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Ultrasound-guided Delivery of microRNA Loaded Nanoparticles into Cancer

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Abstract

Ultrasound induced microbubble cavitation can cause enhanced permeability across natural barriers of tumors such as vessel walls or cellular membranes, allowing for enhanced therapeutic delivery into the target tissues. While enhanced delivery of small (<1 nm) molecules has been shown at acoustic pressures below 1MPa both *in vitro* and *in vivo*, the delivery efficiency of larger (>100 nm) therapeutic carriers into cancer remains unclear and may require a higher pressure for sufficient delivery. Enhanced delivery of larger therapeutic carriers such as FDA approved pegylated poly(lactic-co-glycolic acid) nanoparticles (PLGA-PEG-NP) has significant clinical value because these nanoparticles have been shown to protect encapsulated drugs from degradation in the blood circulation and allow for slow and prolonged release of encapsulated drugs at the target location. In this study, various acoustic parameters were investigated to facilitate the successful delivery of two nanocarriers, a fluorescent semiconducting polymer model drug nanoparticle as well as PLGA-PEG-NP into human colon cancer xenografts in mice. We first measured the cavitation dose produced by various acoustic parameters (pressure, pulse length, and pulse repetition frequency) and microbubble concentration in a tissue mimicking phantom. Next, *in vivo* studies were performed to evaluate the penetration depth of nanocarriers using various acoustic pressures, ranging between 1.7 and 6.9 MPa. Finally, a therapeutic microRNA, miR-122, was loaded into PLGA-PEG-NP and the amount of delivered miR-122 was assessed using quantitative RT-PCR. Our results show that acoustic pressures had the strongest effect on

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cavitation. An increase of the pressure from 0.8 to 6.9 MPa resulted in a nearly 50-fold increase in cavitation in phantom experiments. *In vivo*, as the pressures increased from 1.7 to 6.9 MPa, the amount of nanoparticles deposited in cancer xenografts was increased from 4- to 14-fold, and the median penetration depth of extravasated nanoparticles was increased from 1.3-fold to 3-fold, compared to control conditions without ultrasound, as examined on 3D confocal microscopy. When delivering miR-122 loaded PLGA-PEG-NP using optimal acoustic settings with minimum tissue damage, miR-122 delivery into tumors with ultrasound and microbubbles was 7.9-fold higher compared to treatment without ultrasound. This study demonstrates that ultrasound induced microbubble cavitation can be a useful tool for delivery of therapeutic miR loaded nanocarriers into cancer *in vivo*.

Keywords

Image guidance; ultrasound; cancer; drug delivery; nanocarriers; therapy

1. Introduction

Nanometer scaled particles prepared with natural or synthetic polymers, lipids, or inorganic solids have been widely investigated as therapeutic carriers for anti-cancer drugs [1]. These particles, once injected into the bloodstream, can passively accumulate in tumors due to the leaky tumor vasculature and reduced lymphatic drainage, a phenomenon called enhanced permeability and retention (EPR) effect [2]. However, the delivery efficiency via EPR is low: less than 5% of the administered particles accumulate in the tumor [3]. Furthermore, it has been shown in animal models that the EPR effect is highly heterogeneous in different tumor models, and even within a single tumor [4, 5]. Little is known about the EPR effect in humans. One clinical study found substantial variation of the delivery dose of liposomes via the EPR effect ranging between 2.7 and 53% injected dose/kg in patients with various tumors including breast, lung, and head and neck cancers [6]. Therefore, therapeutic delivery of drugs relying only on the EPR effect likely leads to unreliable and unpredictable clinical outcomes. An active delivery approach augmenting drug delivery into tumors is critically needed.

Drug delivery facilitated by ultrasound (US) and contrast microbubbles (MB) is a promising active delivery approach. This approach utilizes the interactions between US and clinically used MB to create openings on a nearby surface, such as the vessel wall and/or cell membranes, allowing for increased particle permeability across natural barriers, a process termed “sonoporation”. Sonoporation is highly associated with cavitation, which involves expansion, contraction, and/or collapse of gas-filled MB. The expansion and contraction of MB can directly 'push' and 'pull' a nearby surface and induce fluid jets and microstreaming in the surrounding fluid [7, 8]. These MB activities were estimated to produce shear stress as high as 74 kPa, and circumference stress of 0.43 - 6 MPa [9, 10]. Such high stress is sufficient to cause deformation or rupture of the nearby surface (cellular membranes or vessel walls) observed in several previous studies [11, 12]. Sonoporation has been investigated for the treatment of preclinical brain [13], liver [14], pancreatic [15], breast

[16], and ovarian cancers [17], and recently in a first in man clinical case study for adjuvant treatment of locally advanced pancreatic cancer [18].

