# Ultrasonic Cell Therapy via Nanocarrier-Based Nucleus Delivery of Molecular Sonosensitizer

Santanu Shaw, Abu Raihan Sarkar, Ankan Kumar Sarkar, Nayana Mukherjee and Nikhil R. Jana*

School of Materials Science, Indian Association for the Cultivation of Science,

2A & 2B Raja S. C. Mullick Road, Kolkata-700032, India

*Address for correspondence to camnrj@iacs.res.in

## Experimental section

**Materials.** L-aspartic acid, oleylamine, ethylene diamine, chlorocholine chloride, N-hydroxysuccinimide (NHS), sodium azide, deoxy-2-glucose, ivermectin, Dulbecco's modified Eagle medium (DMEM), hoechst 33342, phosphoric acid (88%), methanol, diethyl ether, dimethyl sulfoxide (DMSO), 1,3-diphenylisobenzofuran (DPBF), 2,2,6,6-tetramethylpiperidine (TEMP), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), bovine serum albumin, protoporphyrin IX disodium salt, N-hydroxysuccinimide (NHS), β-actin monoclonal antibody were purchased from Sigma-Aldrich. Para guanidino benzoic acid hydrochloride, and N,N'-dicyclohexylcarbodiimide (DCC) were purchased from TCI chemicals. Fetal bovine serum (FBS), 3-(4,5-dimethylthiazol)-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Hi-media, Chlorin e6 was purchased from Cayman Chemical, rhodamine isothiocyanate, FITC were purchased from Thermo Fischer Scientific. Active caspase-3 rabbit primary antibody and Nrf2 rabbit primary antibody were purchased from ABclonal. HRP tagged horse anti-mouse secondary antibody and HRP tagged goat anti-rabbit secondary antibody were purchased from Vector Laboratories.

**Instrumentation.** Fluorescence spectra were measured using multimode fluorescence microplate reader (Molecular Device, Spectramax M2$^e$) and Perkin Elmer LS55 luminescence spectrophotometer. Hydrodynamic size and zeta potentials were measured by Malvern Nano ZS instrument. EPR spectra were recorded on a MiniScope MS400 spectrometer (equipped with a temperature controller TC H03, Magnettech, Berlin, Germany). Quantitative cellular uptake was measured by BD Accuri C6 Flow Cytometer. In vitro cell imaging was performed by Olympus IX81 microscope with Cell Sense software. Nuclear magnetic resonance (NMR) spectra were recorded by Bruker DPX-400 using $D_2O$ as solvent. Molecular weight of the samples was measured by Quadruple TOF (Q-TOF) microMS system using electrospray ionization technique using Thermo scientific LTQ Orbitrap XL hybrid Fourier transform mass spectrometer. UV-Visible spectra were recorded by Shimadzu UV 2401PC spectrophotometer. Intelect mobile 2 ultrasound (Chattanooga, USA with frequency 1 and 3 MHz with power 0.5-3 W $cm^{-2}$) with planar transducer (35 mm diameter) was used as an ultrasound source.

**Synthesis of functionalized polymeric carrier.** We have synthesized polymeric carrier utilizing a multi-step process as described earlier.[26] At first, amino-benzyl guanidinium ligand was prepared by coupling reaction between DCC (494.4 mg, 2 mmol) and para-guanidino benzoic acid (430 mg, 2 mmol) and NHS (276 mg, 2 mmol). The reaction proceeded in DMF at 4 °C for 6 hours, followed by the addition of ethylene diamine (132 µL, 2 mmol) and triethyl amine (450 µL, 2 mmol) and subsequent precipitation of the product with acetone.

Separately, amino-chlorocholine ligand was synthesized by reacting chlorocholine chloride (79 mg, 0.5 mmol) with ethylene diamine (33 µL, 0.5 mmol) using potassium iodide catalyst. Product was then purified via precipitation.

Next, polysuccinimide was prepared by heating 6 g L-aspartic acid in 14 mL mesitylene solvent in presence of 330 µL phosphoric acid for 4-5 h. Product was precipitated, washed and dried. The resulting polysuccinimide polymer exhibited a molecular weight of approximately 26 kDa.

Finally, the functionalized polymeric carrier was synthesized by reacting 228 mg (2 mmol) polysuccinimide in DMSO with 165 μL (0.5 mmol) oleylamine, 4 mL (1 mmol) amino-benzyl guanidinium and 3 mL (0.5 mmol) amino-chlorocholine. The reactions were conducted in DMSO at 90 °C under an argon atmosphere, followed by precipitation using 1:1 methanol-diethyl ether for purification. This process is repeated three times and the final product was dried in a hot oven and collected.

**Preparation of sonosensitizer loaded nanocarrier.** A sonosensitizer solution (1 mg/mL) in DMSO was prepared. Subsequently, 10 μL of this sonosensitizer solution was mixed with 30 μL of a DMSO solution of polymer (50 mg/mL). The resulting solution was thoroughly mixed using a vortex shaker for 15 minutes. Finally, the entire solution was directly introduced into either a 1.0 mL HEPES buffer solution or cell culture media.

**Determination of singlet oxygen ($^1O_2$).** Briefly, 3 mL of the sonosensitizer-loaded polymer nanomicelle was mixed with 38 μL of DPBF solution (8 mM DPBF) that was prepared in DMSO solvent. This solution was kept in the dark and exposed to ultrasound irradiation (1.0 MHz, 0.5 W $cm^{-2}$, 50 % duty cycle) for various durations. UV−visible absorption spectra were recorded at 5 minute intervals during ultrasound exposure to monitor the generation of singlet oxygen. For the control experiment, a polymer nanomicelle without the sonosensitizer was mixed with DPBF and subjected to the same ultrasound treatment.

Singlet oxygen generation was further verified using an electron spin resonance (ESR) spectrometer with TEMP as the singlet oxygen trapping agent. In this procedure, 400 μL of the sonosensitizer-loaded polymer nanomicelle was combined with 100 μL of 20 mM TEMP in a vial, followed by 5 minutes of ultrasound irradiation. The mixture was then immediately analyzed with the ESR spectrometer.

**Cellular uptake study**. KB cells were cultured in DMEM media supplemented with high glucose, 10 % fetal bovine serum (FBS), and 1 % penicillin-streptomycin antibiotic, maintaining a humidified atmosphere with 5 % $CO_2$ at 37 °C. Initially, a 40 µL aliquot of sonosensitizer-loaded nanocarrier was introduced into one mL of HEPES buffer, followed by a 5-minute incubation period. Subsequently, cells underwent thorough washing with HEPES buffer and were then subjected to incubation with fresh media for 1-2 hours. Following this, a further round of washing with HEPES buffer was carried out, after which the cells were fixed using a 4 % paraformaldehyde solution before being subjected to imaging under a fluorescence microscope.

To investigate cellular uptake mechanisms, cells were pre-incubated individually with various endocytosis inhibitors/conditions for 45 minutes. These inhibitors and their respective final concentrations were sodium azide ($NaN_3$) (10 mM), deoxy-2-glucose (DOG) (50 mM), ivermectin (25 µM), sucrose (450 nM), amiloride (50 µM), and MβCD (10 mM). Following this pre-incubation step, the sonosensitizer-loaded nanocarrier or FITC-tagged nanocarrier was added to the cell culture media and incubated for 5 minutes before proceeding for imaging.

For co-localization studies, KB cells were seeded onto a 4-well chambered slide plate containing DMEM media supplemented with 10 % FBS and 1 % penicillin-streptomycin antibiotic. The sonosensitizer-loaded nanocarrier was introduced into the cell in HEPES buffer, incubated for 5 minutes and then subjected to washing and addition of fresh media. Subsequently, 10 µL of hoechst solution (221 µM) was added and incubated for 10 min before imaging under a fluorescence microscope.

**Intracellular ROS analysis**. KB cells were cultured in a 35 mm confocal disc for 24 hours at 37 °C. Subsequently, cells were washed with HEPES buffer of pH 7.4 and fresh DMEM was replenished. Following this, sonosensitizer-loaded nanocarrier was introduced and incubated for 5 min then the cells were washed again with HEPES buffer and further incubated for 30

minutes after which they underwent ultrasound exposure for 3 min (1 MHz, 0.5 W $cm^{-2}$, 50% duty cycle). Ultrasound exposure was facilitated using a degassed water column. The cells were then further incubated 30 μM DCFH-DA, with an incubation period of 20 min. Afterward, cells were washed with HEPES buffer of pH 7.4, and fresh DMEM was added, followed by imaging under a confocal laser scanning microscope.

For the quantitative analysis of intracellular ROS, cells were detached from the plate through trypsinization, centrifuged to remove the media, and then redispersed in HEPES buffer for the subsequent flow cytometry study.

