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# Ultrastructural Modifications of Cell Membranes and Organelles Induced by Sonoporation

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**Abstract**—Sonoporation increases transiently the native cell membrane permeability. However, the exact mechanism involved in the membrane permeabilization remains to be elucidated. While, no consensus is reached, the pore formation is usually hypothesized as having a central role in the membrane permeabilization induced by sonoporation. In this study, we investigate the sonoporation effect on the plasma membrane and organelles using electron microscopy. Adherent U-87 MG cells were insonated at 1 MHz, 1 W/cm<sup>2</sup> acoustic intensity, 20% duty cycle for 10 or 60 s. BR14<sup>®</sup> microbubbles were added at a bubble/cell ratio of 5. SYTOX<sup>®</sup> Green was used as a permeabilization marker. With these US conditions in combination with BR14<sup>®</sup>, flow cytometry results showed a permeabilization rate of 60%. The ultrastructural modifications of the cells were monitored by scanning and transmission electron microscopy (SEM/TEM) either immediately or 15 min post-sonoporation. Based on SEM images, the control cells (No US) showed a regular plasma membrane with microvilli, while the insonified cells (US+BR14<sup>®</sup>) exhibited circular and dark spots on their surfaces, suggesting pore-like structures. The number of these structures increased in the presence of BR14<sup>®</sup> and with the insonation time. The pore-like structures size distribution is heterogeneous, and ranged from 10 nm to 160 nm. However, these structures might also correspond to caveolae or clathrin endocytic vesicles. To investigate the transient character of these structures, the cells were fixed 15 min after sonoporation. The results showed that the number of these pore-like structures decreased strongly indicating that the cells are still metabolically active. The maximal size of these pores is 100 nm and we suggest that small pores still require more time to reseal. Sonoporation effects on the organelles structure were investigated. Based on TEM images, insonified cells (US+BR14<sup>®</sup>) presented a stimulation of endocytosis pathways compared to control cells. In addition, the sonoporated cells showed less dense cytoplasm, suggesting a decrease of their viscosity, which would facilitate the intracellular traffic of delivered molecules. In conclusion, these microscopic observations reveal that the sonoporation effects are not confined to the membrane only, but also occur at the intracellular level.

**Key words**— Sonoporation, Uptake mechanisms, Microbubbles, Electron microscopy, Glioblastoma cells

## I. INTRODUCTION

It is known that sonoporation leads to a transient permeabilization of the cell membrane. One of the hypotheses is the formation of non-selective and non-lethal perforations [1–5], due likely to mechanical stresses from the oscillating bubbles in the cell vicinity. In fact, bubble oscillation and

collapse may generate micro-streaming, micro-jets, or shockwaves [6], [7] allowing formation of pores in the plasma membrane and / or propelling plasmid molecules into the cells. Current studies report that the pores form with a size varying from few nm to few  $\mu$ m (several nm to 200 nm [9]; < 150 nm to 1  $\mu$ m [10]; 10 - 100 nm in the absence of microbubbles and 1- 3  $\mu$ m when Optison<sup>®</sup> was added [11], and up 75 nm [8], ~ 100 nm to 1.25  $\mu$ m [12]). These studies also suggested that the pore size increases with increasing the acoustic pressure, insonation time and pulse repetition frequency. Moreover, it has been also shown that the cell viability is linearly decreases with the increasing pore size [9].

The objective of this study is to observe the modifications of cell membrane and organelles induced by sonoporation. Hence, we used electron microscopy to visualize whether pores form after sonoporation and to investigate their duration and size.

## II. MATERIAL AND METHODS

The morphological changes of cells were investigated through microscopic observations of the plasma membrane and intracellular organelles.

### Cell Culture

Human glioblastoma cells (U-87 MG) were seeded on 18 mm diameter glass cover slips, placed in 24 well-plates and containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% v/v fetal calf serum (FCS, Gibco-Invitrogen, Carlsbad, CA) and incubated at 37°C under an humidified atmosphere in 5% CO<sub>2</sub> incubator. Cells were cultured until 80% confluence before ultrasound exposure.

### Ultrasound Exposure

Ultrasound waves were transmitted at 1 MHz frequency using 20 cycles per pulse and for 10 s and 60 s at 1 W/cm<sup>2</sup>, (SoniGene<sup>™</sup> systems, VisualSonics). Ultrasound contrast agent, BR14<sup>®</sup> (Bracco Research, Switzerland) was added into the well at a microbubble/cell ratio of 5.

### Cell Permeabilization

SYTOX<sup>®</sup> Green, a small and non-permeant molecule was used, at a final concentration of 1  $\mu$ M, to monitor the membrane permeabilization, by flow cytometry. Just after sonoporation, the cell medium was removed and the cells were

washed with PBS and collected through centrifugation (4 min, 800 g). The cells were resuspended in 500  $\mu$ L. The cell mortality was assessed by flow cytometry after propidium iodide staining. Fluorescence histograms were recorded with a flow cytometer (Beckman-Coulter, Fullerton, CA) and analyzed using the Kaluza software supplied by the manufacturer. A minimum of 10 000 events was analyzed to generate each histogram. The gate was arbitrarily set for the detection of red and green fluorescence. The ultrasound conditions described earlier were reproduced to measure the rate of SYTOX<sup>®</sup> Green uptake in the cells.

