum (v/v), 2 mM L-glutamine, 100 UI/mL penicillin, 100 µg/mL streptomycin and (Sigma, Milano, Italy) in a humidified atmosphere of 5% $CO_2$ air at +37°C. The HT-29 was detached using 0.05% trypsin-0.02% EDTA solution (Sigma), suspended once again in culture medium and seeded at the appropriate cell concentration for ultrasound exposure.

### C. In vitro sonodynamic treatment

In the exponential growth phase HT-29 cells was pre-incubated in the dark for 24 hours with aminolevulinic acid (ALA, 50 µg/mL), a natural precursor of the sonosensitizer protoporphyrin IX (PpIX). The cells were then normalized to $5 \times 10^5$ cells in a polystyrene tube filled with phosphate-buffered saline (PBS), pH 7.4, for the ultrasound irradiation. The *in vitro* experiment was performed under a dim light and the temperature of the medium was overseen to avoid hyperthermia during the experiment (maximum temperature recorded was 33°C).

### D. Cell proliferation assay

The WST 1 assay (Roche Applied Science, Penzberg, Germany) was employed to consider any effects the treatment had on the HT-29 cell proliferation. After the treatments, $2.5 \times 10^3$ cells were seeded in 100 µl of culture medium in replicates ($n$=8) in 96-well culture plates. WST-1 solution (10 µl) was added at 24, 48, and 72 hours, and the plates were incubated at +37°C in 5% $CO_2$ for 1.5 hours. The well absorbance was evaluated at 450 and 620 nm in a microplate reader (Asys UV340; Biochrom, Cambridge, UK). Cell proliferation data were expressed as a percentage of untreated cells.

### E. Ultrasound field characterization

A complete characterization of the ultrasound field in the same condition in operative mode has been carried out. Both the ultrasound power and the spatial distribution of the ultrasound pressure $p(x,y,t)$, impinging on the burette surface only, have been evaluated [9].
The ultrasonic power, $P$, is calculated according to the relation:

$$P = u(T)g\Delta M \quad (1)$$

where: $u(T)$ is the speed of sound in water, $g$ is the gravity acceleration, depending on the water temperature, $T$, and $\Delta M$ is the mass variation induced by the ultrasound field [10].
The uncertainty at measurement conditions is show in TABLE I, where: $f$ is the working frequency of the ultrasound transducer, $T$ is the temperature of the water vessel, $U_{IN}$ is the rms voltage at the transducer input, $P$ is the measured power level and $U(P)$ is expanded uncertainty of the power [11].

TABLE I.

| $f_a$ | $T$ | $U_{IN}$ | $P$ | $U(P)$ |
|---|---|---|---|---|
| MHz | °C | V | W | % |
| 1.866 | 20.8 | 40.1 | 0.7 | 6.0 |

An ONDA type AIMS III scanning tank system with needle hydrophone ONDA type. HNA-0400 and ONDA preamplifier type AH-2020 (HI GAIN configuration) has been used to characterize the ultrasound field [12], as shown in Fig. 2.

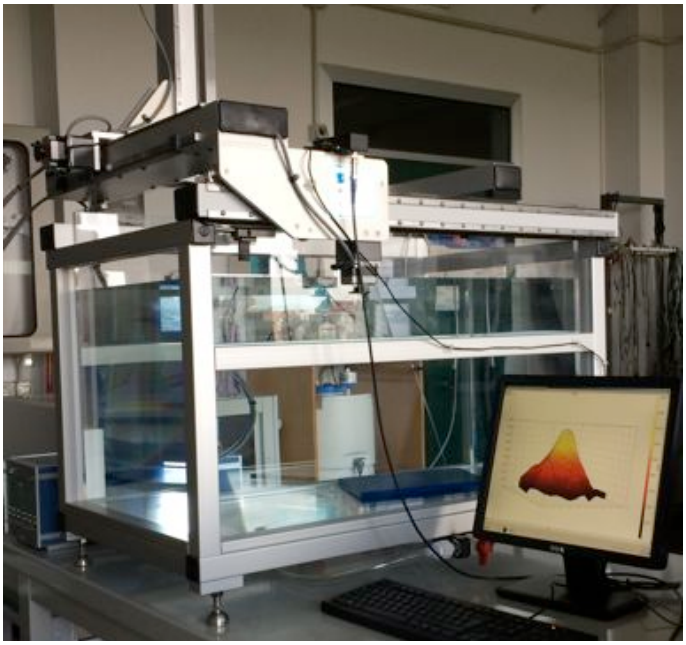

Fig. 2. ONDA AIMSII Scanning tank system.

