Supporting Information

Ultrasound Augments On-demand Breast Tumor Radiosensitization and Apoptosis Through a Tri-responsive Combinatorial Delivery Theranostic Platform

Prateek Bhardwaj[†]*, Jayant Sastri Goda[§], Venkatesh Pai[§], Pradip Chaudhari[‡], Bhabani Mohanty[‡], Trupti Pai[ϕ], Komal Vishwakarma[§], Rahul Thorat[‡], Tabassum Wadasadawala[§]*, Rinti Banerjee[†]*[¥]

[†] Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai 400076, Maharashtra, India

[§] Department of Radiation Oncology, Tata Memorial Centre, Mumbai, & Homi Bhabha National Institute, Maharashtra, India

[‡] Animal house facility, Tata Memorial Centre, Mumbai, & Homi Bhabha National Institute, Maharashtra, India

[ϕ] Department of Pathology, Tata Memorial Centre, Mumbai, & Homi Bhabha National Institute, Maharashtra, India

[¥]Deceased date: 8th July 2021

***Corresponding Authors**

E-mail for correspondence: prateekbhardwaj26@gmail.com, twadasadawala@actrec.gov.in

EXPERIMENTAL SECTION

***In vitro* biocompatibility.** Briefly, 10^4 L929 cells per well were seeded in a 96-well plate. After 24 hours, cells were incubated with different dilutions of blank liposomes and nanobubbles for 72 hours followed by MTT assay. MTT at a concentration of 1 mg/ml was added to the wells for 4 hours. DMSO was added as a solubilizing agent to solubilize the purple formazan precipitate and absorbance was taken at 560 nm using ELISA Plate reader.

***In vitro* qualitative time dependent cellular internalization.** Qualitative analysis of liposomal uptake over a period of time in MDAMB-231 cells was performed using confocal fluorescence microscopy. Briefly, 5×10^5 cells/well were seeded in a 24-well plate. After 24 hours, cells were incubated with curcumin loaded liposomes at 100 μM concentration for 1-4 hours. After incubation, cells were washed with chilled PBS thrice and fixed with 4% paraformaldehyde for 30 minutes. Cells were again washed with PBS and mounted on a glass slide using 50% glycerol for visualisation under confocal laser scanning microscope.

***In vitro* reactive oxygen species (ROS) generation.** Briefly, 5×10^5 MDAMB-231 cells per well were seeded in a 24-well plate. Cells were incubated with DCFH-DA at a final concentration of 100 μM for 2 hours. Different drug loaded liposomes and respective nano-conjugates were incubated at an equivalent PTX concentration of 10 μM for 12 hours. For ultrasound triggered groups, cells were simultaneously subjected with ultrasound of 2 W/cm^2, 100% duty cycle for 30 seconds. Cells were washed twice with chilled PBS and trypsinized. Trypsinized cells were then centrifuged at 3000 rpm for 3 minutes and cell pellet was resuspended in PBS for analysis under FACS.

Orthotopic TNBC model development. Ventral side of mice was shaved and an incision was made between 4th and 5th right nipple. Mammary fat pad was pulled out and 5×10^6 MDAMB-231 cells per 70 µl of PBS was injected followed by resealing of incision with tissue adhesive. Developed tumor was confirmed using diagnostic ultrasound of 7-15 MHz.

