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Magnetic targeting and ultrasound activation of liposome-microbubble conjugate for enhanced delivery of anti-cancer therapies

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**Abstract**

Effective delivery of chemotherapeutics with minimal toxicity and maximal outcome is clinically important but technically challenging. Here, we synthesize a complex of doxorubicin (DOX) loaded magnetoliposome (DOX-ML) microbubbles (DOX-ML-MBs) for magnetically responsive and ultrasonically sensitive delivery of anti-cancer therapies with enhanced efficiency. Citrate stabilized iron oxide nanoparticles (MNs) of 6.8 ± 1.36 nm were synthesized, loaded with DOX in the core of oligolamellar vesicles of 172 ± 9.2 nm, and covalently conjugated with perfluorocarbon (PFC) gas loaded microbubbles to form DOX-ML-MBs of ~4 μm. DOX-ML-MBs exhibited significant magnetism and were able to release chemotherapeutics and DOX-MLs instantly upon exposure to ultrasound (US) pulses. In vitro studies showed that DOX-ML-MBs in presence of US pulses promoted apoptosis and were highly effective in killing both BxPC3 and Panc02 pancreatic cancer cells even at a low dose. Significant reduction in the tumor volume was observed after intravenous administration of DOX-ML-MBs in comparison to the control group in a pancreatic cancer xenograft model of nude mice. Deeply penetrated iron oxide nanoparticles throughout the magnetically targeted tumor tissues in presence of US stimulation were clearly observed. Our study demonstrated the potential of using DOX-ML-MBs for site specific targeting and controlled drug release. It opens a new avenue for the treatment of pancreatic cancer and other tissue malignancies where precise delivery of therapeutics is necessary.

**Keywords:** Doxorubicin, chemotherapy, targeted delivery, magnetic nanoparticles, microbubbles, liposomes, ultrasound
1. Introduction

Pancreatic cancer (PC) is the seventh leading cause of cancer-related death worldwide\(^1\) and the third leading cause of cancer death in the United States\(^2\). PC is an aggressive cancer with an estimated survival of only 3 to 6 months after diagnosis and 5-year overall survival of only 7 %\(^3\). The high mortality rate of PC is associated with its lack of early symptoms, aggressive tumor biology, and lack of effective systemic therapies. Surgical resection provides the best hope for long-term survival among patients with small and localized pancreatic tumors\(^4\). However, only 15-20% of patients are amenable to resection, and the majority of tumors are locally advanced or metastatic at the time of diagnosis\(^5\). For these patients, chemotherapy and possibly chemo-radiotherapy are often used to treat the advanced disease\(^6\). However, patients receiving these therapies have a high incidence of severe adverse and toxic effects to normal tissue. In addition, drug delivery to pancreatic tumors presents a unique challenge as peritumoralstromal proliferation exerts additional barriers to drug penetration\(^7\). Furthermore, clinical efficacy of many chemotherapies is hindered by the development of chemoresistance\(^8\).

These limitations in the systemic treatment of PC present an urgent need for innovative formulations and more effective drug delivery systems. Many efforts have been made to overcome the current drug delivery barriers for increased therapeutic concentration at the tumor site and reduced side effects to normal tissues\(^9-10\). One particular method is to use Fe\(_3\)O\(_4\) magnetic nanoparticles (MNs) for targeted imaging and therapy\(^11\). MNs of approximately <10 nm in size are further encapsulated in PEGylated liposomes in order to develop magnetic liposomes (MLs). Previous studies have demonstrated the targeted accumulation of MLs within the desired region of interest (ROI) under the guidance of an external magnetic field\(^12\). MLs are functionally versatile and could provide a platform to deliver the drug by both active and passive targeting through the use of an external magnetic field\(^13-15\).

Another method of selective drug delivery involves the use of ultrasound (US) pulses. Perfluorocarbon gas (PFC) loaded microbubbles (MBs) can be identified and destroyed through distinct nonlinear acoustic echoes during US imaging, causing release of the loaded agent\(^16-17\). The process of US-mediated drug delivery typically involves stable and inertial cavitation of MBs\(^18\) and consists of complex sonophoresis mechanisms, such as acoustic cavitation, microstreaming, thermal effects, diffusion, and convection\(^19\). Exposing MBs to US pulses with a frequency range of 1-10MHz can effectively break the MBs for the enhanced acoustic contrast\(^20\), for controlled release of anticancer therapies and oxygen\(^21\), and for
induction of oxygen free radicals in sonodynamic therapy. Based on the recent advances in magnetic retention of MLs for targeted drug distribution and US fragmentation of MBs for controlled drug release, we propose to synergize these two drug delivery effects by covalent bonding of drug-laden MLs with PFC filled MBs. Our hypothesis is that the combination of US pulses together with magnetic guidance can produce targeted tumor destruction by allocation of DOX within solid tumor without any incision and with minimal unintended toxicity to nearby normal tissue. Such a ML-MB formulation will be administered intravenously in presence of a magnet (Mg) at the desired region of interest (ROI). As the ML-MBs circulate through the ROI, they will be accumulated by the magnetic field. Further application of US pulses at the ROI will break the MBs to facilitate controlled release of drug-laden MLs. With the enhanced permeability and retention (EPR) effect, the released MLs will penetrate through the leaky vasculature for improved tumor tissue penetration and tumor cell uptake. Previous studies have demonstrated the technical feasibility of self-assembled liposome-microbubble conjugate for US-triggered drug delivery. We are the first to bind magnetic nanoparticles with MBs for further enhanced delivery of therapeutics through consecutive mechanisms of magnetic retention and US fragmentation.

The proposed ML-MB formulation and the effect of dual-mode magnetic and US stimuli were investigated by encapsulating doxorubicin hydrochloride (DOX), a potent chemotherapeutic agent for the treatment of PC. Previous studies have demonstrated the enhanced delivery of DOX using the formulations of PEGylated liposome (Doxil) and liposome-microbubble conjugate. We have further engineered the conjugate of DOX laden magneto-liposome and microbubbles (DOX-ML-MBs) by attaching DOX laden MLs (DOX-MLs) covalently with PFC gas filled MBs. The DOX-ML-MB formulation is designed for controlled accumulation by an external Mg field and selective activation by external US pulses. In this study, the magnetic responsiveness and the US effect of DOX-ML-MBs were evaluated; the formulation design and the process parameters were optimized; the anti-cancer potent of DOX-ML-MBs was evaluated in pancreatic cancer cells, and the drug delivery efficacy of magnetic and US dual-mode stimuli was demonstrated in-vivo.