**Cytotoxicity study.** KB cells were treated with either sonosensitizer-loaded nanocarriers or free drugs and incubated for 5 minutes. After washing with buffer and adding cell culture media, cells were either exposed to ultrasound or left untreated. Following a 24-hour incubation, MTT solution was added. The formation of violet formazan crystals was subsequently observed. After removing the media, the formazan crystals were dissolved using an SDS solution (prepared in a 1:1 mixture of water and DMF). Finally, absorbance readings were taken at 570 nm using a microplate reader. The well without ultrasound was taken as a control with 100 % cell viability.

**Cell apoptosis assay.** KB cells were treated with either sonosensitizer-loaded nanocarriers or free drug and incubated for 5 minutes. Following this initial incubation, some cells were exposed to ultrasound while others were not. All cells were then incubated for an additional 24 hours. Subsequently, cells were washed with HEPES buffer and detached using trypsin, then resuspended in binding buffer with the addition of 1:1 mixture of Annexin V-FITC and PI was added to the cells and incubated for 10 minutes before analysis via flow cytometry to evaluate cell apoptosis.

**Preparation of 3D tumour spheroid model.** To initiate the formation of spheroids using U87 MG glioblastoma cells, first cells were cultured in a T-25 flask containing complete nutrient mixture F-12 Ham medium until they reach approximately 70–80% confluency. Cells were gently washed with HEPES 7.4 buffer and detached them using trypsin-EDTA. Next, trypsin was neutralized by adding complete Ham medium and cell suspension was transferred to a centrifuge tube, and centrifuged at 300 × g for 5 minutes. Supernatant was discarded and the cell pellet was suspended in 1.0 mL of complete Ham medium. About 900-1000 cells were taken into 150 µL of medium in each well of an ultra-low attachment at the U-bottom 96-well plate (Nunclon™ Sphera™, Japan). Next, plate was centrifuged using a Hiper Mini Plate centrifuge (HiMedia) at 2200 rpm for 4 minutes to facilitate initial cell aggregation at the bottom of each well. Next, plate was incubated at 37 °C in a humidified atmosphere with 5% $CO_2$ for 3-7 days, ensuring the plate remains undisturbed to allow the formation of compact spheroids. After 3 days, 50 µL of medium from each well was removed carefully and replaced with 50 µL of fresh medium. This media replacement was continued at every 2–3 days, to support optimal spheroid growth and viability.

**Sonosensitizer uptake and therapeutic study in 3D tumour spheroid model.** Spheroids were initially treated with PPIX/Ce6 loaded nanocarrier (or control nanocarriers). After 2 hrs of incubation, the spheroids were gently washed, and fresh cell culture medium was added. Following overnight incubation, the spheroids were fixed with 4% paraformaldehyde (PFA), and confocal microscopy was performed to evaluate sonosensitizer uptake.

For the therapeutic study, sonosensitizer labelled spheroids were exposed to ultrasound for 5 minutes (1 W/cm², 50% duty cycle, 1 MHz). Next, bright-field images were captured daily from day 1 to day 5 to monitor changes in spheroid size. Spheroid volume ($V = 4/3\pi r^3$, where $r$ represents the spheroid radius) was calculated using ImageJ software and compared across experimental groups to assess therapeutic efficacy.

**Ultrasound irradiation.** Ultrasound treatment was performed using a Chattanooga therapeutic ultrasound system (DJO Global) operating at a frequency of 1 MHz. Ultrasound was applied at an intensity of 0.5-1 W $cm^{-2}$ with a 50% duty cycle for a total exposure time of 3 min per well or spheroid. A planar transducer was positioned externally beneath the culture plate and acoustically coupled to the plate using commercial ultrasound gel. The transducer was not immersed in the culture medium, ensuring sterile conditions and uniform acoustic exposure. Standard cell culture media were used without additional degassing.

**Nuclear/cytoplasmic fractionation and quantification of sonosensitizer.** KB cells were treated with sonosensitizer-loaded nanocarrier, sonosensitizer-loaded control nanocarrier. For nanocarrier-treated groups, cells were incubated with the formulations for 15 min, washed twice with ice-cold PBS, and further incubated in fresh complete medium for 2 h prior to fractionation. Cells were then collected by gentle scraping and pelleted by centrifugation. The cell pellets were resuspended in hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, pH 7.9) and incubated on ice for 15 min, followed by gentle pipetting to disrupt the plasma membrane. The lysate was centrifuged at 1,000 × g for 5 min at 4 °C to obtain the cytoplasmic fraction (supernatant). The nuclear pellet was washed once with hypotonic buffer and extracted using nuclear extraction buffer (20 mM HEPES, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, pH 7.9) with intermittent vortexing for 30 min at 4 °C, followed by centrifugation at 14,000 × g for 10 min. Fluorescence intensities of the sonosensitizer in nuclear and cytoplasmic fractions were directly measured using a microplate reader at the characteristic excitation wavelengths, and relative nuclear and cytosolic distributions were calculated from normalized fluorescence intensities and reported as percentage distribution.

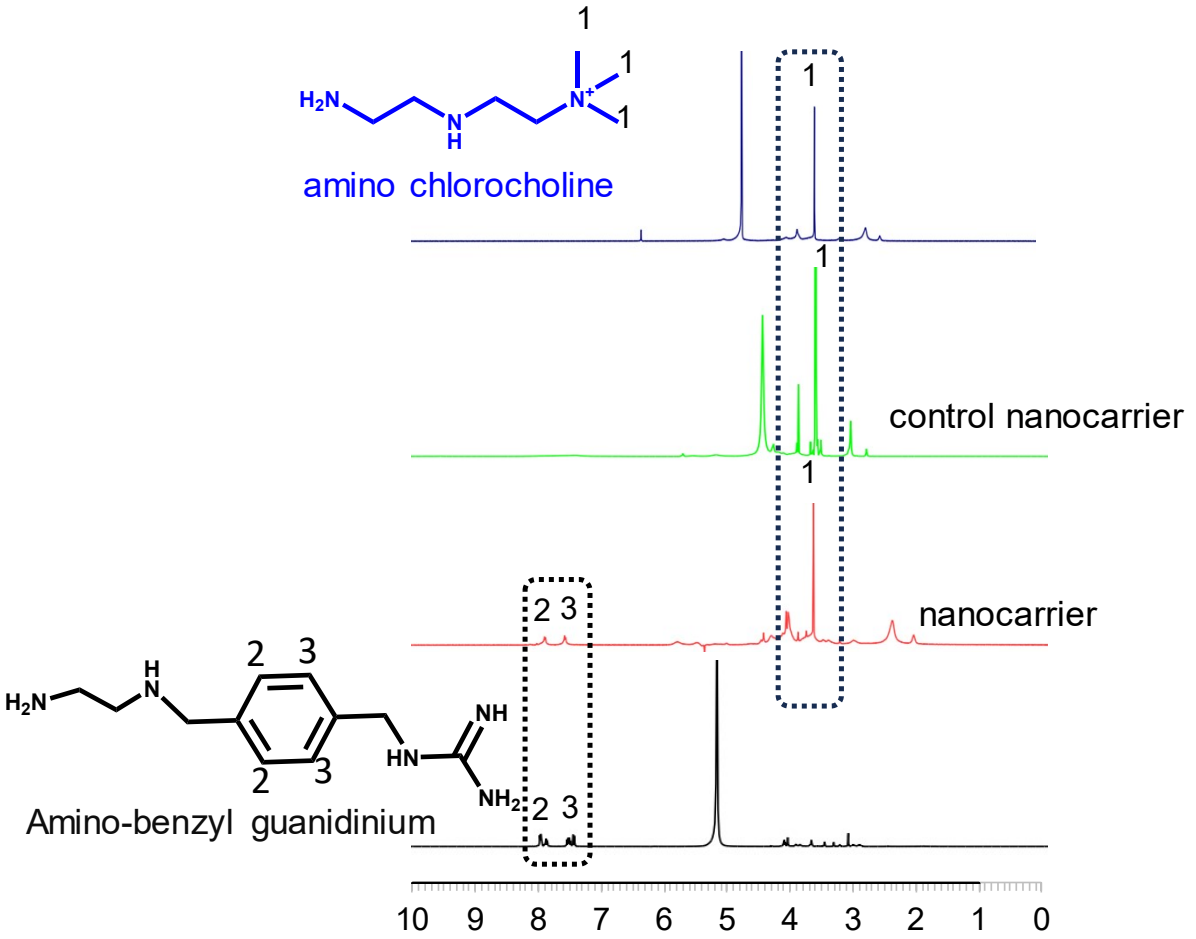


**Figure S1.** The $^{1}$H NMR spectra of the functionalized polymer and the control polymer taken in DMSO-$d_6$ solvent. The $^{1}$H NMR spectra of amino-chlorocholine and amino-benzyl guanidinium ligands was also shown for comparison. Results reveal characteristic signals of the ligands present in the polymer backbone.