#### Scanning and Transmission Electron Microscopy (SEM/TEM)

The ultrastructural modifications of the cells, after sonoporation, were monitored by SEM and TEM. For SEM experiments, the cells were insonified and attached by immersion in 2% paraformaldehyde and 1% glutaraldehyde based fixative at room temperature. Cells were fixed at different time points post-sonoporation, either immediately (0 min) or 15 min post-sonoporation. Regarding TEM experiments, the cells were treated as above, and then ultrathin sections were obtained with a Leica Ultracut UCT ultramicrotome, and observed at 100 kV with a Jeol 1011 transmission electron microscope (Tokyo, Japan).

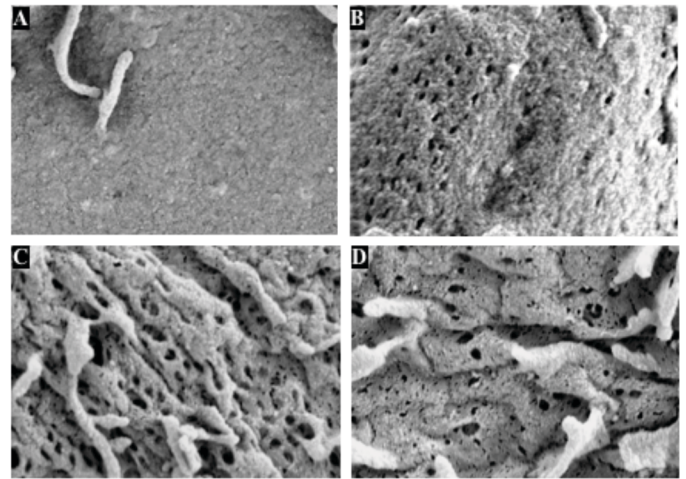
The pore-like structures were counted manually using the same set of images for each condition (control, 0 min, and 15 min post-sonoporation). Ten cells per condition were included in the analysis (n=10).

### III. RESULTS AND DISCUSSION

#### A. Cells membrane permeabilization

The SYTOX<sup>®</sup> Green incorporation is an indicator of membrane permeability. Flow cytometry analysis showed that at the selected US conditions (1 MHz, 1 W/cm<sup>2</sup>, 20% duty cycle, 60 s) and in combination with BR14<sup>®</sup> microbubbles, the cell membrane permeabilization rate reached 60%, against 16% for 10s exposure, while cell mortality did not exceed 1.11%, for all conditions.

The changes in cell membrane and intracellular organelles were investigated after ultrasound exposure, using scanning and transmission electron microscopy. Figure 1 shows SEM images of U-87 MG cells before insonation (control), either immediately after insonation (0 min) with ultrasound alone and in presence of BR14<sup>®</sup> microbubbles or 15 min post-sonoporation in presence of the microbubbles. The images indicate that immediately after sonoporation, pore-like



**Figure 1:** SEM photomicrographs showing the morphology of the U-87 MG plasma membrane, at 50 000 times magnification for four conditions: (A) control cell; (B) ultrasound alone, ultrasound + BR14<sup>®</sup> microbubbles either immediately (C) or 15 min post-sonoporation (D).

structures appear on the cell membrane. Note also that the number of these structures increases in the presence of BR14<sup>®</sup> microbubbles.

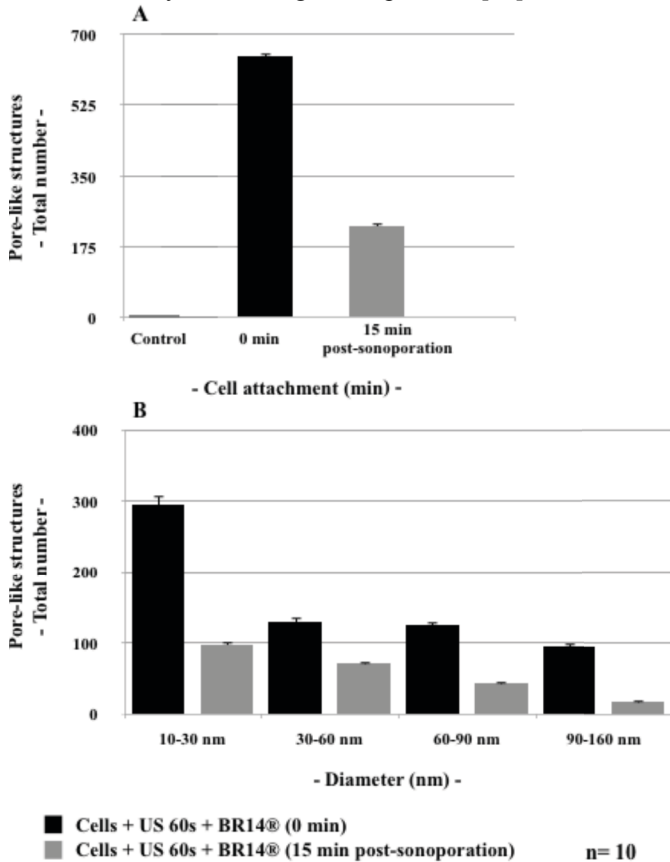
The size distribution of the pore-like structures is rather heterogeneous and varies from tens of nanometers up to 160 nm. Since caveolae represent 10% of the membrane surface and that their size ranges from 50 to 100 nm, it might be suggested that a portion of these structures represent caveolae that are still open, while the other part represents a pore-like structures or membrane invaginations. Clathrin-mediated endocytosis could be mentioned but the latter is more fleeting than caveolae. The cells that were fixed and observed 15 min after sonoporation, showed a significant decrease in the number of pore-like structures (Fig. 2). This suggests that the cells are still active and the pores might close up. In addition, it was observed that small pore-like structures, in the range of (10-50 nm), remain open and take more time to reseal, while larger ones (70-160 nm) disappear rapidly, which lead to suppose that cells mend large membrane disruption first, thus a gradual resorption of the pore-like diameter.