2. Materials and Methods

2.1. Materials
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG2000-MAL), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-
distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000](DSPE-MPEG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[PDP(polyethylene glycol)-2000] (DSPE-PEG2000-SPDP) were purchased from Biocoen Biotechnology CO., Ltd (Beijing, China). Cholesterol (Chol), glycerine and propylene glycol were purchased from Sigma-Aldrich. Perfluoropropane (C₃F₈) was purchased from Jietong Gas Technology Co., Ltd. (Guangzhou, China). Sephadex G-50 was purchased from YuanYe Biological Technology Co Ltd. (Shanghai, China). Ferrous chloride tetrahydrate was purchased from Alfa Aesar. Ammonium Sulphate and ferric chloride hexahydrate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). MTT assay kit was purchased from Beyotime Biotechnology (Jiangsu, China). Fetal bovine serum (FBS), Trypsin-EDTA solution, Roswell Park Memorials Institute(RPMI)-1640 medium and Penicillin-streptomycin solution were supplied by Sangon Biotech (Shanghai, China). Triple distilled water from Milli-Q Plus System, Millipore Corporation (Bedford, USA) was used.

2.2. Method

2.2.1. Preparation and characterization of DOX laden and maleimide functionalized magneto-liposomes (DOX-MLs)

Citric acid stabilized magnetic nanoparticles (Fe₃O₄-NPs, MNs) were prepared by a co-precipitation method as reported previously. The process and characterization details have been provided in the supporting information. The produced MNs were used for the subsequent loading to liposomes during dehydration-hydration preparation of MLs. In more detail, a homogeneous thin dry lipid film of DSPC, cholesterol, and DSPE-PEG2000-maleimide (molar ratio- 60:20:20) was prepared by dissolving the lipids in pure chloroform and drying them as a thin film by rotavapour (IKA, RV8, Germany). Further, the dry film was hydrated with the aliquot of MNs in 250 mM ammonium sulphate solution using rotary evaporator for 30 min while rotating at 90 rpm followed by freeze-thaw cycle. The size of obtained magneto-liposomes (MLs) was further reduced using a mini extruder (LIPEX liposome extruder, ATS Engineering Inc., Canada) and a Whatman 200 nm pore size polycarbonate membrane (Whatman). The quantity of MNs was optimized to obtain the maximal loading of MNs in MLs.

DOX-MLs were prepared by incubating 5 mL suspension of MLs with 10 mg/mL DOX solution at 50-60°C for 4 h. The non-entrapped MNs and DOX were removed by size exclusion chromatography (SEC) using Sephadex G50 before characterization. The hydrodynamic diameter, the size distribution and the ζ-potential of DOX-MLs were characterized by dynamic light scattering (DLS) using a Zetasizer Nano Particle
Characterization System (Malvern Instruments Ltd., Malvern, Worcestershire, UK). The MN loading in MLs and the surface morphology of MLs were verified by transmission electron microscopy (TEM, Hitachi H-7650). Before TEM analysis, the samples were diluted with ultrapure water, and a drop was placed on carbon coated copper grids for imaging. Cryo-TEM (Tecnai G2 Spirit Biotwin 120 KV) was used to image blank and DOX loaded liposomes without MN. The actual Fe content in MLs was determined using an analytical method of inductively-coupled plasma/atomic emission spectrometry (ICP-AES) analysis (Thermo Jarrell Ash, USA)\(^{31}\). The encapsulation efficiency and the loading rate of DOX in DOX-ML were analysed by High Performance Liquid Chromatography (Agilent, HPLC system)\(^{32}\). The phospholipid content of MLs was estimated by the method of Stewart-Marshall\(^{33}\). The magnetic properties of MLs were characterized by a superconducting quantum interference device (SQUID) in comparison with MNs.

We further developed the indocyanine green (ICG) and fluorescein (FITC) labelled MLs (ICG-MLs, FITC-MLs) for fluorescent labelling of MLs by the method described above with slight modification\(^{12}\). Briefly, 0.5 mg/mL ICG or 0.1 mg of FITC was added to the thin film hydrating solution along with MN to obtain ICG-MLs/FITC-MLs. The prepared particles were characterized and further utilized to fabricate ICG-ML-MBs and FITC-ML-MBs for animal experiments. The concentrations of ICG and FITC in MLs were quantified by fluorometry. Both ICG and FITC were used as model drugs in this study due to their different fluorescence characteristics. On the one hand, ICG was used for near infrared fluorescence imaging of drug distribution in deep tissue in vivo. On the other hand, FITC is used for microscopic imaging of drug distribution in tissue specimen.

**2.2.2. Fabrication and characterization of DOX-laden magneto-liposome-microbubble (DOX-ML-MBs)**

Thiol functionalized MBs were prepared using the lipid solution in glycerine-propyleneglycol-water (GPW, 1:1:8) and PFC gas (C\(_{3}\)F\(_{8}\)). Activated thiol functionalized MBs were prepared using DSPE-PEG2000-SPDP. The disulphide bond in DSPE-PEG2000-SPDP was reduced with 100 mM dithiothreitol (DTT) solution (0.5 mL DTT per 1 mL lipid solution) in sodium phosphate buffer (pH 8.0) at room temperature for 2 h to obtain DSPE-PEG2000-SPDP with sulphydryl group after centrifugation for 15 min at 5000 rpm. Initially a thin lipid film was prepared by dissolving DSPC and DSPE-PEG2000-SPDP (molar ratio: 80:20) in chloroform and evaporating the mixture to a thin layer by a rotary evaporator. The clear lipid solution was obtained by dispersing the film in GPW to obtain the lipid concentration of 1 mg/mL. The solution was then transferred into a 3 mL glass vial with the headspace filled
with PFC gas. Finally, functionalized MBs were prepared by rapidly shaking the solution at high speed for 20 s using an YJT-2 shaker (Shanghai Medical Instruments Co., Ltd., Shanghai, China) and collected by centrifugation.

DOX laden ML-MB complex (DOX-ML-MB) and ICG/FITC laden ML-MB complex (ICG-ML-MB/FITC-ML-MBs) were prepared by mixing the aforementioned lipid solution having activated thiol group with DOX-ML or ICG-ML/FITC-ML, respectively and shaking the mixtures at high speed\textsuperscript{25}. The resulting DOX-ML-MBs were stored at 4 °C in a refrigerator until further use. The bonding of DOX-ML with MBs was confirmed by detecting the red fluorescence emission of DOX using a confocal fluorescence microscope (Nikon Instruments Inc., Japan). Also the covalent bond formation was further confirmed by Fourier transform infrared (FTIR, Thermo Nicolet Avatar 6700 FTIR) spectrometry.

The DOX loading rate in DOX-ML-MBs was maximized by varying the thiol and the maleimide lipid concentrations\textsuperscript{25}. The DOX concentration in DOX-ML-MBs was analysed using a reverse-phase HPLC method as reported earlier\textsuperscript{32}. The size of the MBs and their concentration were determined by an optical microscope (Model DSZ2000X, Chongqing UOP photoelectric technology Co. Ltd, China) using a haemocytometer (Bright-Line, Hausser Scientific, Horsham, PA, USA) and an image-processing program (MATLAB, R2016a, Mathworks, USA). The DOX concentration in DOX-ML-MBs was further adjusted according to the required dose of DOX for further studies. The iron (Fe) content in DOX-ML-MBs was also determined by ICP-AES analysis. The release of DOX from DOX-ML-MBs after the exposure of US at different time points was studies and compared with those from free DOX, DOX-ML and DOX-ML-MBs in the release medium (PBS; pH 7.4) without US stimulation. The detailed method has been described in the supporting information.