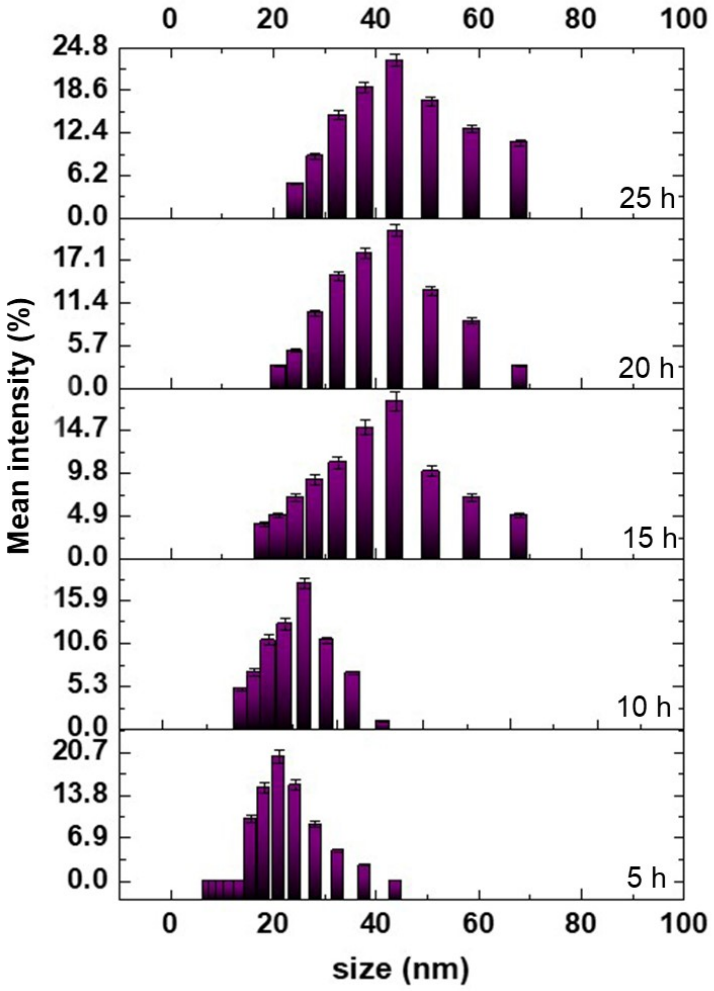


**Figure S2.** DLS-based size distribution of PPIX-loaded nanocarriers measured at 5 h intervals at physiological pH (7.4) for up to 25 h, indicating a minor increase in hydrodynamic size over time. Data represent n = 3 independent experiments.

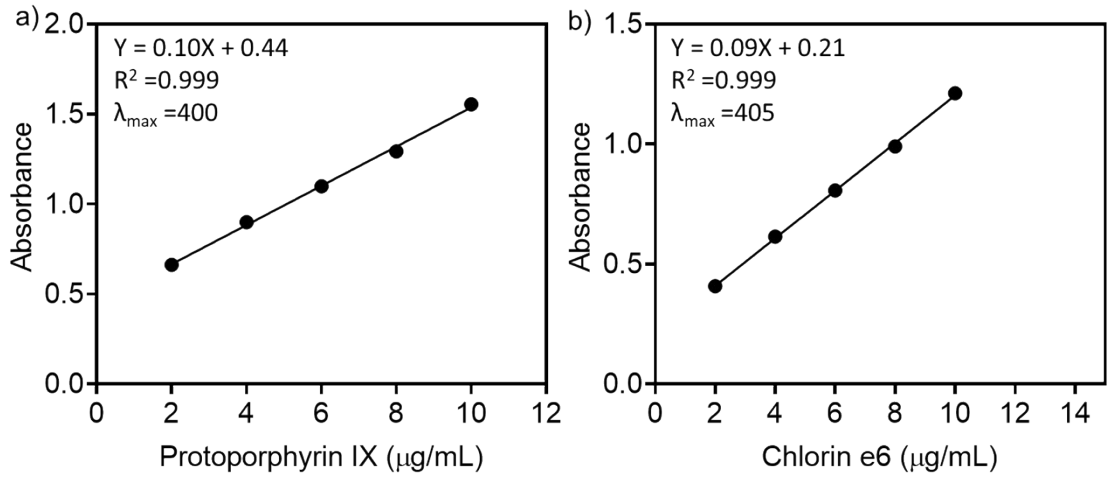


**Figure S3.** Calibration curves of protoporphyrin IX and chlorin e6, using their absorbance in DMSO at 400 nm and 405 nm, respectively. Here Y represents absorbance and X denotes concentration (μg/mL).

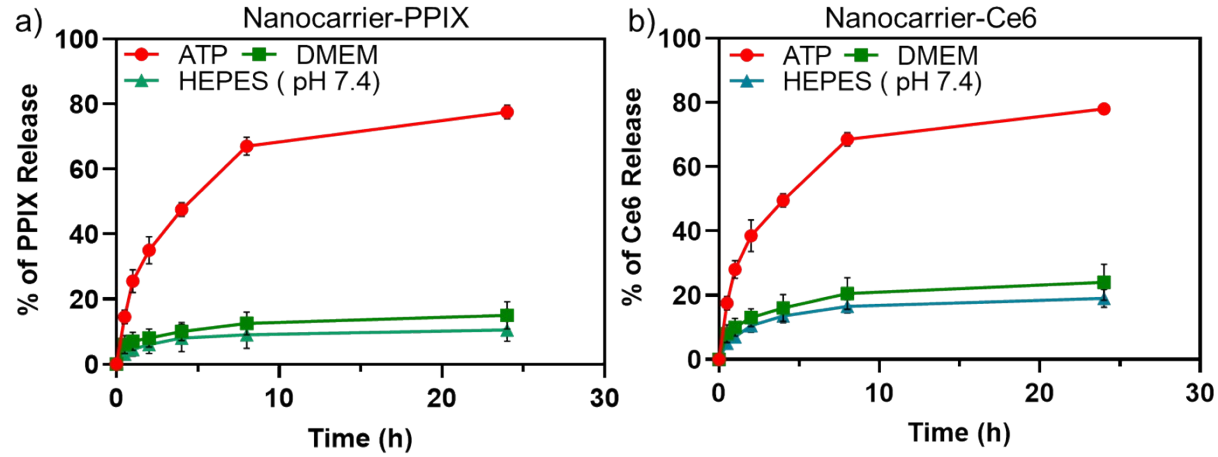


**Figure S4.** In vitro drug release profiles of drug-loaded nanocarriers in HEPES buffer (pH 7.4), DMEM, and ATP-containing media, demonstrating extracellular stability and enhanced competitive release under intracellular-mimicking conditions.