The feasibility of US and MB facilitated delivery has been demonstrated using model drugs with sizes varying from < 1 nm to $10\text{ }\mu\text{m}$, with the majority being less than 20 nm [19]. Delivery of larger (>100 nm) therapeutics or therapeutic carriers is more challenging because higher energies may be needed to create larger openings for the particles to pass through [20]. However, delivery of large therapeutics has great clinical significance. Many therapeutic agents are either quickly degraded or rapidly cleared by the reticuloendothelial system after free intravenous administration. Encapsulating these therapeutic agents in carriers can increase their circulation time and chance to reach the target tissue. In addition, encapsulation of cytotoxic drugs such as chemotherapeutics can significantly reduce systemic toxicity. Currently, commercially available nanocarriers are produced with sizes ranging between 90 and 300nm (for examples, Doxil is 90 nm [21], Epaxel is 150 nm [22], and Verteporfin is 150 to 300 nm [23] in diameter) to ensure sufficient drug loading capacity.

MicroRNAs (MiRs) are regulatory molecules of cells that regulate the expression of various cellular genes involved in several pathways related to the onset of tumor development, proliferation, drug-resistance and metastasis. It has been found that the MiRs are abnormally expressed in multiple cancer types. Restoration of the miR levels during cancer pathogenesis can affect cancer development, thereby acting as a potent anti-cancer drug [24]. In addition, certain miR can be downregulated in several cancer types [24], thereby potentially having patients with different cancer types benefit from successful delivery of a particular miR. In this study, we investigated the delivery of miR-122, an endogenous miR associated with the pathogenesis of cancers. The down-regulation of miR-122 is responsible for enhanced tumor proliferation and resistance to conventional chemotherapy [25]. Over-expression of miR-122 can induce apoptosis and/or cell cycle arrest [26], inhibition of tumorigenesis and angiogenesis [27], and re-sensitizes various types of cancers, including colon [28] and liver [29] cancers to chemotherapy. Successful delivery of sufficient amounts of miR-122 into tumors to act as new anti-cancer agent is of paramount importance. However, miRs are quickly degraded by nucleases once injected intravenously. Here we proposed to encapsulate the miR-122 into nanocarriers to prevent nuclease degradation. Delivery of miR-122 loaded nanocarriers at the tumor site was then facilitated by US and MB.

To investigate US and MB assisted delivery of nanocarriers, two types of nanocarriers were investigated in this study. The first type was semiconducting polymer nanoparticles (SPN). SPN are fluorescent nanomaterials made with completely organic and biologically nontoxic components [30]. The controllable size, good biocompatibility, and stable fluorescence under physiological conditions make them an ideal biosensor for disease monitoring *in vivo* [31]. SPN were synthesized with a size and shell composition similar to FDA approved therapeutic carriers, such as pegylated poly(lactic-co-glycolic acid) nanoparticles (PLGA-PEG-NP), and served as model nanocarriers. The high fluorescence of the SPN allows for investigation of the amount and spatial distribution of the nanocarriers in tumors. The second type of nanocarriers were FDA approved PLGA-PEG-NP [32], with a similar size

compared to SPN model nanocarriers and were loaded with a tumor suppressor therapeutic microRNA, miR-122 [26, 27, 29, 33].

We hypothesized that successful delivery of nanocarriers can be achieved by onset of inertial cavitation. Experiments were first performed on tissue mimicking phantoms to evaluate the acoustic parameters for successful cavitation. *In vivo* experiments were then conducted to explore a combination of appropriate acoustic parameters resulting in successful delivery of SPN model nanocarriers into human colon cancer xenografts in mice with minimal tissue damage. Finally, the optimal acoustic parameters were applied for the delivery of miR-122 loaded PLGA-PEG-NP into human colon cancer xenografts.