FIGURES

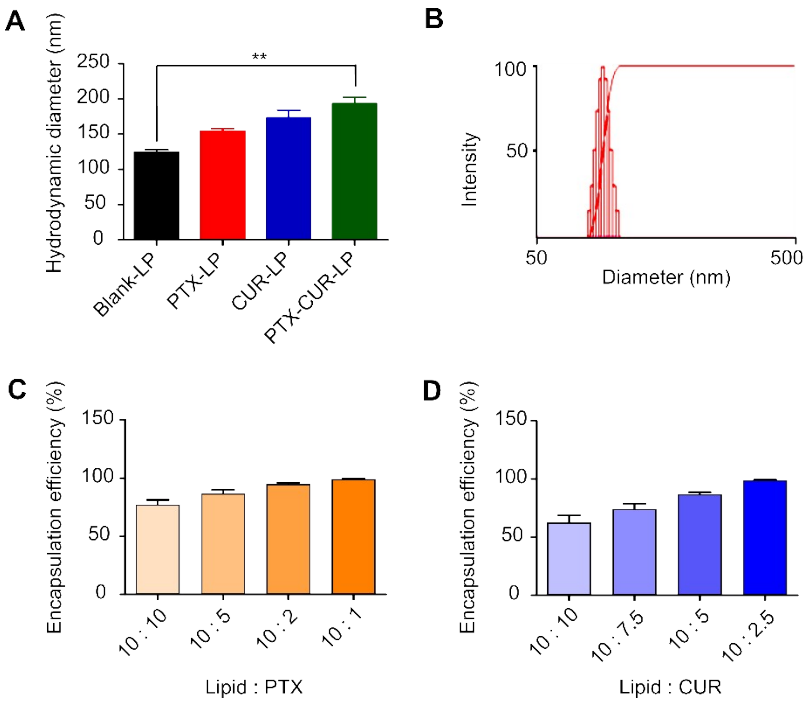

Figure S1. A) Comparative hydrodynamic diameter of blank and drug loaded liposomes distribution. B) Hydrodynamic diameter distribution of dual drug loaded liposomes (PTX-CUR-LP). Encapsulation efficiency of liposomes for different ratios of C) PTX and D) CUR. Data are represented as mean ± SEM (n=3). **$p < 0.01$.

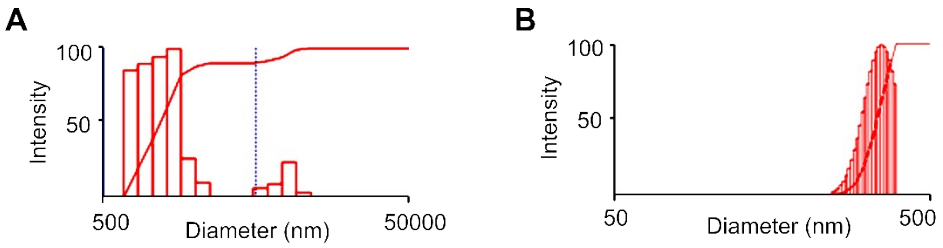

Figure S2. Hydrodynamic diameter distribution of A) upper-most layer and B) middle layer nanobubbles.

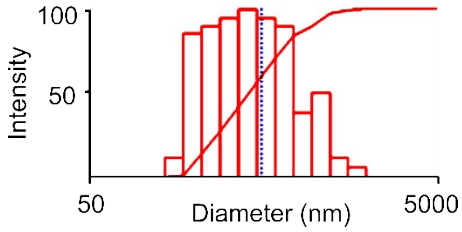

Figure S3. Hydrodynamic diameter distribution of nano-conjugates.

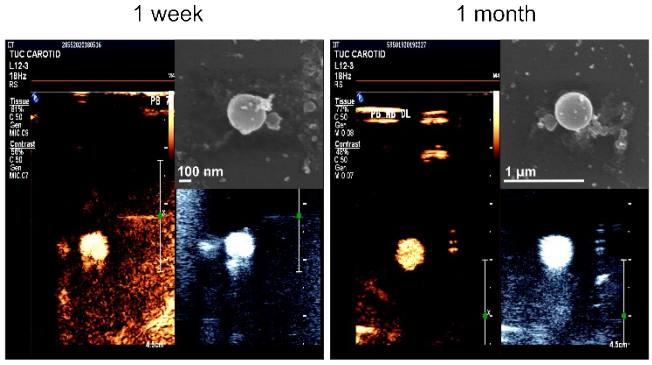

Figure S4. Contrast-enhanced and B-mode ultrasonographs of nano-conjugates with inset showing their SEM image after a storage period of 1 week and 1 month at 4ºC.