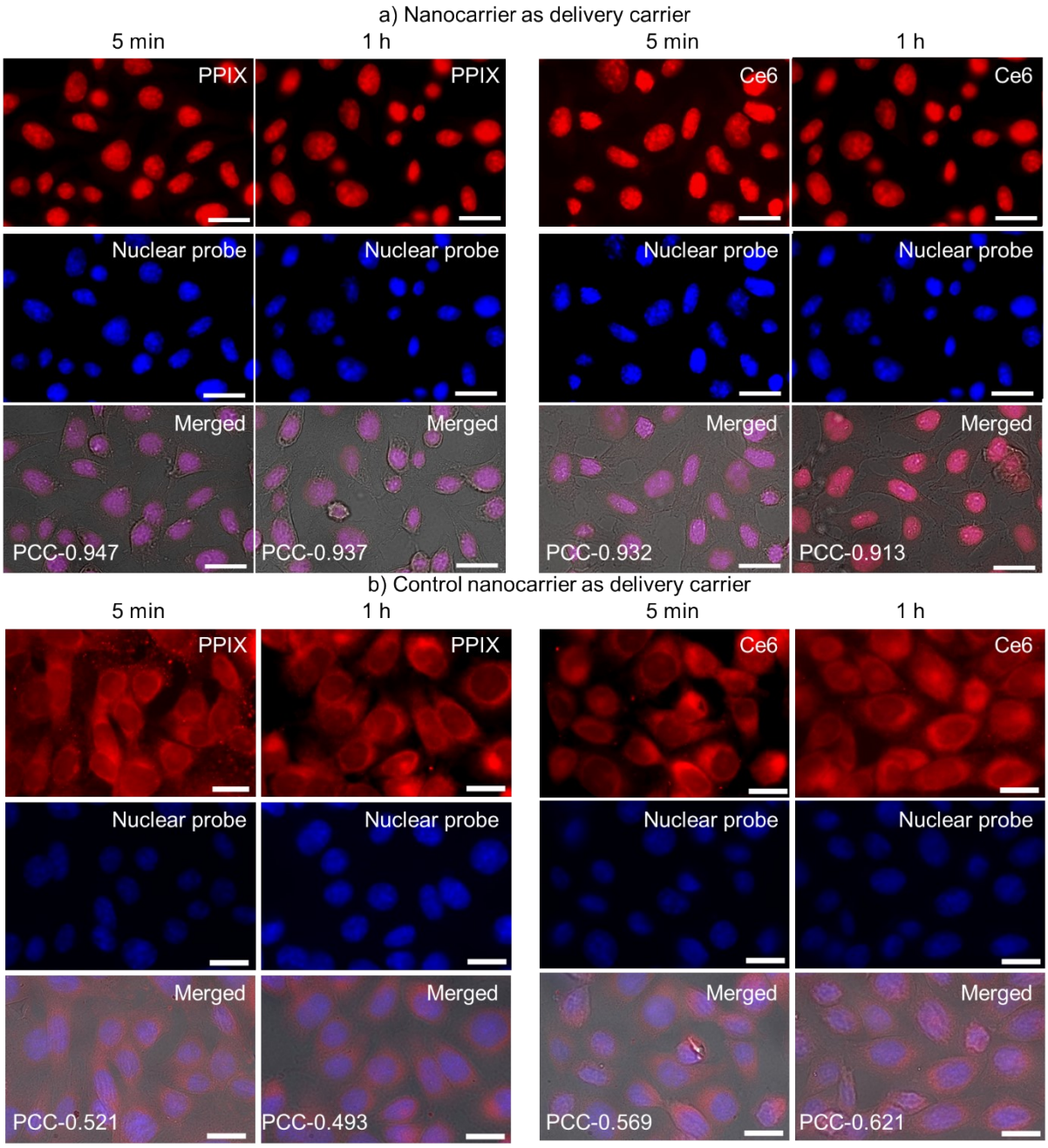


**Figure S5.** Evidence of nuclear delivery of PPIX and Ce6 using nanocarrier (a) compared with cytosolic delivery using a control nanocarrier (b). KB cells were incubated with sonosensitizer-loaded nanocarrier and control nanocarriers in HEPES buffer for 5 minutes, following washed with HEPES buffer, and then fresh media was added. Next, cells were incubated with the nuclear probe and imaging was performed after 5 min and 1 hour under a fluorescence microscopy. Pearson correlation coefficient (PCC) for co-localization was between 0.91 to 0.95 for nanocarrier, as compared to control nanocarrier with PCC in between 0.49 to 0.6. Scale bar represents 20 μm.

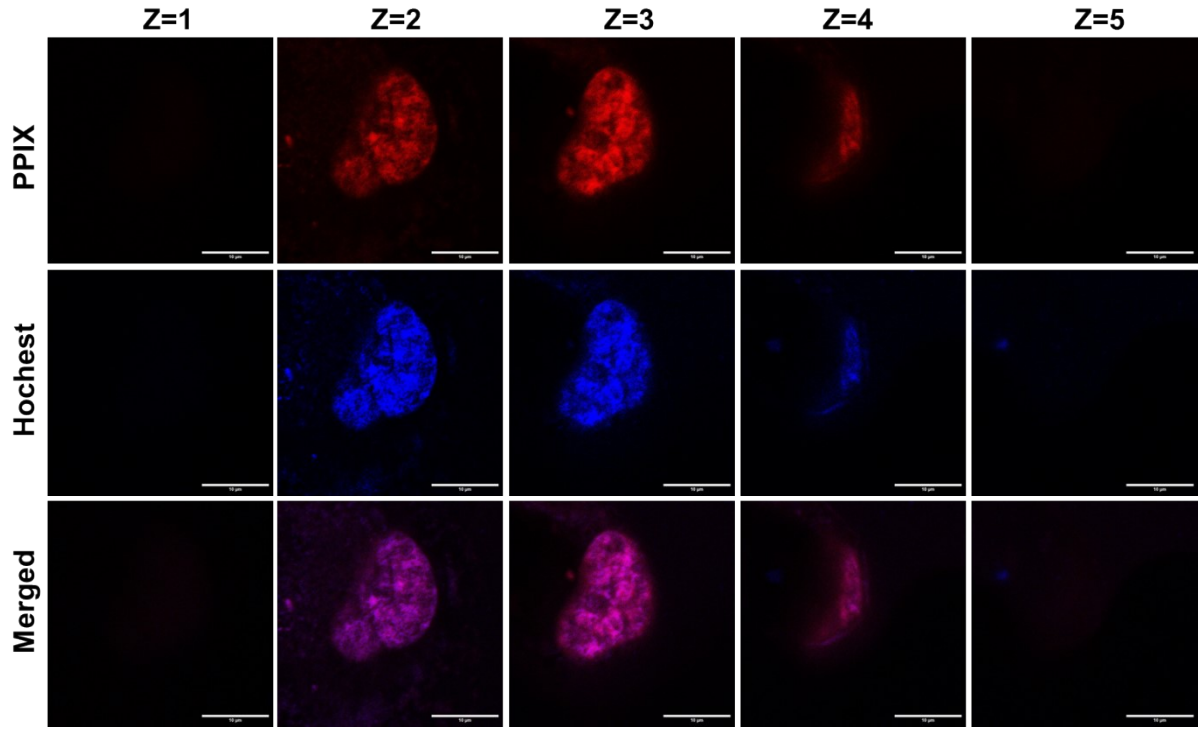


**Figure S6.** Z-stack confocal images of PPIX-loaded nanocarrier after ultrasound exposure, showing the nuclear accumulation of the sonosensitizer. Merged images at different Z planes clearly reveal red fluorescence of PPIX colocalized with the nucleus dye hoechst. Red excitation was used for detecting PPIX fluorescence, and UV excitation for nuclear staining. Scale bar: 10 µm.

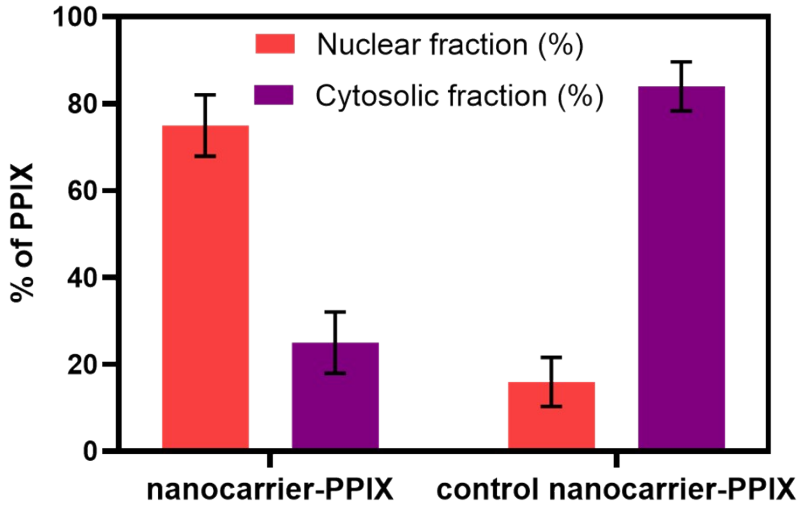


**Figure S7.** Quantitative nuclear and cytoplasmic distribution of the sonosensitizer in KB cells after nuclear/cytoplasmic fractionation. Cells were treated with active or control nanocarriers for 15 min, washed, and incubated for 2 h before analysis. Fluorescence quantification shows

enhanced nuclear accumulation for the nanocarrier and predominant cytosolic localization for the control nanocarrier.

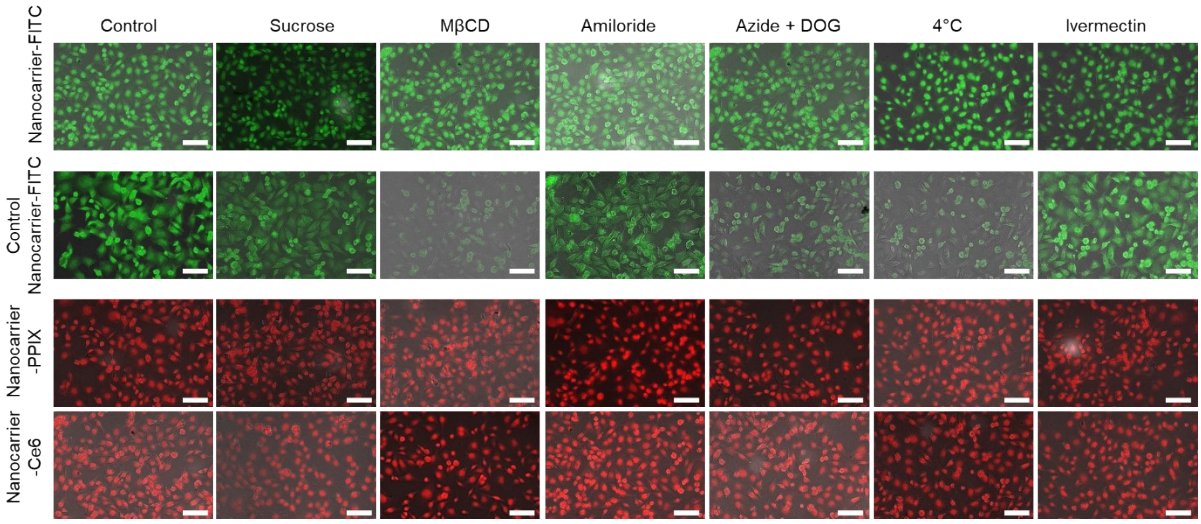


**Figure S8**. Evidence of direct membrane translocation and non-endocytosis-based delivery of sonosensitizers to the cell nucleus. Cells are initially incubated with endocytosis inhibitors or exposed to specific conditions (such as 4 °C or in the presence of azide-DOG) for 45 minutes. Subsequently, the cells are treated with the FITC-tagged nanocarriers or sonosensitizer-loaded nanocarrier for 5 minutes. After this incubation, the cells are washed and then incubated in fresh media for an additional 2 hours. The cells are then imaged under blue excitation for FITC -tagged nanocarriers and green excitation to detect sonosensitizer. The scale bar represents 20 μm.