2.2.3. Magnetic retention of DOX-ML-MBs in a microfluidic vascular phantom

Magnetic retention of DOX-ML-MBs was verified in a polydimethylsiloxane (PDMS) microfluidic vascular phantom. The phantom was prepared by a soft lithography method following the process as described in the supporting information. It was embedded with three parallel micro-channels of 0.5-mm wide, 0.1-mm deep, and 50-mm long. The middle channel had a reservoir at the centre connected with the left and the right channels through openings of 50 µm.

The DOX-ML-MB suspension at a concentration of 5×10^7/mL was primed through the middle micro-channel of the phantom at a flow rate of 0.25 mL/min. A neodymium-iron-boron (Nd$_2$Fe$_{12}$B) Mg disc of 0.2 T was placed 2 mm away from the right micro-channel. The left micro-channel was used as control without the magnetic field. DOX-ML-MBs flowing
through the middle channel were collected and counted by a haemocytometer (XB-K-25 Qiu Jing, Shanghai). After 1 mL of DOX-ML-MBs was primed through the middle channel, the DOX-ML-MBs collected from the left and the right channels were counted in order to evaluate the magnetic retention of the DOX-ML-MBs.

The aforementioned microfluidic vascular phantom was used to investigate the destruction threshold of DOX-ML-MBs when exposed to US pulses at different mechanical indexes from 0.05 to 1. The US transducer was aligned at 45° to the micro channel to minimize the standing waves. The DOX-ML-MB suspension at 1×10^6/mL was infused at a constant speed of 0.25 mL/min.

2.2.4. Culture of Panc02 and BxPc-3 pancreatic cancer cell lines, COS-7 cells and cytotoxicity studies

A murine pancreatic adenocarcinoma Panc02 cell line and a human primary pancreatic adenocarcinoma BxPc-3 cell line were kindly provided by Dr. Longping Wen and Dr. Jun Wang (School of Life Sciences, University of Science and Technology of China, Hefei, China) respectively. The COS-7 fibroblast-like cells was obtained from China Center for Type Culture Collection (CCTCC). The cell lines were cultured using RPMI medium flourished with 10% (v/v) foetal bovine serum and 1% (v/v) penicillin-streptomycin and were maintained in an incubator at 37 °C humidified with 5% CO_2 atmosphere.

The anticancer effect of DOX-ML-MBs was evaluated on pancreatic cancer Panc02 and BxPc-3 cells and was compared with COS-7 cells. The cells were seeded at a concentration of 5×10^3 cells/well in 96-well tissue culture plates. After overnight incubation of cells at 37 °C in an incubator with 5% CO_2, the medium was changed and the following treatments were applied: (1) plain media (“Control”), (2) US only without MBs (“US only”), (3) MBs followed by US activation (“MBs(+US)”), (4) free DOX without MB formulation (“Free DOX”), (5) DOX laden magneto-liposome (“DOX-ML”), (6) complex of DOX laden magneto-liposome and microbubbles followed by US pulses (“DOX-ML-MBs (+US)”), and (7) complex of DOX laden magneto-liposome and microbubbles without US pulses (“DOX-ML-MBs (-US)”). For the treatments involving DOX, the equivalent DOX concentrations from 0.5 µg/mL–20 µg/mL were used. For the treatments involving US, 1 MHz US pulses at a 20% duty cycle and an intensity of 2 W/cm^2 were applied to each well for duration of 20 s. 4 h after treatments, the cells were washed and the fresh media were replaced. Subsequently 24 h later, 10 µL of the MTT reagent (5mg/mL) was added and incubated for an additional 4 h. Afterward, the solubilization reagent DMSO was added to the media and the absorbance was measured at 570 nm using a microplate spectrophotometer (Thermo Fisher Scientific,
USA) after gentle agitation at room temperature. The experiments were performed in triplicate.

### 2.2.5. Intracellular uptake

The uptake efficiency of DOX encapsulated in different formulations by Panc02 cells under the influence of US was evaluated by confocal fluorescence microscopy and FACS analysis. For FACS and confocal fluorescence imaging study Panc02 cells were seeded in 6-well culture plate and 15 mm glass bottom cell culture dish, respectively, at $1 \times 10^6$ cells/well density.

The cells plated in 15 mm glass bottom cell culture dish were incubated with an equal amount of DOX in Free DOX, DOX-ML, DOX-ML (+US), DOX-ML-MBs (+US), and DOX-ML-MBs (-US) for 4 h. For the treatments involving US, 1 MHz US pulses at a 20% duty cycle and an intensity of 2 W/cm$^2$ were applied to each well for duration of 20 s. After incubation, the cells were carefully rinsed with PBS to remove excess formulations. The cells samples were fixed at $-80$ °C with ice cold methanol for 60 minutes. The cells nuclei were stained with a staining dye DAPI, at concentration of 10 μL of 1 mg/mL. The cells were imaged by a confocal fluorescence microscope (Nikon Instruments Inc., Japan).

Following the incubation with Free DOX, DOX-ML, DOX-ML (+US), DOX-ML-MBs (+US), and DOX-ML-MBs (-US) and US treatment as described above, the treated cells plated in 6-well plates, were rinsed with PBS, trypsinized and diluted by RPMI medium without phenol red. The cells were centrifuged and the pellets were resuspended with flow buffer and the fluorescence emission was measured using BD Biosciences FACS Calibur flow cytometer.

### 2.2.6. Apoptosis

The cell apoptosis was determined by using Annexin V and propidium iodide (PI) kit (Invitrogen, CA, USA) with set protocol. Panc02 and BxPc-3 cells were seeded at density of $1 \times 10^6$ cells/well in 6-well plate and treated for 24 h with (1) plain media (“Control”), (2) US only without MBs (“US only”), (3) MBs followed by US activation (“MBs(+US)”), (4) Free DOX, (5) DOX-ML, (6) DOX-ML-MBs (+US), and (7) DOX-ML-MBs (-US) containing DOX (2 μg/mL equivalent concentration). The US, 1 MHz US pulses at a 20% duty cycle and an intensity of 2 W/cm$^2$ were applied to corresponding cells for 20 s immediately after the treatment. Further, the cells were trypsinized and centrifuged; the collected cells were rinsed with PBS and re-suspend in binding buffer (500 μl). Finally, 5 μl of annexin V-FITC and propidium iodide were added into cells and stored in dark for 30 min prior to flow cytometry analysis.
2.2.7. Animal models