## III. Results

### A. *In vitro biological effects of the sonodynamic treatment*

The sonodynamic treatment with the ALA as a pro-drug of the sonosensitizer PpIX (50 μg/ml for 24 hours) was able to significantly decrease the HT-29 cell growth (Fig. 3). Noteworthy is the fact that the sonosensitizer and ultrasounds alone unaffected the HT-29 cell growth. Indeed, observation under the microscope showed a significant increase of necrotic cells only in the HT-29 cells treated with ALA and ultrasounds 24 h hours after the treatmets (Fig. 4).

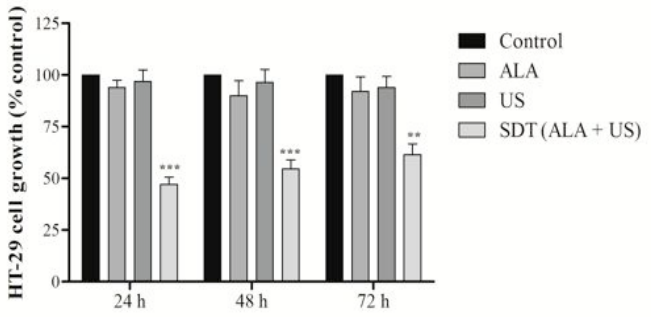


Fig. 3. Effect of different treatment conditions on HT-29 cell growth as a function of time. HT-29 were treated with US (1.8 MHz for 5 minutes) or ALA (50 μg/mL for 24 hours) or both as SDT. Statistically significant difference versus untreated cells: ** $p < 0.01$; *** $p < 0.001$.

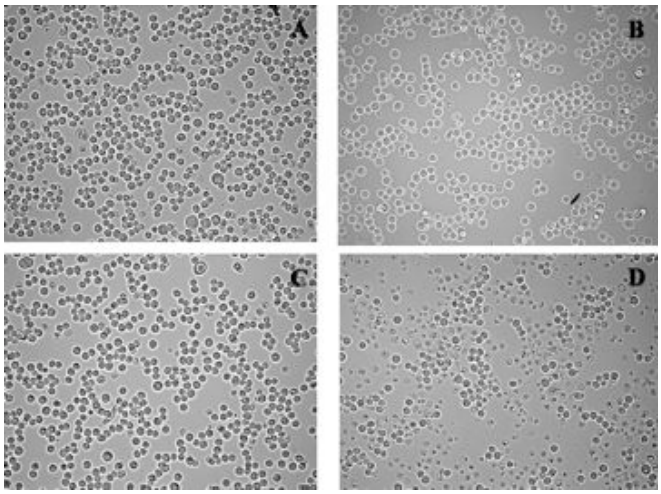


Fig. 4. Representative pictures of HT-29 cells 24 hours after the sonodynamic treatment with ALA and US. A) untreated cells; B) cells treated with ALA; C) cells treated with US; D) cells treated with ALA and US (magnification 10x).

### B. *Ultrasound fileds*

The planar scan of the ultrasound field in operating conditions (transducer, mechanical adaptor and burette), at distance $d \cong 80$ mm from the transducer surface, is displayed in Fig.5.

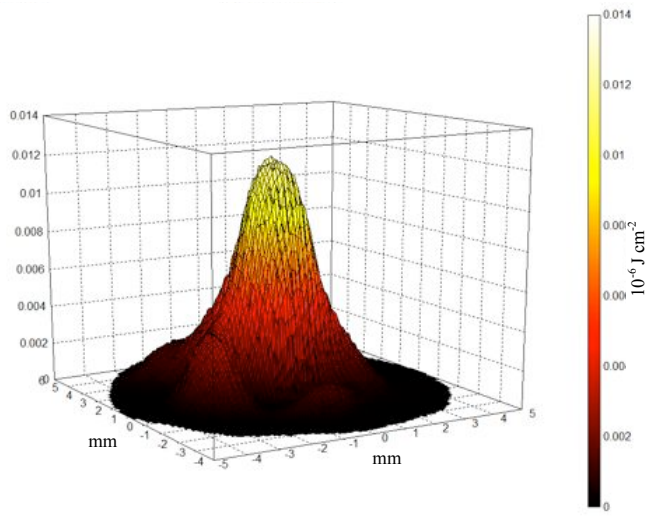


Fig. 5. Planar scan of the ultrasonic field used during the insonation.

With the aforementioned measurement setup and data analysis, the real energy that hit the cellular culture in the burette has been evaluated [13].

During the 300 s insonation process, with an ultrasound power $P = 0.7$ W, the energy supplied to the cells is about $5\ 10^{-6}$ J.

## IV. DISCUSSION

The capability of measuring, with a low uncertainty, the energy released to cells allows the refinement of the technique by the modulation of the power during the specified treatment.

This capability is even more useful in *in vivo* experiments, where a complete control of ultrasound field parameters is essential to limit hyperthermia effects caused the ultrasound beam interaction.

## ACKNOWLEDGMENT

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