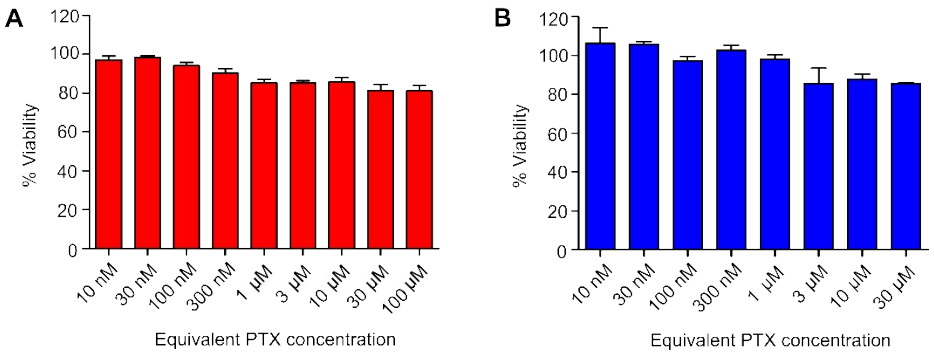

Figure S5. Biocompatibility of A) liposomes and B) nanobubbles on L929 mouse fibroblast cell line. Data are represented as mean ± SEM (n=3).

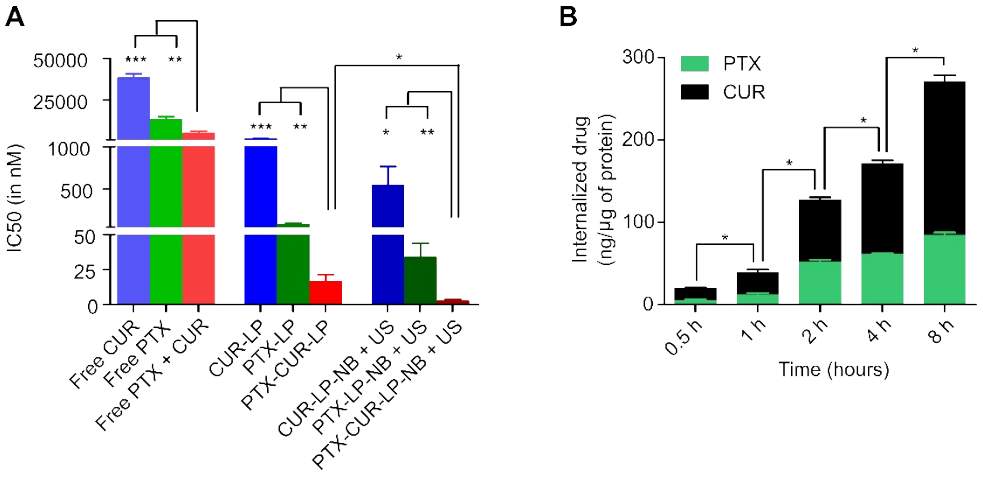

Figure S6. A) Comparative IC50 of free curcumin (free CUR), free paclitaxel (free PTX), free paclitaxel and curcumin combination (free PTX+CUR), CUR loaded liposomes (CUR-LP), PTX loaded liposomes (PTX-LP), dual drug loaded liposomes (PTX-CUR-LP), CUR-LP conjugated with nanobubbles (CUR-LP-NB), PTX-LP conjugated with nanobubbles (PTX-LP-NB), PTX-CUR-LP conjugated with nanobubbles (PTX-CUR-LP-NB) in the presence of ultrasound on MCF-7 cells. B) Quantification of internalized PTX and CUR upon time dependent incubation of PTX-CUR-LP with MCF-7 cells. Data are represented as mean ± SEM (n=3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