Athymic male nude mice at an age of 6-weeks were purchased from Shanghai slack laboratory animal Co., LTD, Shanghai and housed at the Laboratory Animal Center of USTC. The study was approved by USTC Animal Care and Use Committee (Protocol No: USTCACUC1702045). The animals were treated according to the ethical guidelines in the Guide for the Care and Use of Laboratory Animals. To evaluate the drug delivery outcome after magnetic accumulation and US stimulation, we used healthy animals without tumor xenograft since tumor xenograft may induce additional variables in drug distribution dependent upon vascular perfusion, tumor size and tissue heterogeneity. To facilitate fluorescence assessment of drug distribution, we used ICG and FITC labelled ML-MBs. ICG and FITC are fluorescence dyes with emission peaks at 814 nm and 519 nm, respectively. Drug distribution in the animal model was imaged in vivo by a small animal imaging system where ICG was used as a model drug due to its emission peak in the near infrared wavelength range that facilitated deep tissue imaging. Drug distribution in the animal model was also evaluated in vitro by a fluorescence imaging microscope where FITC was used as a model drug considering the filter set availability and the achievable spatial resolution in fluorescence microscopy.

To evaluate the anti-cancer outcome after magnetic and US mediated delivery of drug-laden ML-MBs, we used a pancreatic cancer xenograft model. Panc02 cells were maintained in RPMI-1640 medium supplemented with 10% foetal calf serum as described above. Cells (1 × 10⁶) were re-suspended in PBS and implanted into the hind leg muscle of male Balb/c nude mice. The therapeutic studies were carried out 21 days after implantation when the tumors grew to the size of around 150 mm³, as measured by a calliper.

2.2.8. In-vivo study of drug delivery efficiency

The healthy male mice without tumor xenograft were randomly divided into four groups with six mice in each group for tail vein administration of different treatments under anaesthesia. Group I were administered with ICG-ML only; group II were administered with ICG-ML in presence of US pulses; group III were administered with physical mixture of ICG-ML and MBs in presence of US; and group IV were administered with ICG-ML-MBs in the presence of US pulses. In order to evaluate the effect of magnetic stimulation on drug distribution, an Nd₂Fe₁₂B permanent Mg of 0.2 T was placed beneath the lower left flank of each animal. As control, no Mg was placed on the lower right flank of the animal and also the tissues were not exposed to the US. For all the treatments involving US, 1 MHz US pulses at a duty cycle of 30% and a power intensity of 2 W/cm² were applied to the region of interest (ROI) on the
lower left flank muscle tissue for 2 min. ICG distributions in the ROI (left flank) and the control tissue (right flank) of each animal were monitored by a Xenogen IVIS® spectrum system and analysed by a Living Image 4.5.2 software package. After the treatments, the animals were sacrificed and the effect of magnetic and US stimuli on the treated mice was evaluated by collecting and imaging the control tissue. The collected tissues were sectioned and stained by hematoxylin and eosin (H&E) staining to observe any effect of treatment on the tissues.

Moreover, four other mice were treated with FITC-ML-MBs as described above. After the treatment, the sample tissues from the lower left flanks and the control tissues from the lower right flanks were collected, washed, sectioned using cryostat, and imaged by a confocal fluorescence microscope (Nikon Instruments Inc., Japan). The fluorescent uptake of the tissue were taken under consideration to examine the effect of magnetic and US stimuli after administration of FITC-ML-MBs.

2.2.9. In vivo anticancer and toxicity studies

The pancreatic tumor xenograft mice were randomly distributed into 5 groups (n = 5). Following induction of anaesthesia (isoflurane), a 100 μL mixture of PBS containing DOX-ML-MBs (MB = 1.6 × 10^8), was administered by tail vein injection to Groups 2 & 3 while Group 1 received vehicle only. For Group 2, US (frequency = 1 MHz, US power density = 3.5 W/cm², duty cycle = 30% was directed to the tumor region for 3.5 min during and after injection. For Group 3, in addition to US using the above parameters, a stack of permanent Mg was directed to the tumor region for 3.5 min during and after injection, resulting in an approximate magnetic field intensity of 0.10 T and gradient of 14.9 T/m at the tumor site.

Group 4 mice received DOX-ML in presence of US and permanent Mg with the same parameters as above. Group 5 mice received free DOX without US. Treatments using the above conditions were repeated on Days 1, 5 and 9 and the animals were sacrificed at the end of study.

Before the animals were sacrificed at the end of tumours regression study, blood samples were drained from retro orbital plexus of the mice and collected in pre-heparinized tubes. The whole blood sample was used for haematological analysis and serum free blood was applied for biochemical parameter evaluation with the help of hematoanalyzer (XT-1800i, Sysmex Corporation, Japan). The biochemical analysis was carried out with the help spectrophotometry by using commercial kits (Rayto Life and Analytical Sciences Co., Ltd. China) in automated biochemical analyser (Chemray-240).
Following the blood collection, the mice were sacrificed and the organs such as liver, heart, kidney and spleen along with tumor were collected and fixed with paraformaldehyde (10% solution) for further histology analysis. Hematoxylin and eosin (H&E) staining were used to observe the histological change of these vital organs.

The tumor sections were also analyzed for the presence of Fe by using Prussian blue staining method. The tumor sections were immersed in a freshly prepared solution containing equal parts of 20% HCl and 10% potassium ferrocyanide for 20 min at room temperature. Following which the slides were rinsed using ultrapure water. Furthermore, the slides were stained with nuclear fast red for 5 min. We also analysed the hemocompatibility of DOX-ML-MBs in fresh blood of mice in presence/absence of US. The detail method has been described in the supporting information.

### 2.2.10. Statistical Analysis

The experiments were conducted at least in the set of three in attempt to achieve statistical significance. Statistical analysis was performed with Graph pad Prism 5; a minimal level of significance was 0.05.

### 3. Results

#### 3.1. Preparation of MN and DOX-ML

The citric acid stabilized Fe$_3$O$_4$-NPs (MNs) were fabricated following a previously established protocol$^{34}$. The size and the distribution of the MNs were confirmed by scanning electron microscopy (SEM; Leica Stereoscan 410, Wetzlar, Germany), TEM and dynamic light scattering (DLS), whereas the crystalline structure of MNs was evaluated using X-ray diffraction (XRD) pattern (Philips X’pert PRO X-ray diffractometer). The TEM and the SEM images in Figure S1A and S1B show a uniform nanostructure for the prepared MNs. The DLS results in Figure S1C show that the MNs have an average size of 6.8 ± 1.36 nm and ζ-potential of -23.65 ± 2.96 mV. Figures S1D represents the MNs in solution and Figure S1E represent the influence of an external Mg on the MNs in an aqueous solution. The XRD pattern revealed the high crystallinity diffraction peaks similar to the standards (JCPDS card no. 01-075-0449) as shown in supplementary Figure S2.