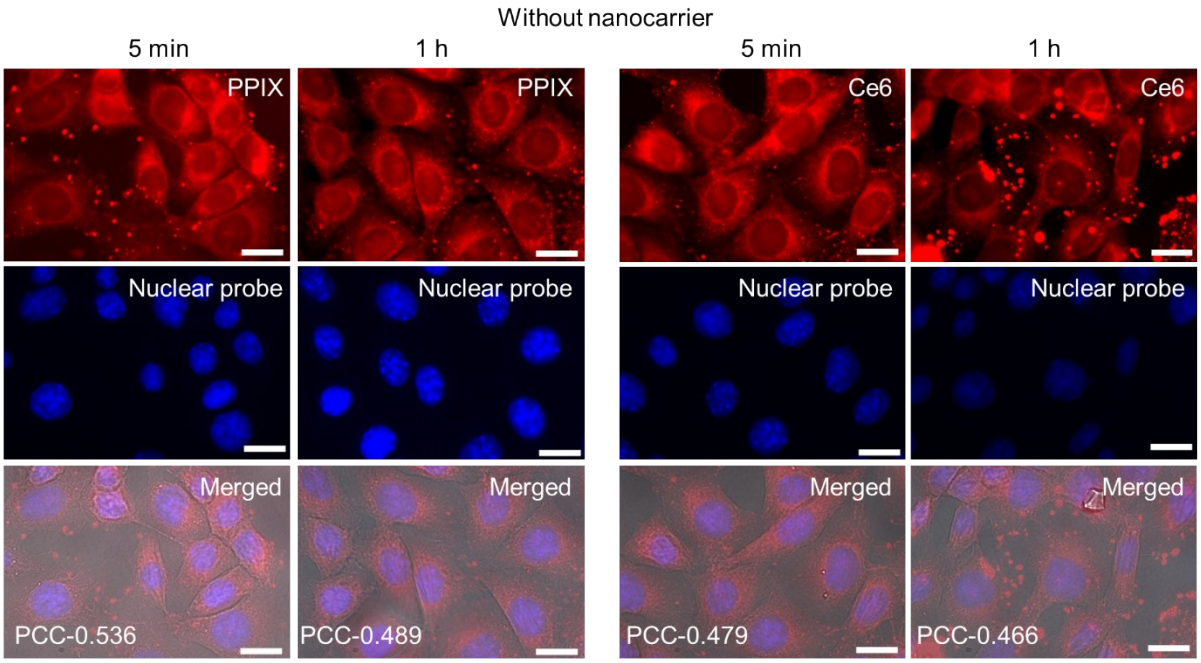


**Figure S9.** Evidence of cytosolic delivery of PPIX and Ce6 without using any carrier. KB cells were incubated with PPIX and Ce6 in HEPES buffer for 5 minutes, washed with HEPES buffer, and then fresh media was added. Next, cells were incubated with nuclear probe and imaging was performed after 5 min and 1 hour under a fluorescence microscope. Pearson correlation coefficient (PCC) for co-localization was between 0.49 to 0.54 indicating poor colocalization. Scale bar represents 20 µm.

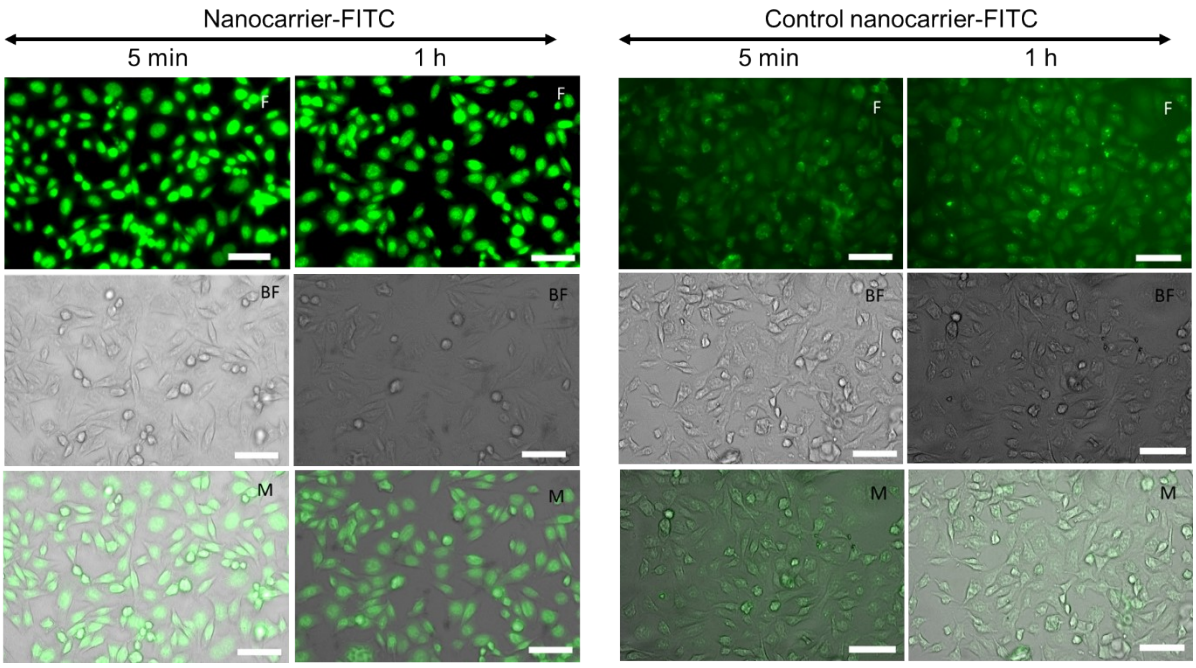


**Figure S10**. Evidence of nuclear localization of nanocarrier as compared with control nanocarrier. KB cells were incubated with FITC-tagged nanocarriers in HEPES buffer for 5 minutes, washed with HEPES buffer, and then fresh media was added. Imaging was performed after 5 min/1 hour under a fluorescence and bright field mode and then merged. Scale bar represents 50 µm.

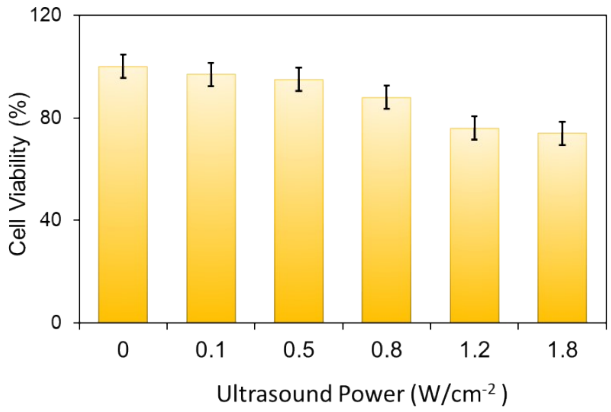


**Figure S11.** Power-dependent cell viability analysis is used to optimize ultrasound parameters. Cells were exposed to varying ultrasound intensities in the absence of nanocarriers, and conditions showing minimal cytotoxicity were selected for subsequent experiments.

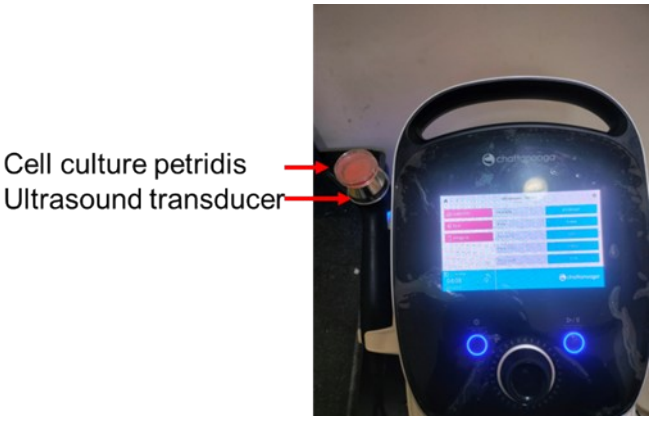


**Figure S12.** Digital photograph of the ultrasound exposure setup showing ultrasound transducer exposed on the bottom of the cell culture petridish coupling with acoustically active ultrasound gel.