#### B. Transmission Electron Microscopy

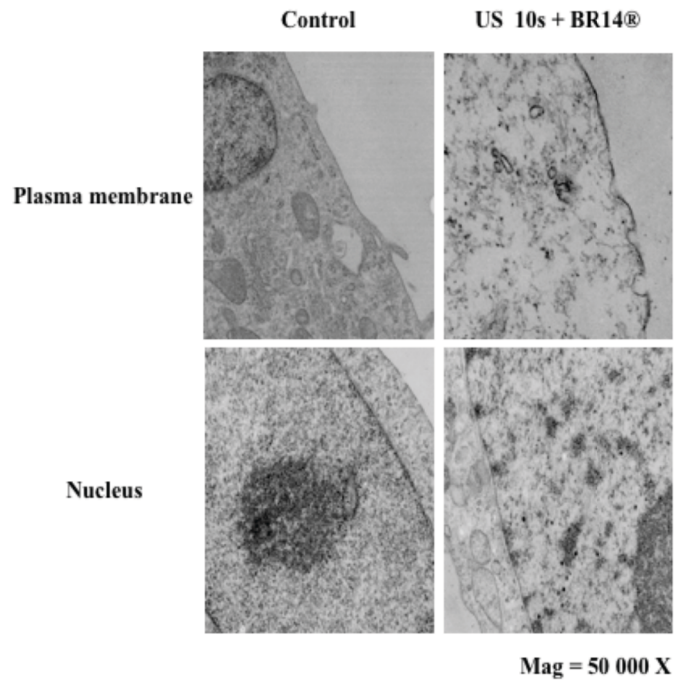
To complete this SEM study, we investigated the sonoporation effect at the intracellular level by investigating the intracellular organelles. The TEM micrographs given in Fig. 3 show a chromatin condensation after sonoporation, in the presence and also in absence of microbubbles. The cytoplasm along with mitochondrial matrix appears lighter, compared to control cells, suggesting a loss of cytoplasm viscosity, which would facilitate the intracellular traffic of delivered molecules. The cytoplasmic viscosity is regulated by proteins including actin-binding, indicating that the cell cytoskeleton should be altered after ultrasound exposure. As the mitochondrial matrix appears lighter, and since mitochondria is the energy currency of the cells, it might be suggested a disruption of energetic metabolism, or a modulation of intracellular oxidative stress, increasing cell membrane permeability [13]. Untreated cells showed

continuous plasma membrane morphology, while treated cells exhibit membrane invaginations which are more pronounced when the cells are insonified in the presence of microbubbles. Clathrin and caveolin dependent endocytosis were observed in the presence of microbubbles.

Moreover, cells insonified in the presence of microbubbles exhibited dark spots in the nucleus due probably to a “chromatin condensation”. It is well known that the condensed form of chromatin makes it unattainable to transcription factors, suggesting transcriptional inhibition, thus the cell division activity as well as genes expression [14].



**Figure 2:** Histograms of pore-like structures counting based on the analysis of 10 cells per condition. **A.** Total number of pore-like structures (control cells, insonified cells fixed immediately or 15 min after sonoporation). **B.** Total number of pore-like structures versus the diameter.



**Figure 3:** Transmission electron microscopy observations of U-87MG cells at control and after exposure to ultrasound and microbubbles.

#### IV. CONCLUSIONS

The electron microscopy observations reveal that sonoporation effects are not limited to the cell membrane level but are also seen at the intracellular level. Sonoporation induces likely the formation of pore-like structures on the cell plasma membrane with a size ranging from 10-160 nm. These membrane alterations are transient, since 15 minutes post-sonoporation, 65% of the pore-like structures disappeared. At the intracellular level, the images indicate the appearance of caveolae dependent pathways and likely a change in the viscosity of the cytoplasm.

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