After successful synthesis and characterization of MNs, we prepared the MLs with fast magnetic responsiveness by encapsulating MNs in lipid vesicles with and without DOX loading. Figure 1A shows the schematic representation of DOX-MLs. The averaged diameter of DOX-MLs was 172 ± 9.2 nm, the ζ-potential was -29 ± 3.7 mV, and the polydispersity index (PDI) was 0.129. The HPLC analysis revealed that the DOX encapsulation efficiency in DOX-MLs was 82 %. The Fe content in the DOX-MLs was 0.86 mole/mole of lipid.
Clearly, the presence of Fe did not affect the loading of DOX in ML. Figure 1B shows the size distribution of DOX-MLs. Figure 1C shows the TEM image of MLs that reveal the well encapsulation of MNs within the core of lipid vesicles. Figure S3 (A&B) represents the cryo-TEM images of blank liposomes and Dox-laden liposomes.

Figure 1: A) Schematic illustration of the DOX loaded magnetic liposomes. B) DLS data of particle size distribution of ML. C) TEM image of ML at 200 nm scale bar. D) Magnetization curves of MNs and DOX-MLs at 300 K.

3.2. Magnetic responsiveness of MNs and DOX-MLs

The superparamagnetic properties of the MNs and DOX-MLs were analysed using a superconducting quantum interference device (SQUID) at room temperature. Figure 1D shows the magnetization loops for MNs and DOX-MLs at 300 K, respectively. According to the figure, MNs show a greater magnetization in comparison to that of DOX-MLs, possibly attributed to the entrapment of MNs in the core of lipid bilayer. However, the superparamagnetic DOX-ML scan be effectively controlled by an external Mg.

3.3. Synthesis and characterization of DOX-ML-MBs

The DOX-ML-MBs were prepared by self-assembly of covalent bonding of the thiol group in activated MBs and the maleimide group in DOX-MLs in the presence of PFC gas. Figure 2A
represents the schematic diagram of self-assembled DOX-ML-MBs, whereas Figure 2B illustrates the covalent bond formation between two lipids.

DOX-ML-MBs with an average diameter of ~4 µm and a concentration of $1.76 \times 10^9$/mL were synthesized, as shown in Figure 3A. The amount of DOX loaded in 1 mL of DOX-ML-MBs was found to be 74.8 µg; the Fe content in $5 \times 10^7$ DOX-ML-MBs was 0.207 mg. The unloaded DOX-MLs were separated by centrifugation and the formation of DOX-ML-MBs was further confirmed by confocal microscopic imaging. Figures 3B, 3C and 3D represent the bright-field, overlay and fluorescence images of DOX-ML-MBs suspension, respectively. In Figure 3D, microscopic imaging of fluorescence emission by DOX-MLs at the outer layer of DOX-ML-MBs further confirmed the effective bonding of DOX-MLs with MBs. In addition the results obtained from FTIR analysis (as presented in Figure S4) further confirmed the formation of the thiol-maleimide covalent bond. DSPE-PEG2000-MAL in ML and thiol group in activated DSPE-PEG2000-SPDP MBs to form thioether bond. DSPE-PEG2000-MAL showed a weak C=O stretch band at 1686.21 cm$^{-1}$, and a broad N-H stretch band at 3600–3200 cm$^{-1}$, centered at 3403.63 cm$^{-1}$. The two stretch bands are characteristic absorption of the secondary amide groups in the structure of DSPE-PEG2000-MAL. Characteristic weak peak of disulphide between 2000-2300 cm$^{-1}$ was diminished in thiol activated lipid representing reduction of disulphide bond in DSPE-PEG2000-SPDP with a generation of new peak at 2552 cm$^{-1}$ representing presence of SH bond. FT-IR spectra of thiol-maleimide conjugate revealed the vibration absorption peak of the C–S bond at 648 cm$^{-1}$, whereas the peak at 2552 cm$^{-1}$ was diminished by the reaction of DSPE-PEG 2000-MAL with activated DSPE-PEG 2000-SPDP.

Figure S5A describes the amount of DOX from DOX-ML-MBs after activation using US and Figure S5B shows the release pattern of DOX from free DOX, DOX-ML and DOX-ML-MBs without stimulation of US. The results clearly demonstrated that the US highly promotes the DOX release from ML. It was observed that, even after the exposure of US for 120 s, complete release could not be attained which reveals that the drug was still entrapped in the ML. These ML with the assistance of Mg could still result in higher accumulation of DOX at ROI after disruption of endothelial barrier. However, without US the release of DOX-ML-MBs was much slow then DOX-ML. The result might be attributed to the covalent bonding of ML and MBs which could have reduced the surface area of ML exposed to the release medium, further reducing the diffusion of DOX from the ML.
Figure 2: A) Schematic illustration of the DOX loaded magnetic microbubbles. B) thiol-maleimide covalent linkage between liposomes and microbubbles

Figure 3: A) Particle size distribution of magnetic liposomes-microbubbles complex (DOX-ML-MBs). B) Bright field image of DOX-ML-MBs. C) Confocal overlay image of DOX-ML-MBs. D) Fluorescent image of DOX-ML-MBs (an enlarged image of a single microbubble at the top right corner) showing the red fluorescent generating from DOX-ML confirming the conjugation of liposomes to the surface of microbubbles.
3.4. Magnetic responsiveness of DOX-ML-MBs

Figure 4A illustrates the experimental setup for testing the magnetic responsiveness of DOX-ML-MBs, with the design details of the microfluidic device described in Figure S6. Figure 4B compares the amount of DOX-ML-MBs retained in the microfluidic channels due to the magnetic force along with the unaffected DOX-ML-MBs flushing out with the flow. DOX-ML-MBs have a significantly higher affinity toward the Mg in comparison with absence of Mg (p < 0.01). Figure 4C shows the microscopic view of the DOX-ML-MBs accumulated inside the microfluidic channel by the magnet field.

![Figure 4: A) Experimental setup microfluidic devise to evaluate the magnetic property of DOX-ML-MBs. B) Magnetic microbubbles retained after introducing them into the microfluidic chip in the presence and absence of a fixed magnet. C) Microscopic view of microfluidic chip after introducing the magnetic microbubbles with or without influence of Mg.](image)

3.5. Cytotoxic effects of DOX-ML-MBs on cells

Figures 5 A and 5B compare the anti-cancer outcomes after different treatments in Panc02 and BxCp-3 cancer cells, respectively. Both cell lines show similar patterns of therapeutic differences after these treatments. Compared with control, applying US pulses induces no significant cell death or cell detachment, whereas applying US pulses at the same intensity in presence of blank MBs (“MBs(+US)”) induces minor cytotoxicity. We further evaluated the anti-cancer potencies of different DOX formulations at different doses. At equivalent concentration of DOX, DOX without microencapsulation (“free DOX”) and DOX laden magneto-liposome (“DOX-ML”) yield similar level of anticancer potency, while applying
DOX-ML-MBs followed by US pulses (“DOX-ML-MBs(+US)”) increases the anticancer potency. In comparison, applying DOX-ML-MBs without US pulses (“DOX-ML-MBs (-US)”) yields only slight cytotoxicity, attributed by the reduced DOX release and cellular uptake of the DOX-ML-MB formulation. The therapeutic differences of these treatments were statistically significant (p < 0.01). With the increased level of the equivalent DOX dose, all the DOX formulations show the increased anticancer potency. However, the anticancer potency of “DOX-ML-MBs (+US)” has the largest increase rate so that a much lower therapeutic dose will be needed for effective treatment. These \textit{in vitro} study results imply that localized delivery of DOX-ML-MBs followed by US stimulation could be an effective method for anticancer therapy, superior to other current drug delivery methods.

