Supplementary

Systematic Analysis of Ultrasound Parameters for Gene Delivery Efficiency and Cell Viability in 4T1 Cells

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Table of Contents

Method S1. Mechanical index calculation.
Figure S1. Effects of baseline control groups on luciferase activity and cell viability.
Table S1. Acoustic pressure and mechanical index (MI) at varying ultrasound intensities with a well plate.
Table S2. Total acoustic energy calculations according to changes in acoustic intensity.
Table S3. Total acoustic energy calculations according to changes in duty cycle.
Table S4. Total acoustic energy calculations according to changes in exposure time.

Method S1. Mechanical index calculation.

To define appropriate ultrasound exposure conditions for subsequent gene delivery experiments, the mechanical index (MI) was calculated using the measured peak negative pressure. Acoustic pressure was measured with a hydrophone in the presence of the well plate, and the peak negative pressure was used to calculate MI according to the following equation:

$$MI = \frac{P}{\sqrt{f}}$$

Where P is the peak negative pressure (MPa) measured by the hydrophone and f is the center frequency (MHz) of the transducer. By measuring acoustic pressure at different intensity settings, the corresponding MI value was determined for each condition. The ultrasound intensity range used in this study was then selected based on the calculated MI values to establish exposure conditions suitable for MB-assisted gene delivery while minimizing excessive acoustic exposure.

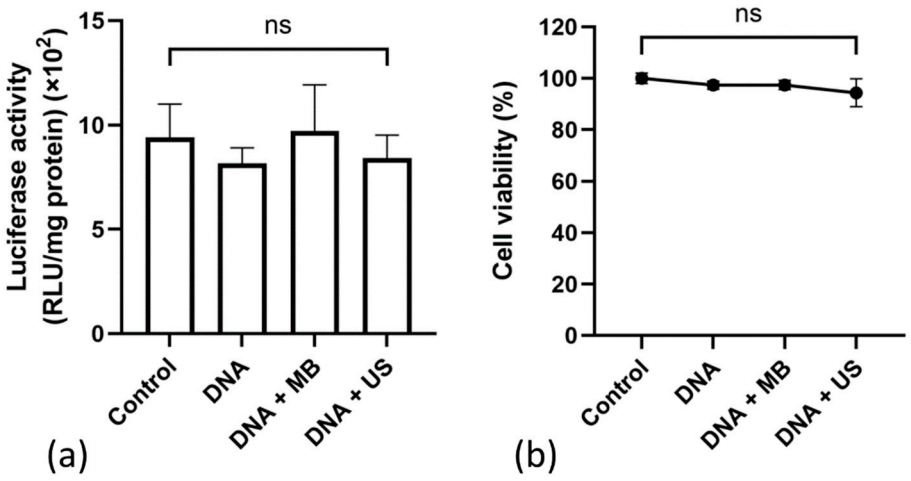


Figure S1. Effects of baseline control groups on luciferase activity and cell viability.

(a) Luciferase activity and (b) cell viability were evaluated in 4T1 cells under baseline control groups, including untreated control, DNA alone, DNA + MB, and DNA + US groups. The DNA + US group was exposed to ultrasound at 1 W/cm² intensity, 100 Hz PRF, 20% duty cycle, and 60 s exposure time. No significant differences were observed among the groups, indicating that DNA, MBs, or ultrasound alone did not substantially affect luciferase activity or cell viability under the tested conditions. Data are presented as mean ± SD (n = 4). ns, not significant.

Table S1. Acoustic pressure and mechanical index (MI) at varying ultrasound intensities with a well plate.

Intensity, Spatial average temporal peak (I_{SATP}, W/cm²)	0.2	0.5	1	1.5	2	2.5	3
Peak negative pressure (MPa)	0.125	0.205	0.293	0.355	0.412	0.470	0.504
Mechanical index	0.125	0.205	0.293	0.355	0.412	0.470	0.504

Table S2. Total acoustic energy calculations according to changes in acoustic intensity. Total acoustic energy was calculated by varying acoustic intensity while keeping all other acoustic conditions constant. Duty cycle was converted from percentage to fractional form (20% = 0.2) for energy calculations, and the resulting total acoustic energy values were expressed in J/cm².

Parameter	0.5 W/cm²	1 W/cm²	1.5 W/cm²
Intensity (W/cm²)	**0.5**	**1**	**1.5**
PRF (Hz)	100	100	100
Duty cycle	0.2	0.2	0.2
Exposure time (sec)	60	60	60
Total acoustic energy (J/cm²)	6	12	18

Table S3. Total acoustic energy calculations according to changes in duty cycle. Total acoustic energy was calculated by varying duty cycle while keeping all other acoustic conditions constant. Duty cycle was converted from percentage to fractional form (20% = 0.2) for energy calculations, and the resulting total acoustic energy values were expressed in J/cm².

Parameter	10 %	20 % (Duty cycle)	50 %
Intensity (W/cm²)	1	1	1
PRF (Hz)	100	100	100
Duty cycle	**0.1**	**0.2**	**0.5**
Exposure time (sec)	60	60	60
Total acoustic energy (J/cm²)	6	12	30

Table S4. Total acoustic energy calculations according to changes in exposure time. Total acoustic energy was calculated by varying exposure time while keeping all other acoustic conditions constant. Duty cycle was converted from percentage to fractional form (20% = 0.2) for energy calculations, and the resulting total acoustic energy values were expressed in J/cm².

Parameter	30 sec (Exposure time)	60 sec	90 sec
Intensity (W/cm²)	1	1	1
PRF (Hz)	100	100	100
Duty cycle	0.2	0.2	0.2
Exposure time (sec)	**30**	**60**	**90**
Total acoustic energy (J/cm²)	6	12	18