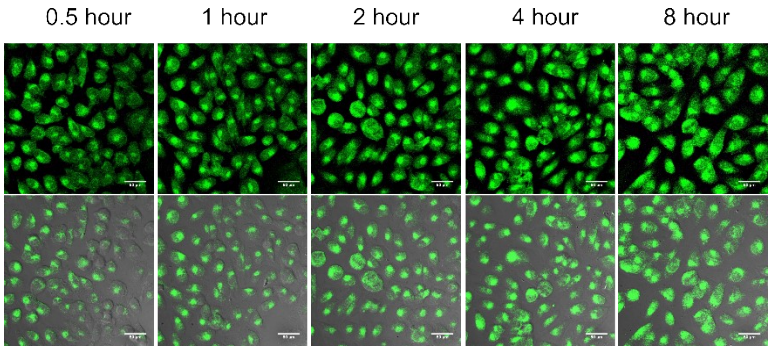

Figure S7. Confocal laser scanning microscopic images of MDAMB-231 cells upon incubation with curcumin loaded liposomes at different time points (Error bars: 50 µm).

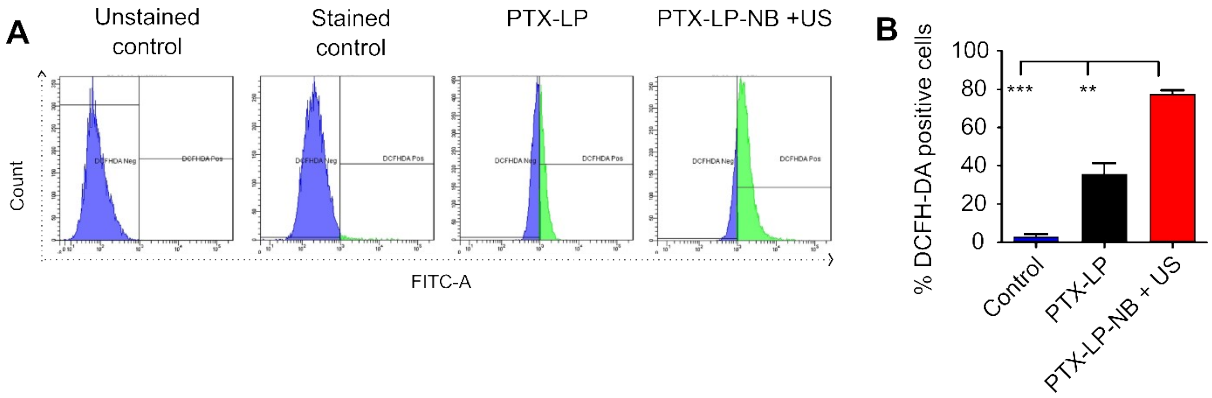

Figure S8. Effect of ultrasound triggered formulation on the generation of reactive oxygen species in MDAMB-231 cells. Data are represented as mean ± SEM (n=3). **p < 0.01, ***p < 0.001.