The cytotoxic effect in COS-7 cells was then evaluated after the treatment with equivalent concentration of DOX in different formulations along with free DOX. The DOX-ML-MBs induced significant (p < 0.01) cytotoxicity to the cells in comparison with free DOX and DOX-ML when US was applied (Figure S7). In addition, no significant toxicity was observed in the cells treated with ML or ML-MBs (without encapsulated DOX) in comparison to the untreated cells. Also, the application of US (parameters as described before) did not cause any significant toxicity (data not shown). The results clearly suggest that the ML-MBs or ML itself were non-toxic to the cells. However, the presence of DOX could cause concentration depended toxicity to the cells which could be significantly enhanced in presence of MBs and ultrasound.

![Graph showing cell viability vs. DOX concentration](image-url)
Figure 5: Plot of % cell viability for (A) Panc02 (B) BxPC-3 after treatment with (i) control (untreated) (ii) US only (iii) MBs + US (iv) free DOX (v) DOX-ML (vi) DOX-ML-MBs (vii) DOX-ML-MBs + US. *: p < 0.01, #: p < 0.05 in comparison with DOX-ML-MBs + US.

3.6. Intracellular uptake

The intracellular uptake of DOX-ML-MBs in Panc02 cells assisted with US was evaluated using FACS and confocal fluorescent microscope studies. The results were compared with uptake of free DOX and DOX-ML at equivalent concentration of DOX.
Figure 6: A) Cellular uptake of (i) Control (ii) Free DOX (iii) DOX-ML-MBs – US (iv) DOX-ML (v) DOX-ML + US (vi) DOX-ML-MBs+ US using Fluorescence-activated cell shorter (FACS) in Panc02 cells. PTX-Hyb-MPs. B) The corresponding mean fluorescent intensity of data represented in A, *, p < 0.05 DOX-ML-MBs + US vs DOX-ML + US; #, p < 0.01 DOX-ML-MBs + US vs DOX-ML. ‡,p < 0.05 DOX-ML vs DOX-ML-MBs - US; †, p < 0.05 DOX-ML vs free DOX. C) Confocal microscopic image of Panc02 cells following by incubation of Free DOX, DOX-ML, DOX-ML+US, DOX-ML-MBs – US, DOX-ML-MBs+ US at an equal concentration of DOX (0.5 μg/mL).
The uptake of DOX by the cells using FACS and confocal microscope has been illustrated in Figure 6 (A&B) and Figure 6C respectively. The results obtained by both FACS and confocal microscope were complimentary to each other, where the effect of US could be seen clearly. The DOX-ML-MBs resulted in significantly (p < 0.01) higher uptake in presence of US when compared with free DOX, DOX-ML and DOX-ML-MBs in absence of US. Also, the uptake of DOX-ML-MBs was negligible without US. The results clearly demonstrate the efficiency of US in cellular uptake which could be attributed to the sonoporation effect caused by US resulting in higher uptake of DOX-ML-MBs.

3.7. Apoptosis analysis

Next, we performed a widely used Annexin V-FITC binding-based flow cytometric assay for a quantitative determination of cancer cell selective apoptosis inducing properties of the formulations containing equivalent amount of DOX (2 μg/mL). Panc02 cells treated with i) plain media (“Control”), (ii) US only without MBs (“US only”), (iii) MBs followed by US activation (“MBs(+US)”), (iv) Free DOX, (v) DOX-ML, (vi) DOX-ML-MBs (-US), and (vii) DOX-ML-MBs (+US) containing DOX (2 μg/mL equivalent concentration) for 24 h. The result showed significantly higher population of apoptotic cells for DOX-ML-MBs (+US) compared to free DOX and DOX-ML (Figure 7). Treatment with DOX-ML-MBs (+US), showed around 55.91 % apoptosis including early and late apoptosis, while treatment with free DOX and DOX-ML resulted in around 16.84 % and 37.2 % of apoptosis. The apoptosis induced by US treatment to the cells in absence and presence of blank MBs was also evaluated which resulted in 5.56 % and 9.81 % respectively. However, DOX-ML-MBs (-US) resulted in minimum effect to the cells resulting in 5.85 % of apoptosis to the cells. The results clearly demonstrate the effect of US which significantly enhance the apoptosis effect in the cells. The results might be attributed to the higher penetration of drug in the cells due to the effect of US.
3.8. In vivo study of magnetic and US mediated drug delivery

The drug delivery effects of different formulations upon magnetic and US stimuli were studied in vivo. ICG was used as a model drug for the study and its bio-distribution was evaluated by acquiring fluorescence images using a Xenogen IVIS® spectrum system. Healthy mice without tumor xenografts were used for the drug delivery study in order to eliminate the sample-to-sample deviations in tumor size and heterogeneity. For each animal,
magnetic and/or US stimuli were applied in the ROI (left hind leg), while the counterpart, the right hind leg was used as a blank control without any stimuli.

Figure 8: Bioluminescence images of mice after the intravenous administration of different treatments. A) US induced ICG deposition in mice injected with (i) Group-I ML + Mg (ii) Group–II ML + US + Mg (iii) Group–III physical mixture of ML + MBs + US + Mg (iv) Group–IV ML-MBs + US + Mg. The mice receiving magnetic microbubbles showed the highest ICG delivery. B) Average radiant efficiency of different groups after initial and 1 day. The ML-MBs + US + Mg revealed significant increase #: p < 0.05 †: p < 0.05 *: p < 0.05 in radiant efficiency compare to ML + Mg, ML + US + Mg, ML + MBs + US + Mg groups respectively.

Figure 8A shows the representative fluorescence images acquired immediately after treatment at Day 0 and at Day 1 for four treatment groups. Fluorescence intensities on the left hind legs
are significantly higher than those on the right hind legs and fluorescence emission consistently lasts for more than one day, indicating the enhanced drug delivery and the sustained drug release upon exposure to magnetic and US stimuli. Figure 8B plots the averaged fluorescence intensities within ROIs of the left hind legs acquired Day 0 and Day 1 after all the treatments. The results shows that tail vein injection of the ML-MB formulation in the presence of magnetic field followed by US stimulation dramatically increases the drug distribution and its superiority to other drug delivery methods was statistically significant.