2. Material and Methods

2.1. Experiments in tissue mimicking phantoms

2.1.1. Tissue mimicking phantoms—Agarose-based hydrogel was prepared as tissue mimicking phantom by melting 1% w/v agarose in water. Extra fine graphite powder was added to the phantom during the melting process at 3% w/v, acting as acoustic scatterers for ultrasound imaging. The melted solution was poured in a polycarbonate mold with acoustic windows for both the cavitation induction and detection transducers. A plastic rod of 3 mm diameter was placed in the solution and removed after the gel was completely solidified, creating a cavitation chamber.

2.1.2. Microbubbles—Clinical grade lipid-shelled perfluorobutane-and-nitrogen-filled MB [34] (BR38; kindly provided by Bracco Research, Geneva, Switzerland), were used as the cavitation nuclei in this study. MB were lyophilized and stored in septum-sealed vials with perfluorobutane and nitrogen gas. Prior to each use, MB lyophilisates were resuspended in sterile 0.9% saline. MB had a mean diameter of $1.4 \pm 0.1 \mu\text{m}$ and were neutrally charged (zeta potential $-0.3 \pm 0.3 \text{ mV}$) as assessed by the manufacturer.

2.1.3. Ultrasound apparatus—MB cavitation was induced by US pulses of 1.8 MHz generated by an array transducer (P4-1, Philips Healthcare, Andover, MA) connected to a research platform (V1, Verasonics, Redmond, WA). The pulses were calibrated in degassed water using a needle hydrophone (HNR-0500, Onda, Sunnyvale, CA). The full width at half maximum (FWHM) beamwidths on the pressure profile were calibrated to be 1.4, 10.1, and 12.6 mm in the transducer's X, Y and Z directions, respectively.

Experimental setup of the phantom study is shown in Figure 1. The cavitation initiation transducer was placed on top of the phantom such that the lateral axis was in parallel with the cavitation chamber with a 30 mm standoff distance. MB were then injected into the chamber and exposed to US. During the exposure, cavitation was detected passively by detecting broad band noise emitted from MB collapse and actively by imaging of the destruction of MB using US. For passive cavitation detection, a 10-MHz single element transducer (V312, Panametrics NDT, Waltham, MA) with a -6 dB bandwidth of 5.3-10.7 MHz was used to collect the acoustic scattering from the cavitation chamber with a standoff distance of 45 mm. The two transducers were perpendicular and co-focused at the cavitation chamber. The collected acoustic scattering was amplified by a pulser/receiver (PR5072,

Panametrics, Waltham, MA) and recorded by a digital oscilloscope (DSO8104A, Agilent Technologies, Santa Clara, CA) at a sampling rate of 100 MSamples/s. For active cavitation detection, the 10-MHz passive cavitation detection transducer was replaced with a 5-MHz US imaging transducer (L7-4, Philips Healthcare, Andover, MA) operated with a second Verasonics system (V1, Verasonics, Redmond, WA). B-mode images were acquired before, during, and after the treatment using the second Verasonics system at a low power setting (pressure 0.3 MPa, frame rate 10 Hz) which did not destroy MB. Uncompressed image data of the B-mode images were collected for data analysis off-line.

Effects of four parameters on cavitation were studied: peak negative pressure, pulse length, pulse repetition frequency (PRF), and MB concentration (Table 1). In all series in the phantom studies, the center frequency of US was fixed at 1.8MHz, and a total of 200 pulses were delivered in each exposure. The duration of 200 pulses was selected because the MB in the focal region are completely destroyed within this duration.

2.1.4. Cavitation signal processing—Cavitation activities in the tissue mimicking phantom were analyzed using both passive and active cavitation detection methods (described in the supplementary information SI.1). In brief, for passive cavitation, inertial cavitation dose (ICD) was quantitatively evaluated by measuring the broadband noise emitted during inertial cavitation, as described previously [35]. For active cavitation imaging, the ultrasound imaging signal intensity changes were analyzed and compared for the different US conditions.