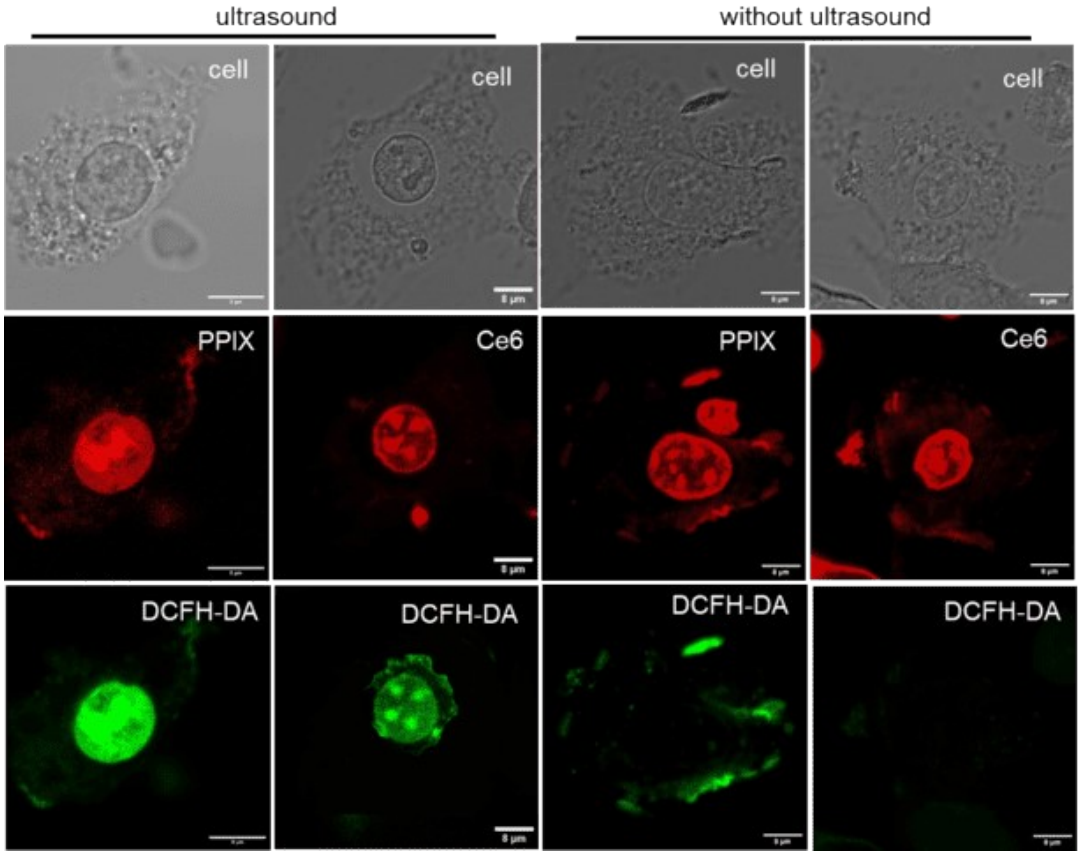


**Figure S13**. Confocal microscopic images of nanocarrier-loaded sonosensitizer before and after ultrasound exposure, along with DCFH-DA treatment for ROS generation analysis. The sonosensitizer-loaded nanocarrier was incubated with cells for 5 minutes, followed by washing

and the addition of fresh culture media. After an additional 30-minute incubation, ultrasound treatment was applied to the nanocarrier-loaded sonosensitizer. Both ultrasound-treated and untreated samples were then treated with DCFH-DA, and images were captured after 20 minutes of incubation. The green emission of DCFH-DA is collected between 505 to 535 nm by exciting at 488 nm. Additionally, red emission of sonosensitizer is captured by exciting at 561 nm and collecting emission above 570 nm. Scale bar represents 8 μm.

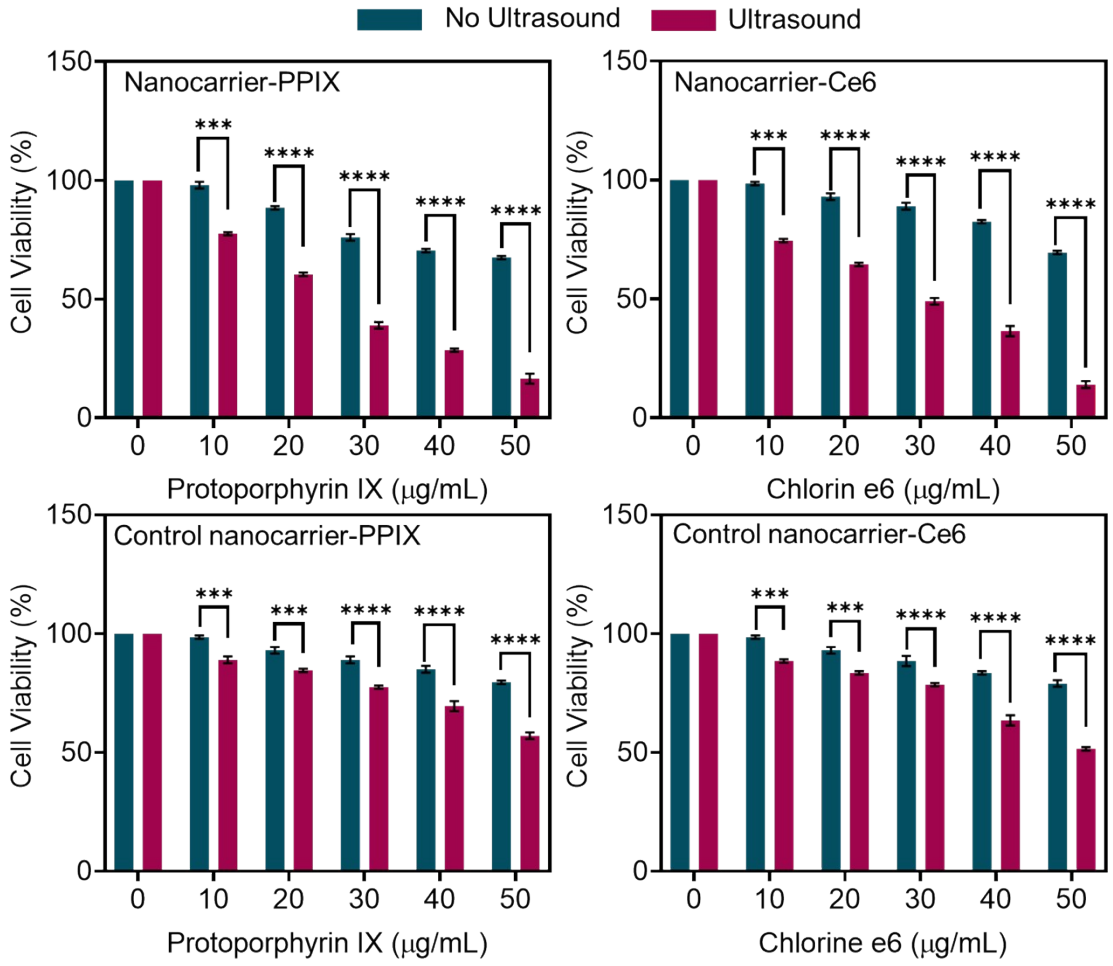


**Figure S14.** Evidence of high cytotoxicity of sonosensitizer via nanocarrier-based nuclear delivery as compared with cytosolic delivery using control nanocarrier. KB cells were incubated with sonosensitizer loaded nanocarriers for 5 min and then treated with ultrasound and kept it for 24 h following cell viability assessed using MTT assay. Error bars represent mean ± S.D for 3 experiments and asterisks indicate statistically significant differences (*P < 0.01, **P < 0.001, ***P < 0.0001, and ****P < 0.00001) determined using Sidak's multiple comparison test.