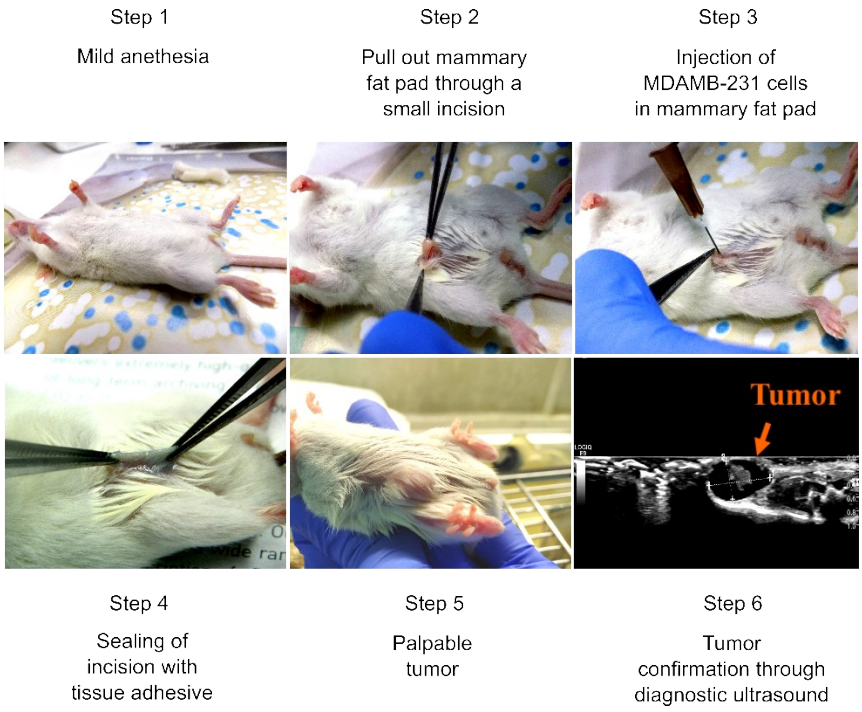

Figure S9. Development of orthotopic triple negative breast cancer xenograft model in NOD-SCID mice.

Table 1. IC50 of different formulations on MDAMB-231 and MCF-7 cells. Values are represented as mean ± SEM.

Formulation	**IC50 on MDAMB-231**	**IC50 on MCF-7**
Free CUR	42.2 µM ± 3.7	38.1 µM ± 1.5
Free PTX	16.4 µM ± 1.1	13.0 µM ± 1.0
Free PTX+CUR	5.1 µM ± 0.9	4.7 µM ± 0.9
CUR-LP	3.4 µM ± 0.6	1.4 µM ± 0.11
PTX-LP	521.1 nM ± 115.0	76.7 nM ± 10.1
PTX-CUR-LP	134.9 nM ± 34.0	16.3 nM ± 2.9
CUR-LP-NB + US	1.4 µM ± 0.3	540 nM ± 130.1
PTX-LP-NB + US	156.9 nM ± 42.4	33.7 nM ± 5.9
PTX-CUR-LP-NB + US	32.2 nM ± 6.7	2.4 nM ± 0.84

Table 2. Fold reduction in the ^{18}F-FDG uptake in mice treated with dual drug loaded liposomes (PTX-CUR-LP) in comparison to untreated control, Taxol®, and single drug loaded control formulations.

PTX-CUR-LP	Fold reduction w.r.t Control	Fold reduction w.r.t Taxol®	Fold reduction w.r.t CUR-LP	Fold reduction w.r.t PTX-LP
PET ^{18}F-FDG SUV	12.89	5.81	10.65	3.73
CT volume	6.44	2.15	4.87	2.20

Table 3. Fold reduction in the ^{18}F-FDG uptake in mice treated with dual drug loaded nano-conjugates in the presence of ultrasound (PTX-CUR-LP-NB + US) in comparison to untreated control, Taxol®, and non-triggered control.

PTX-CUR-LP-NB + US	Fold reduction w.r.t Control	Fold reduction w.r.t Taxol®	Fold reduction w.r.t PTX-CUR-LP
PET ^{18}F-FDG SUV	34.75	15.68	2.69
CT volume	22.11	7.40	3.42

Table 4. Fold reduction in the ^{18}F-FDG uptake in mice treated with dual drug loaded nano-conjugates in the presence of ultrasound and radiation (PTX-CUR-LP-NB + US + R) in comparison to untreated control, Taxol®, and radiotherapy treated clinical controls.

PTX-CUR-LP-NB + US + R	Fold reduction w.r.t Control	Fold reduction w.r.t Taxol®	Fold reduction w.r.t Radiation
PET ^{18}F-FDG SUV	59.50	26.72	15.19

Table 5. Scoring of lungs, liver, and spleen of mice treated with different formulations for observed histopathological changes by clinical pathologist. Scoring system: 0 (0%), 1 (0-25%, mild), 2 (25-50%, moderate), 3 (50-75%, high) and 4 (75-100%, very high).

	Inflammation			**Necrosis**	**Metastasis**	**Morphological changes**
	Lungs (Lu)	**Liver (Li)**	**Spleen (S)**	**Lu, Li, S**	**Lu, Li, S**	**Lu, Li, S**
Control	0	1	2	0	0	0
Taxol®	1	1	2	0	0	0
Radiation	0	0	2	0	0	0
PTX-CUR-LP-NB + US	0	1	2	0	0	0
PTX-CUR-LP-NB + US + R	1	0	2	0	0	0