2.2. *In vivo* delivery of model nanocarriers

To explore appropriate acoustic conditions for optimal nanocarrier delivery and minimal tissue damage *in vivo*, experiments were conducted to study the delivery profile of the model nanocarriers under different acoustic conditions. Here we focused on studying the effects of varying acoustic pressures on the amount of delivery and the penetration depth, since it was found in the phantom experiments that acoustic pressure had the most significant effects on MB cavitation.

2.2.1. Animal model—All experimental procedures involving animals were approved by the Institutional Administrative Panel on Laboratory Animal Care. *In vivo* experiments were performed on subcutaneous LS174T human colon cancer xenografts established in nude mice (a total of 79 tumors in 50 mice; see supplementary information SI.2).

2.2.2. SPN model nanocarriers—The synthesis of fluorescence SPN is described in the supplementary information (see supplementary information SI.3). The diameter of the SPN was 116 ± 5 nm. These highly fluorescent SPN allowed for assessment of the spatial distribution of nanocarriers delivered into xenografts.

2.2.3. *In vivo* treatment protocol—A total of 41 tumors were treated with SPN. Mice were positioned on the US scanning table and gas anesthetized with 2% isoflurane in 2 L of oxygen per minute. A catheter (MicroMarker Tail Vein Access Cannulation kit; VisualSonics, Toronto, Ontario) was placed into a lateral tail vein for administration of MB and nanocarriers. A total of 350 μ L (3.5×10^8) MB and 150 μ L of SPN (5.6 nM; 5×10^{11})

were mixed into a total solution of 500 μL prior to tumor treatment. The treatment consisted of 5 repetitive cycles of 100 μL MB/SPN mixture administrations combined with US exposures (Figure 2). In each cycle, the MB/SPN mixture was first administered for 1 minute at a constant rate of 100 $\mu\text{L}/\text{min}$ using an automated infusion pump (GeniePlus, Kent Scientific, Torrington, CT) to achieve steady state. This resulted in an estimated concentration of 3.3×10^7 MB/mL in the bloodstream, assuming that the total blood volume of a mouse is 2 mL [36] and that the MB are completely destroyed by US in each treatment cycle. Administration was then paused and US pulses were delivered to the target tumor sites for 1 minute. The US focal beam was electronically steered across 6 locations, 1.5 mm apart each (Figure 2), to allow complete anatomical coverage of the tumors. Each treatment location was exposed to 1000 pulses of 5 cycles in pulse length delivered at a PRF of 100Hz with varying pressures described in Table 2. The exposure duration of 1000 pulses was longer than that in the phantom experiments (200 pulses) because in the *in vivo* condition, the tissue was constantly replenished with MB from the bloodstream. The longer exposure duration allowed for sufficient interaction between cavitation and the vessels, thus increasing the likelihood of sonoporation.

The MB administration rate of 100 $\mu\text{L}/\text{min}$ was selected based on previous experience that MB can be intravenously delivered without being destroyed at that rate [37]. At this administration rate, the rise time of the perfusion curve (defined as the time for the image intensity in the tumor to reach the peak intensity from when the image intensity increase was first detected) was 61 ± 13 sec ($n=4$) as measured by contrast enhanced US imaging using a 21-MHz transducer (MS250, VisualSonics, Toronto, Ontario, Canada) connected to a dedicated small animal US system (Vevo 2100, VisualSonics, lateral and axial resolution of 165 and 75 μm , respectively). Therefore, an administration period of 1 minute was selected to ensure high concentrations of MB at the tumor treatment site. To minimize the overall injection volume to a total of 500 μL in each mouse, MB administration was paused during US exposure.

To provide image guidance during tumor treatment, real-time US imaging was performed in all animals using the same transducer (MS250, VisualSonics) and small animal US system (Vevo 2100, VisualSonics). Contrast-enhanced US imaging was performed using the nonlinear contrast mode [38] at a frame rate of 5 frames per second. Image data were collected from 10 sec before to 30 sec after the treatments. The perfusion and destruction of MB as indicated by the increase and decrease of the image intensity were visually inspected during the treatments as well as quantitatively assessed post treatments. The image data acquired during the treatment were processed using dedicated software (VevoCQ, VisualSonics). The temporal change in the mean image intensity within the tumor region was obtained for the entire treatment process. The mean image intensity was further normalized to the baseline image intensity before the treatment. The maximum image intensity and the post-treatment image intensity were compared among groups treated with different pressures.