Figure 9: Section of muscle tissue and skin tissue after intravenous administration of FITC-ML-MBs of the same mouse. “Targeted muscle tissue” and “Targeted skin” represent the left limb and the respective skin exposed to US and Mg. “Control muscle tissue” and “Control-skin” represent the muscle tissue and the respective skin of the right limb without any exposure to US or Mg. The fluorescence images reveal the enhanced penetration of FITC-ML due to magnetic accumulation and US activation. (Scale bar 500 μm).

The magnetic and US mediated drug delivery effect was further analyzed at the microscopic level by using FITC as the model drug. Figure 9 shows the fluorescence images of the tissue
samples after magnetic and US mediated accumulation of FITC-ML-MBs (left hind leg), in comparison with the control tissue samples (right hind leg). The magnetic and US stimuli significantly increase the non-invasive and deep penetration of the model drug, much superior to the control tissue without stimuli. The results reveals the combinatory effect of sonoporation and magnetic accumulation that might play a pivotal role in enhanced drug delivery to the selected tissue target after intravenous administration.

Figure 10: HE staining microscopic images of skin and muscle tissues after the intravenous administration of DOX-ML-MBs in the same mouse. Targeted tissue represents the left limb and respective skin exposed to US and Mg whereas control tissue represents the muscle tissue and respective skin of right limb which do not have any exposure to US or Mg. A and B represent the skin tissues whereas C and D represent the muscle tissues of control (non-targeted tissues) and targeted tissues respectively. The images are shown at 40× magnification. The arrow represents the tissue damage and necrosis in the treated tissues due to US.

To further evaluate the possible tissue damage induced by magnetic and US mediation of ML-MBs, we collected the skin tissue directly in contact with the US probe and the targeted muscle tissue underneath the skin for pathologic analysis. H&E stained microscopic images in Figure 10 show slight abnormalities with the sign of necrosis in epidermis, dermis and muscle tissues after exposure to magnetic and US stimuli. In comparison, control tissues
without external stimuli show no sign of abnormalities. No other sign of tissue damage or abnormalities was observed in other vital organs after the treatments (data not shown).

3.9. In vivo anti-cancer efficiency

DOX-ML-MBs potential for in vivo efficacy in pancreatic xenograft model of Balb/c nude mice after intravenous injection was investigated. The treatment was started when the tumor volume reached \(\sim 150 \text{ mm}^3\) in mice. Change in body weight of mice and tumor volume were measured throughout the study and animals were sacrificed after four weeks following the treatment initiation. The tumor volumes versus time curve and difference in body weight after treatment are presented in Figure 11 (A&B). Figure 11C represents the morphology of tumor isolated from different groups at the end of the study. There was no sign of any obvious side effect for any of the control, free DOX, DOX-ML, DOX-ML-MBs (-US) and DOX-ML-MBs (+US) treated Groups. The body weights of mice increased with the time and slight effect was observed after the treatment. Reduction in the tumor volume was observed during the treatment in all the groups except the control group. At the end of the study, it was found that the volume of tumor in mice treated with DOX-ML-MBs (+US+Mg) were significantly lower in comparison with the other aforementioned groups. The final tumor volume in mice treated with DOX-ML-MBs (+US+Mg) was lower than control (p < 0.001), free DOX (p < 0.05), DOX-MLs (+US+Mg) (p < 0.05) and DOX-ML-MBs (+US) (p < 0.05) treated group respectively. DOX-MLs (+US+Mg) did reduce the tumor volume; however, DOX-ML-MBs (+US+Mg) tumor inhibition was significantly higher.

![Graph A](image1.png)  ![Graph B](image2.png)
Figure 11: In vivo antitumor efficacy in a Panc02 cells xenograft nude mice after the treatment with i) plain PBS (“Control”), (ii) Free DOX, (iii) DOX-ML + US + Mg (iv) DOX-ML-MBs +US, and (v) DOX-ML-MBs +US + Mg. A) Change in tumor volume during the treatment B) Change in bodyweight during the treatment and C) morphology of tumor harvested after the completion of study (scale bar: 2cm).

The results indicated that the US plays a significant role in the treatment of tumor. The treatment was much more effective in the presence of a Mg. The results could be attributed to the sonoporation effect produced by the US. The results also suggest that the use of Mg might have enhanced the accumulation of DOX loaded in magnetic particles at the tumor site resulting in high accumulation of DOX. The presence of iron oxide NPs, in tumor sections further confirmed the influence of US and Mg. As represented in Figure 12 (A-D), the iron oxide NPs are seen in blue in the groups other than the control and free DOX which do not contain any iron oxide NPs. The results revealed that under the influence of US and Mg the DOX-ML-MBs (+US + Mg) treated group have deeply penetrated iron oxide NPs and could be observed throughout the tumor tissue. The tumor isolated from mice treated with DOX-ML-MBs (+US) and DOX-ML (+US+Mg), also revealed the presence of iron oxide NPs. However, the occurrence was not obvious as in DOX-ML-MBs (+ US + Mg). The results clearly suggest that US could play a significant role in deeply penetrating the tumor tissue, which could result in much higher concentration of chemotherapeutics. The use of Mg also significantly influences the localized accumulation of DOX containing iron oxide NPs.

Hematological and biochemical parameters were also evaluated to study the effect of treatment in the mice and were compared with control group. The results have been illustrated in supporting information Table S1. Further toxicity was evaluated by examining the histology of the organs of treated mice by H&E staining. Figure 12E represents the
histology images of tumor, heart, liver, spleen and kidney after H&E staining. The major variations in different groups were observed in tumor sections, where the control group have healthy tumor cells with normal physiology. In comparison, the treated group tumor sections revealed the clear sign of apoptosis with highest in DOX-ML-MBs (+US + Mg) group. The arrow head represents the region of apoptosis in the figure. A very slight toxicity was observed in the heart tissue of free DOX treated group, however other groups did not show any sign of toxicity in heart tissue. The results might be attributed to the non-targeted distribution of DOX. Also, it has been reported that the DOX could be associated with the cardiotoxicity. However, no signs of toxicity were present in liver, spleen, lung, and kidney. The results could be well ascribed to the accumulation of DOX at the tumor site preventing systemic toxicity.