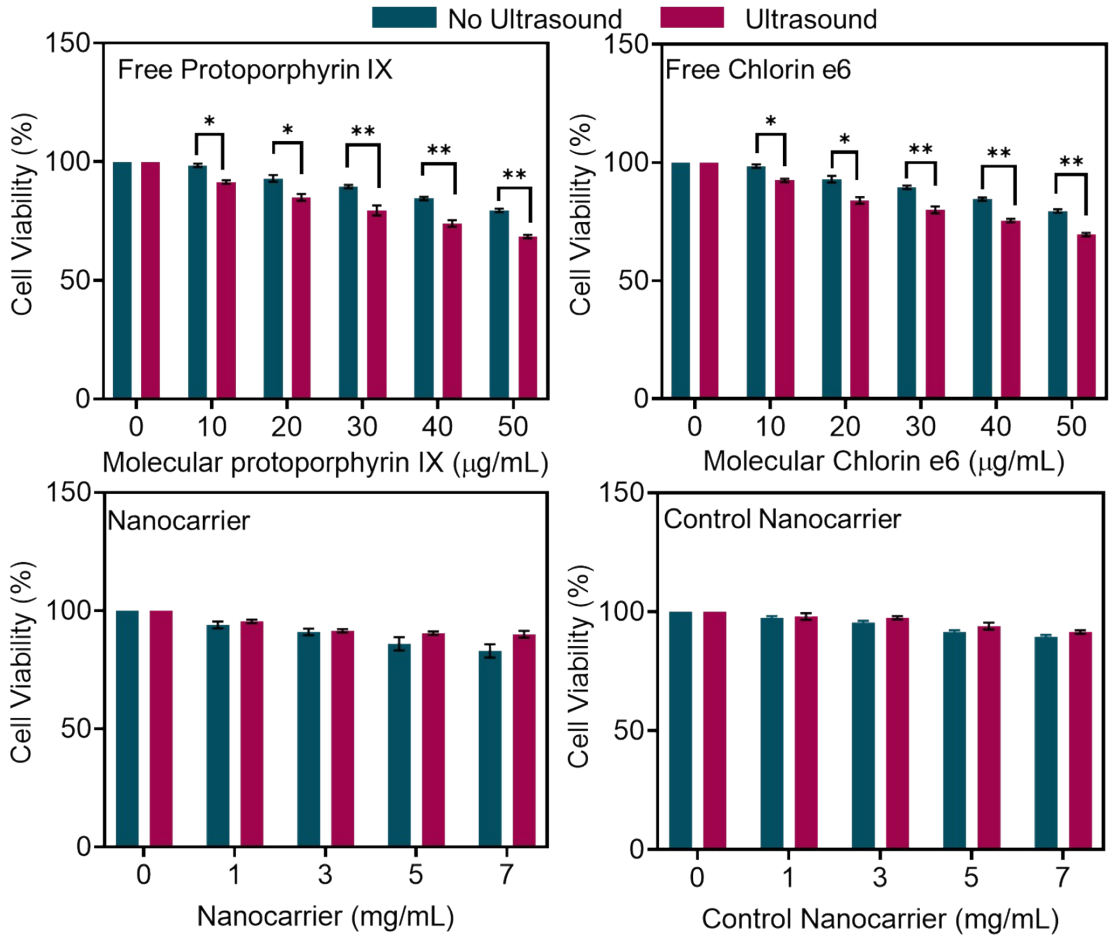


**Figure S15.** Evidence of insignificant cytotoxicity of molecular protoporphyrin ix, chlorin e6, nanocarrier and control nanocarrier under the exposure with ultrasound or without ultrasound. KB cells were incubated with these materials for 5 minutes, followed by a medium change. The cells were then irradiated with ultrasound for 3 minutes and incubated for 18 hours before performing the MTT assay to assess cell viability. Error bars represent mean ± S.D for 3 experiments and asterisks indicate statistically significant differences (*P < 0.01, **P < 0.001, ***P < 0.0001, and ****P < 0.00001) determined using Sidak's multiple comparison test.

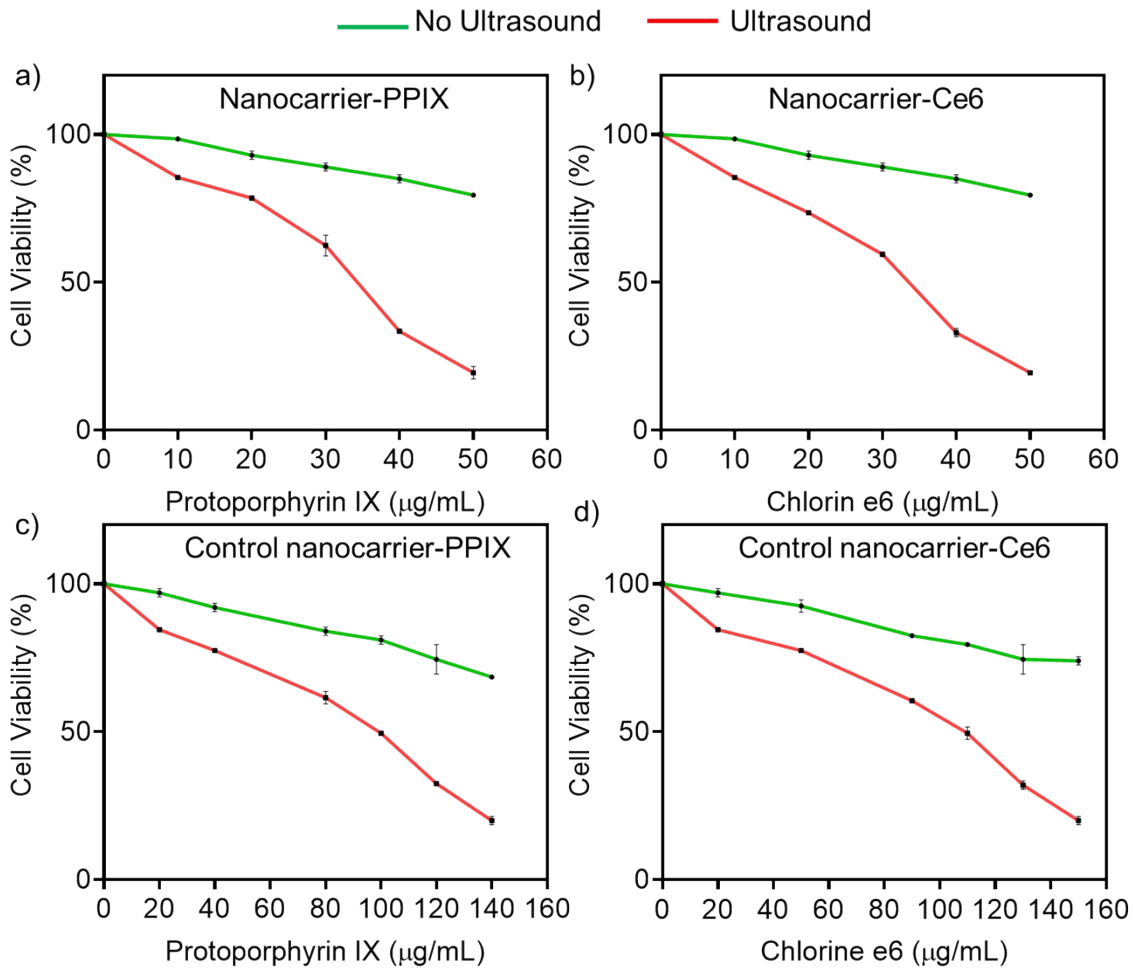


**Figure S16.** Determination of $IC_{50}$ values of sonosensitizer-loaded nanocarriers and control nanocarriers from the cell viability data. The plot represents percentage cell viability (Y-axis) against sonosensitizer concentration (μg/mL) (X-axis). The $IC_{50}$ values are as follows a) nanocarrier-PPIX: 26 μg/mL, b) nanocarrier-Chlorin e6: 34 μg/mL, c) Control nanocarrier-PPIX: 98 μg/mL, d) Control nanocarrier-Chlorin e6: 109 μg/mL.