2.2.4. Ex Vivo Analysis—All animals were euthanized 4 hours post treatment and all tumors were excised for *ex vivo* analysis. Excised tumor tissues were fixed in 4% paraformaldehyde overnight at 4°C and then cut in halves at the center of the xenografts.

One half was immersed in a 30% sucrose solution for cryoprotection, and then embedded in optimal cutting temperature (OCT) media for immunofluorescence analysis of model nanocarrier tumor delivery. The other half was immersed in 70% ethanol, and then embedded in paraffin for histological analysis of tissue damage following Hematoxylin and Eosin (H&E) staining.

Immunofluorescence Analysis of Model Nanocarrier Tumor Delivery: To study the delivery profile of the model SPN nanocarriers, confirm extravasation of the nanocarriers into the tumor parenchyma, quantify the extent of parenchymal delivery including the distance between nanocarriers and tumor vessels, quantitative immunofluorescence analysis was performed on tissue sections stained for both the vascular endothelial cell marker CD31 and the cytoskeleton F-actin marker.

Two slices, 10- μ m and 50- μ m-thick, were cut from each tumor halves. The 10- μ m slices were used to quantitatively assess the extent of nanocarrier delivery across the entire tumor diameter; the 50- μ m slices were used to volumetrically quantify the distance of nanocarrier delivery in relation to tumor vessels. The slices were stained for CD31 and F-actin (see supplementary information SI.4). Tissue slices were imaged with a wide field fluorescence microscope (AxioImager microscope, Carl Zeiss GmbH, Jena, Germany) at 20X magnification. Images of the SPN, CD31, and F-actin were captured in separate color channels (excitation/emission wavelengths were 470/525nm, 550/605nm, and 640/690nm, for SPN, CD31, and F-actin channels, respectively). Raster scans were obtained across the tissue using a motorized stage controlled by the microscope image acquisition software (AxioVision, Carl Zeiss GmbH, Jena, Germany). This scan allowed for reconstruction of the immunofluorescence image of the entire tumor section.

To evaluate the amount of SPN delivered into the tumor, quantitative image analysis was performed using Matlab (MathWorks Inc, Natick, MA). Images of the SPN, CD31, and F-actin channels were separated. In each channel, a pixel intensity threshold was set to be mean + 5 $\times$ standard deviation of the pixel intensity in a background area where no vessel/cellular structures existed. This threshold was chosen to include fluorescent pixels from SPN or positive staining of CD31/F-actin and exclude pixels with autofluorescence, as described previously [39]. Pixels with intensities below this threshold were considered interference from autofluorescence and were excluded. The pixel area with positive signals was calculated for each channel and integrated over all images captured for each tumor, producing a total area of SPN, CD31 or F-actin. The total area was assumed proportional to the amount of SPN, vessels, or cells in each tumor. Next, two ratios were calculated: The first was the ratio of total area of SPN to the total area of CD31, and the second was the ratio of total area of SPN to the total area of F-actin. The first ratio normalized the amount of SPN to the amount of vessels in a tumor, allowing for comparison between tumors regardless of various degrees of vascularization. The second ratio normalized the area of SPN to the area of cells, indicating the portion of a tumor that may be exposed to the therapeutic carriers.

To confirm extravasation of nanocarriers into tumor parenchyma and to measure the distance of nanocarriers from the vessels, 3D confocal laser microscopy was performed on 50- μ m-thick sections (LSM510 Meta confocal microscope, Carl Zeiss GmbH, Jena,

Germany). Z-stack imaging was performed through the entire section with a 1 μm increment. Measurements of the location of SPN with respect to the vessels were made on the reconstructed 3D images using a 3D image processing software (Imaris, Bitplane, South Windsor, CT, USA). The penetration depth was measured as the distance from the SPN to the closest vessel. To study effects of the acoustic pressures on the penetration depth, a total of 54 measurements were made for each acoustic pressure.