The hemolytic potential of DOX-ML-MBs was evaluated using fresh RBCs obtained from fresh blood of mice. The DOX-ML-MBs, free DOX and DOX-ML revealed negligible (< 4.92 ± 0.41%) hemolysis at highest equivalent concentration of DOX (5 μg/mL) in the absence of US. However, we observed that the application of US exhibited enhanced % hemolysis, which increased with the increase in acoustic pressure. The US in presence of DOX-ML-MBs causes significant increase in % hemolysis, which was observed to be 24.79 ± 2.81 at 1 MPa acoustic pressure, whereas in absence of DOX-ML-MBs it was 7.61 ± 0.85%, respectively. The results of hemolytic potential have been illustrated in Figure S8 (A&B).
Figure 12: The tumor sections when treated with Prussian blue staining, A) Control group with no stain, B) DOX-ML (+US), C) DOX-ML-MBs (+US) and D) DOX-ML-MBs (+US + Mg). The substantial amount of iron oxide was observed in DOX-ML-MBs (+US + Mg) group revealing the significant role of US and Mg in the accumulation of NPs. Scale bars: 20 μm. E) The sections of the tumor and other vital organs of the mice after the treatment were examined for any change in histology using H&E staining. The apoptotic section of tumor isolated from different groups were indicated by the arrow head. A slight toxicity was also observed in the heart section of the mice treated with free DOX. However, other organs were normal in histology. The scale bar represents the 50 μm

4. Discussion

The present work reports design and validation of a novel ML-MBs formulation for systemic delivery and localized release of anticancer therapeutics mediated by both magnetic and US fields. Efforts were made to design the DOX-ML-MB formulation for reliable magnetic retention of MLs and effective US fragmentation of MBs. First, citrate acid stabilized MNs were loaded in liposomes for adequate paramagnetic properties, functionalized with maleimide, which could covalently attach with activated thiol-lipid to form a stable conjugate. Second, PFC gas was filled in MBs for stably long circulation life time and showed appropriate acoustic responsiveness. In preparation for DOX-ML-MBs, different lipid components contribute to the overall performance of the conjugate. For example, cholesterol provides the rigidity to the structure whereas PEGylated lipids provides the stearic stability.
and make them stealthy\textsuperscript{36}. Third, conjugation of MLs on the surface of MBs forms a stable complex of multifunctional drug delivery carrier with modified interfacial tension and diffusivity of a gas bubble in liquid\textsuperscript{37}. Finally, entrapment of DOX and MN in DOX-ML-MBs provides the tools for targeted therapy. Although the MRI properties of these microparticles have not been explored yet, we believe that the DOX-ML-MBs we developed can also be used as a contrast enhancement agent in MRI\textsuperscript{38}.

In the synthesis of DOX-ML-MBs, the major physical and chemical properties of the conjugate were characterized through benchtop, in vitro and in vivo experiments. However, precise measurement of the exact amount of liposomes on the surface of MBs was technically difficult since both the liposomes and MBs synthesized at the current stage had broad size deviations. Instead, a rough estimation about the amount of MLs present on the surface of MBs after covalent bonding was made based on the volume and surface area method as reported elsewhere\textsuperscript{39}. Similarly estimation of the amount of drug, MNs, and lipid contents were made wherever required. The release experiments clearly show the need of ML, as after the US activation entire drug loaded in DOX-ML-MBs could not be released. The presence of external magnetic field and US induced sonoporation could accumulate the ML deep in the desired tissue and subsequently release the drug causing high toxicity to the intended target.

We previously developed multiple microencapsulation techniques, such as co-axial flow focusing\textsuperscript{40}, co-axial electrospray\textsuperscript{41}, and co-axial interface shearing\textsuperscript{42}, for production of drug-laden micro and nanoparticles with almost 100\% encapsulation efficiency, uniform size distribution, and high productivity. By applying these novel microencapsulation techniques, we are expected to optimize the physical and chemical characteristics of the DOX-ML-MBs formulating for further improved drug delivery outcome.

In the benchtop experiment, we demonstrated magnetic accumulation of MLs in a microfluidic vascular phantom. For proof of concept, the phantom design was simplified and did not resemble the physical and physiologic properties of biologic tissue. We have previously fabricated multilayered phantoms that simulate both structural and functional heterogeneities of tumor tissue\textsuperscript{43}. We have also synthesized artificial red blood cells to simulate oxygen dynamics in biologic tissue\textsuperscript{44}. These tissue-simulating platforms will allow us to further optimize the process parameters and performance characteristics of the proposed DOX-ML-MBs formulation for the maximal drug delivery outcome.

In the in vitro experiment, we revealed that applying US pulses alone induced minimal cytotoxicity to cancer cells, while cytotoxicity and apoptosis increased significantly in the presence of both DOX-ML-MBs and US, even at low drug concentrations. This result might
be attributed to the enhanced drug distribution and cell permeability caused by US fragmentation of MBs. As the result of multiple sonophoresis mechanisms, US pulses facilitated the entry and accumulation of DOX and DOX-MLs in cancer cells.

In the animal experiment, we administered ICG-ML-MBs in mice under the influence of external Mg in the presence and absence of US. Instead of DOX, fluorescence dyes were used as model drugs in order to facilitate fluorescence imaging evaluation of drug delivery efficiencies for different formulations. Our study revealed significant allocation of fluorophore labeled ML-MBs at the targeted site with significantly greater concentration and much longer life time in comparison with other formulations. Further, we conducted the in vivo anti-cancer activity in xenograft nude mice with Panc02 cancer cells implanted. DOX-ML-MBs treated group along with US and external Mg revealed significant reduction in the tumor volume in comparison with all the other groups. These phenomena might be attributed to acoustic cavitation of MBs that disrupts the endothelial layer at the particular site and causes the enhanced penetration and retention of therapeutics. Additionally, when external magnetic field was applied, the maximum drug from ML which was not released during the acoustic cavitation could also be accumulated in the desired tissue. However, in the absence of magnetic field, acoustic cavitation of MBs was not that effective. The investigation of the influence of applied US and MBs disruption on skin and the targeted tissue revealed necrosis, whereas the controls were normal and revealed no damage. In the long term, it is hoped that this approach will lead to earlier treatment of pancreatic tumors with precise targeting.

5. Conclusion

In summary, we designed and validated a novel DOX-ML-MBs formulation for magnetic and US mediated drug delivery in targeted tissue. DOX was loaded in MLs and covalently conjugated with PFC gas loaded MBs to form DOX-ML-MBs. In vitro studies showed that DOX-ML-MBs in presence of US pulses were highly effective in killing both BxPc3 and Panc02 pancreatic cancer cells. In-vivo administration of DOX-ML-MBs in mice revealed high accumulation at the magnetically targeted tissue with significant tumor regression in presence of US and Mg. Our study demonstrated the technical potential for site specific targeting and controlled release of anti-cancer drugs with increased efficiency and reduced side effects. Although the current study focuses on the treatment of pancreatic cancer, the proposed drug delivery technique is transferrable to the treatment of many other tissue malignancies where triggered release of therapeutics is required.
Supporting Information
The supporting information is available with this article which includes synthesis method and characterization of superparamagnetic nanoparticles, acoustic release study of DOX from DOX-ML-MBs, design and fabrication method of the microfluidic device, % hemolysis caused by free DOX, DOX-ML, DOX-ML-MBs and hematological parameters of control and treatment groups of mice.

Disclosure statement
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