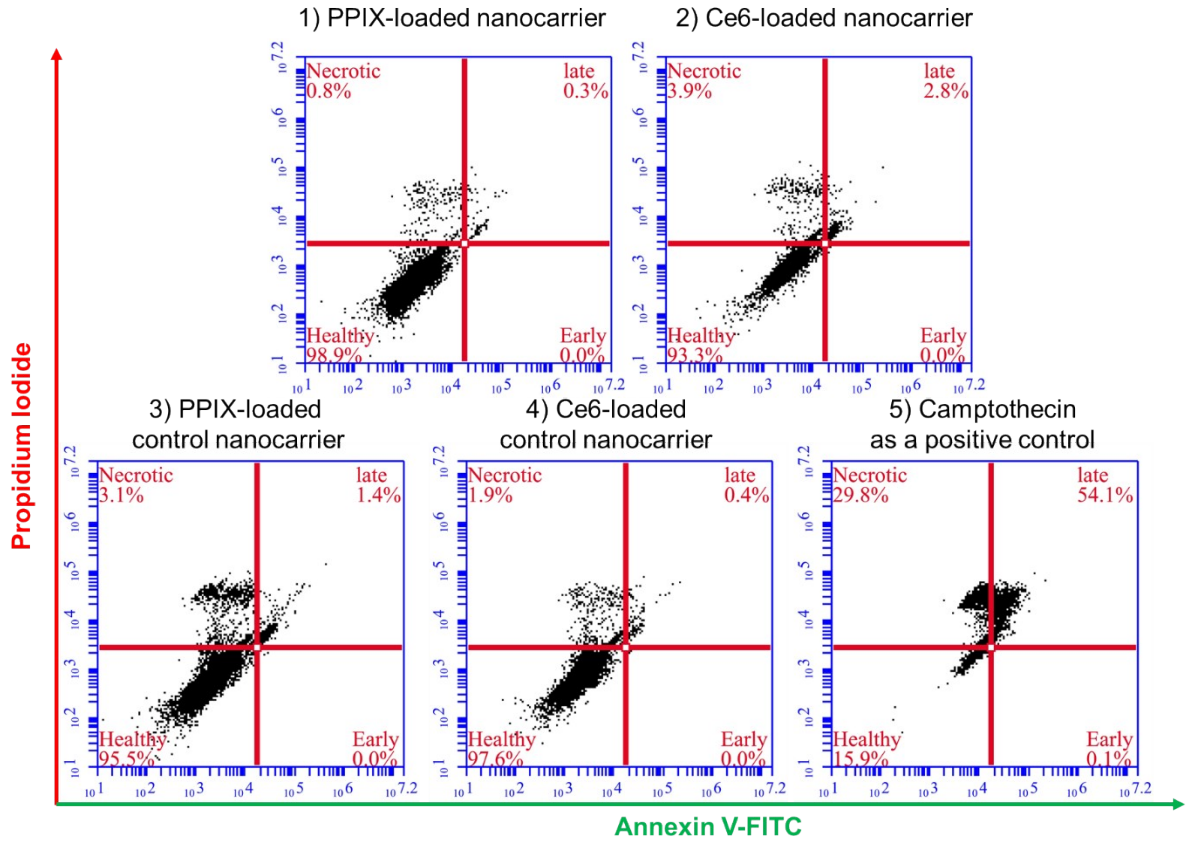


**Figure S17.** Annexin V-FITC and propidium iodide analysis of cells treated with sonosensitizer-loaded nanocarrier without any ultrasound exposure. Other conditions are same as Figure 6. 1) PPIX-loaded nanocarrier, 2) Ce6-loaded nanocarrier, 3) PPIX-loaded control nanocarrier, and 4) Ce6-loaded control nanocarrier, 5) Camptothecin as a positive control.

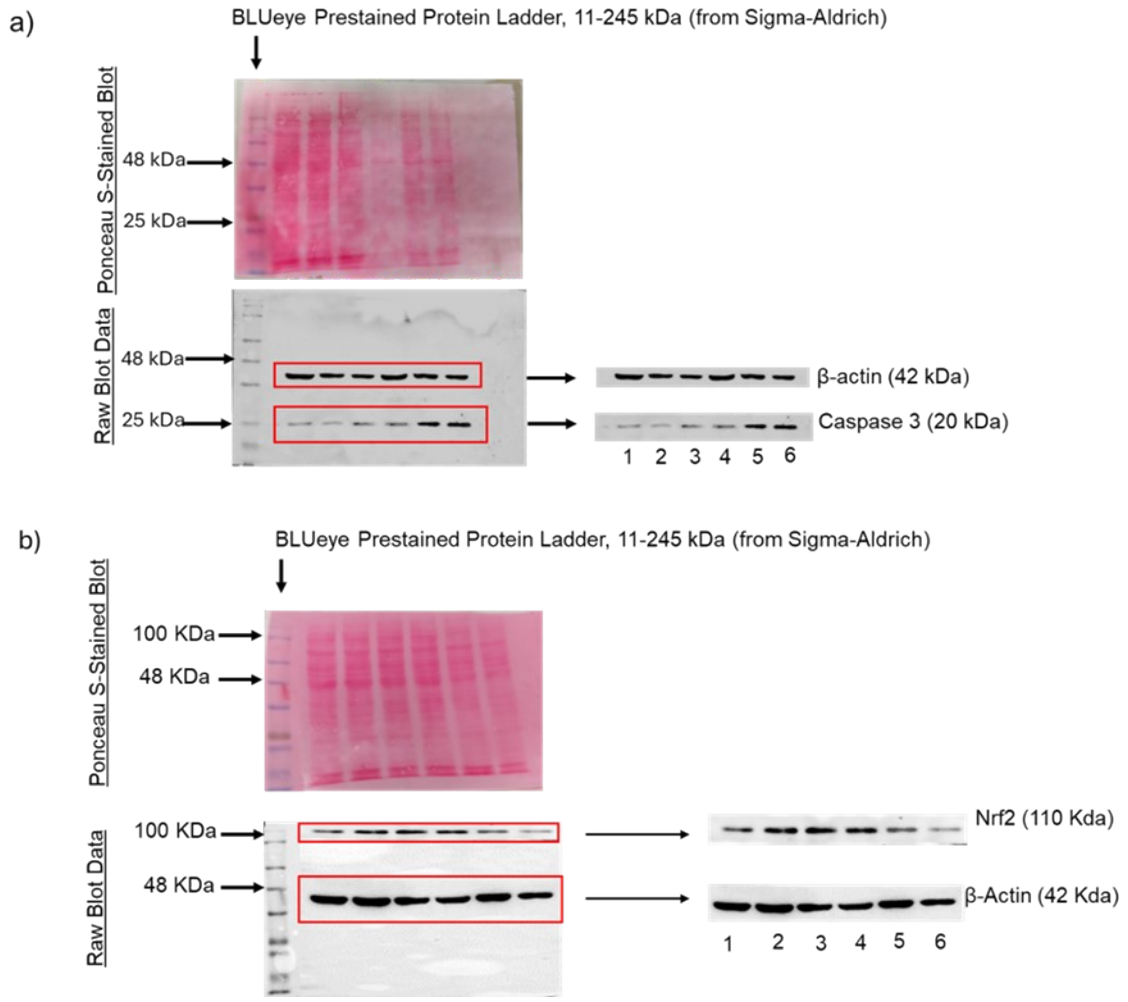


**Figure S18.** Raw western blot data corresponding to the immunoblots shown in Figure 5c (panel a), Figure 5d (panel b). Ponceau S staining, the target protein band, and the actin loading control are all presented on the same blot to demonstrate uniform protein transfer and accurate normalization. The BLUeye Prestained Protein Ladder (11–245 kDa, Sigma-Aldrich) was used to determine the molecular weight of the detected proteins. The multicolored prestained ladder allowed visualization of protein migration during SDS-PAGE and verification of proper separation during electrophoresis and immunoblotting.

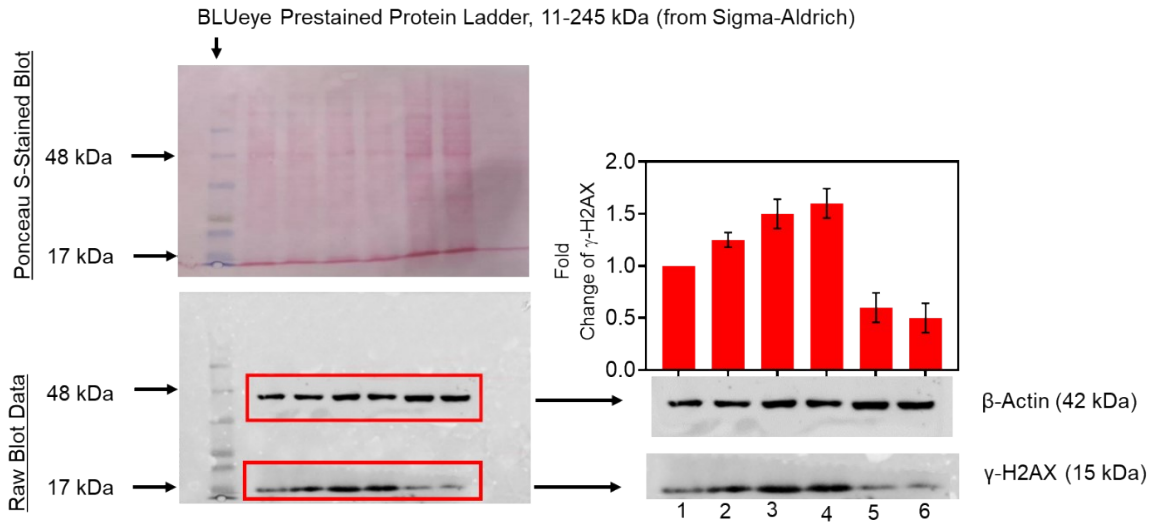


**Figure S19.** Western blot analysis of γ-H2AX expression in KB cells after 12 h incubation with (1) control cells, (2) ultrasound only, (3) control nanocarrier-PPIX, (4) control nanocarrier-Ce6, (5) nanocarrier-PPIX, and (6) nanocarrier-Ce6. γ-H2AX levels were normalized to β-actin. Control and ultrasound-only groups showed minimal expression, control nanocarrier-loaded sonosensitizers showed elevated γ-H2AX, whereas nanocarrier-loaded PPIX and Ce6 exhibited reduced γ-H2AX expression, indicating rapid DNA damage progression and remarkable apoptosis.