Histological Analysis of Tissue Damage: After fixation in 10% neutral buffered formalin, tumor tissues were cut into 4- μm sections, stained for H&E using a routine protocol [40], and analyzed for tissue damage by a board-certified veterinary pathologist using an Olympus BX-41 microscope (Olympus, Center Valley, PA). The degree of tissue damage within (intratumoral) and around (peritumoral) each tumor was assessed in a blinded fashion (blinded to the US treatment conditions applied to each tissue sample) and was defined as the percentage of the total cross-sectional area that was affected by acute hemorrhage (extravasation of red blood cells out of blood vessels) and graded using a 5-grade Likert score (Table 3). The intratumoral hemorrhage grade was assessed in the entire tumor region while the peritumoral hemorrhage grade was obtained from a surrounding region within 0.5 mm from the tumor border. Representative digital photomicrographs were taken using an Axioscope 2 Plus microscope (Carl Zeiss, Thornwood, NY) with a Nikon DS-Ri1 digital microscope camera (Nikon, Melville, NY) and NIS-Elements imaging software (Nikon, Melville, NY).

2.3. Delivery of microRNA-122 encapsulated in PLGA nanoparticles

2.3.1. Synthesis of PLGA-PEG nanoparticles—Control PLGA-PEG-NP and PLGA-PEG-NP loaded with miR-122 were synthesized as described in the supplementary information SI.5. The diameters of control PLGA-PEG-NP and miR-122 loaded PLGA-PEG-NP were 110.6 ± 23 and 115.3 ± 18 nm, respectively. The surface charge, miR-122 loading percentage, and number of miR molecules loaded per nanoparticle in PLGA-PEG-NP can be found in supplementary information SI.5.

2.3.2. *In vivo* tumor delivery of miR-122-loaded PLGA nanoparticles—Optimal acoustic parameters resulting in maximum nanocarrier delivery at minimal tissue damage were chosen to deliver the miR-122 loaded PLGA-PEG-NP *in vivo* (detailed parameters listed in Table 2). The experimental setup and treatment protocols were the same as those described for the SPN model nanocarriers (Figure 2).

Nine mice with a total of 18 tumors (one on each side) were treated. US treatment was performed in one of the tumors and in the same mice the tumor on the other flank served as an untreated intra-animal control. Three mice received control PLGA-PEG-NP without miR-122, and remaining 6 mice received miR-122 loaded PLGA-PEG-NP.

Four hours after treatment, animals were perfused with phosphate buffered saline for 5 minutes under anesthesia in order to remove excess PLGA-PEG-NP in the systemic circulation. The tumors were excised and snap frozen on dry ice for quantitative reverse transcription polymerase chain reaction (RT-PCR) process for miR-122 quantification. The

RT-PCR protocol and miR-122 quantification method are described in the supplementary information (SI.6).

2.4. Statistical analysis

Values are expressed as mean $\pm$ standard deviation. Linear regression analysis, ANOVA, and the Wilcoxon rank sum test were performed for different experimental series (details are provided in supplementary information SI. 7). A p -value of <0.05 was considered statistically significant.

3. Results

3.1. Experiments in tissue mimicking phantoms

3.1.1. Passive cavitation detection—Effects of the parameters on the cavitation dose measured for the MB in the tissue phantoms are shown in Figure 3. The cavitation increased significantly with increasing pressure ($p < 0.001$), although no significance was found between the two lowest pressures ($p = 0.407$).

The pulse length had negligible effects on the ICD within the tested range ($p = 0.409$). The PRF had a positive influence on cavitation ($p = 0.005$). A PRF of 100Hz resulted in significantly higher ICD compared to PRFs of 10, 20 or 50 Hz ($p < 0.001$) while no significant difference was found among PRFs of 10, 20, and 50 Hz ($p = 0.425$).

Increasing MB concentration resulted in increased ICD ($p < 0.001$). However, no significant difference was found between the ICDs produced at $1 \times 10^8/\text{mL}$ and $2 \times 10^8/\text{mL}$ ($p = 0.138$), suggesting that the increasing trend saturated when MB concentrations exceeded $1 \times 10^8/\text{mL}$.

Acoustic pressure had the most significant effect on cavitation. While increasing PRF and increasing MB concentration caused 3- to 4-fold increase in ICD, increasing pressure from 0.7 to 6.9 MPa caused nearly 50-fold increase in the ICD. This substantial increase in the ICD resulted from the longer duration of enhanced broadband noise within the entire 200-pulse exposure duration. It was observed that for pressures below or equal to 5.4 MPa, the broadband noise level was higher within the first 50 pulses and dropped to the baseline level when more pulses were delivered. This suggested that the MB were destroyed within the first 50 pulses. At the highest pressure of 6.9 MPa, the broadband noise level was higher than the baseline level throughout the entire 200-pulse exposure. This prolonged broadband noise enhancement indicated a prolonged cavitation post MB destruction, which was likely induced from remnants of destroyed MB. Since the ICD was a time integral of the broadband noise, the prolonged broadband noise enhancement resulted in a substantial increase in the ICD.

3.1.2. Active cavitation monitoring—Since the most significant effect on cavitation was induced using varying acoustic pressures, the following *in vitro* and *in vivo* experiments focused on effects of acoustic pressures. Representative US images of the MB solution in the tissue phantoms after exposure to US of varying acoustic pressures are shown in Figure 4a. The image intensity in the focal region decreased with increasing pressures ($p < 0.001$), suggesting more MB being destructed with increasing pressures. An interesting phenomenon

was noted during the exposure of US at the highest pressure, 6.9 MPa. A hyperechoic flickering cluster, likely the cavitating MB, was seen at the focus of the transducer throughout the entire exposure duration (Figure 4b).

3.2. *In vivo* delivery of model SPN nanocarriers

In the total of 41 tumors experimented in this study, no significant difference existed in the sizes of the tumors among groups treated with different parameters ($p = 0.66$).

3.2.1. Ultrasound image monitoring—The perfusion and destruction of MB in the tumor was visualized on contrast enhanced US images (Figure 5). During the administration period, the image intensity in the tumor region increased to a peak of approximately 1.6- to 3-fold higher than the baseline intensity before MB perfusion. No significant difference in the peak enhancement was found among groups treated with different pressures ($p = 0.28$). During the US exposure period, the image intensity decreased and returned to the baseline intensity at the end of the exposure. No significant difference in the post-treatment image intensity was found among groups treated with different pressures ($p = 0.59$). This indicated that most MB were destroyed within the US exposure period, regardless of the pressure levels.

3.2.2. *Ex vivo* immunofluorescence analysis—Immunofluorescence images of tumors treated with increasing US pressures are shown in Figure 6a. In control tumors with no US exposure, small amounts of extravasated SPN could be seen in the vicinity of the vessel area. With increasing acoustic pressures, an increasing amount of extravasated SPN was seen. Quantitative analysis of delivered SPN is summarized in Figure 6b and c.

Both the ratios of the total area of delivered SPN to the total area of CD31 stained tumor vessels as well as to the total area of F-actin stained tumor tissue significantly increased at the lowest US pressure used (1.7 MPa) compared to no US ($p = 0.025$) and both ratios further increased with increasing pressures ($p < 0.0001$; Figure 6b and c). The ratio of the total area of SPN to the total area of CD31 increased by 3.9 fold at 1.7 MPa compared to the no US control, and it further increased up to 12.6-fold at 6.9 MPa. The ratio of total area of SPN to the total area of F-actin was increased by 2.5-fold at 1.7 MPa and 13.8-fold at 6.9 MPa compared to no US.

3D confocal microscopy further confirmed extravasation of SPN (Figure 7a). With increasing US pressures, the penetration depth of SPN significantly increased ($p < 0.0001$; Figure 7b). The median penetration depth produced by 6.9-MPa US was approximately 3-fold higher than that observed without US. A maximum penetration depth of nearly 45 μm was observed in the tumors treated with the highest pressure, 6.9 MPa.

3.2.4. Histological examinations—US induced MB cavitation caused only minimal histologically detectable tissue damage, visualized as intra- and peri-tumoral hemorrhage (Figure 8a). Hemorrhage was present in both non-treated and treated tumors. Increasing acoustic pressures had no significant effect on the intratumoral hemorrhage grade ($p = 0.48$), but caused increased peritumoral hemorrhage grade ($p = 0.03$). The overall hemorrhage

grades increased with increasing pressures ($p = 0.04$), which was mainly attributable to the peritumoral hemorrhage (Figure 8b).

3.3. Delivery of miR-122 encapsulated PLGA-PEG nanoparticles

Quantitative RT-PCR of miR-122 delivered into human colon cancer xenografts showed significantly enhanced levels of miR-122 in animals administered with miR-122 loaded PLGA-PEG-NP compared to the control settings (treatments with US and MB but without injection of either type of PLGA-PEG-NP, and treatments with US and MB and injection of control PLGA-PEG-NP), and were further increased when US and MB were applied (Figure 9). The levels of miR-122 in tumors treated with US were increased by an average of 1310 ± 962 fold (range: 270 to 3100 fold), while the levels of miR-122 in contralateral tumors without US were only increased by an average of 224 ± 164 fold (range: 20 to 508 fold) compared to the endogenous levels of miR-122 in untreated animals. Treatments with US and MB significantly increased the levels of miR-122 ($p = 0.004$). Within an animal, the levels of miR-122 in the tumor exposed to US were on average 7.9-fold higher than the contralateral tumor without exposure to US.

4. Discussion

Our study showed that FDA-approved PLGA-PEG-NP loaded with therapeutic miR-122 can be successfully delivered into the extravascular compartment of human colon cancer xenografts using an optimized US-guided drug delivery protocol. To our knowledge, this is the first study to demonstrate efficient delivery of miRs into tumors using US and MB mediated sonoporation.

Once injected intravenously, however, miRs are quickly degraded by nucleases. Therefore, miR-122 was encapsulated into PLGA-PEG-NP to protect them from nuclease mediated degradation in our study. However, due to their size, passive accumulation of PLGA-PEG-NP through the EPR effect only yields in minimal accumulation in tumors [41]. We showed that despite their size >100 nm, miR-loaded PLGA-PEG-NP delivery to the extravascular compartment can be successfully enhanced in tumors through sonoporation. To quantify successful miR delivery into the extravascular compartment only, mice were perfused before excising the tumors to ensure that all circulating PLGA-PEG-NP were cleared from the tumor vessels. Using this approach, miR-122 delivery in human colon cancer xenografts was increased by on average 7.9 fold compared to passive accumulation as assessed by quantitative RT-PCR.

Using 3D confocal microscopy, we further explored the penetration depth of nanocarriers in relation to tumor vessels in tumors. Four hours following intravenous administration, we found that nanocarriers penetrated from $5 \mu\text{m}$ through passive accumulation to up to $45 \mu\text{m}$ using sonoporation. To our knowledge, our study is the first to examine the volumetric distribution of nanocarriers within tumors using 3D confocal microscopy, allowing for a more accurate measurement of the penetration depth of extravasated nanocarriers compared to 2D microscopy. While 3D confocal microscopy captures the volumetric distribution of nanocarriers among tortuous vascular structures, 2D microscopy only images a thin slice of the tissue, often overestimating the penetration depth because the shortest distance between

nanocarriers and the vessels may not fall on the imaging plane. With the volumetric information reconstructed from 3D confocal microscopy, the shortest distance between nanocarriers and surrounding vessels could be assessed in our study by projecting the nanocarriers to the nearest vessel in the 3D volume.

Another objective of our study was to assess whether sonoporation using different acoustic pressures causes macroscopic tissue damage. For this purpose, we analyzed treated tumor tissues for the presence of intra- and peri-tumoral hemorrhage as a biomarker of vessel destruction with consecutive blood extravasation. While there was a trend towards slightly increased hemorrhage at higher acoustic pressures, intra- and peri-tumoral hemorrhage were also observed in non-treated control tumors; even at highest tested pressures there was overall small hemorrhage on histology. Similar trends have been reported previously [43]. Notably, overall increased hemorrhage was mainly attributable to peri-tumoral hemorrhage in our study. This is likely because vessel density is often higher at the tumor periphery than at the tumor center [44]. In addition, cavitation may preferentially occur at an interface, such as that between the coupling gel and the superficial subcutaneous tumors used in this study [45]. This interface may not apply to other tumor models where the tumors are established in a more contiguous tissue environment, such as orthotopic tumors. Peri-tumoral hemorrhage may increase the risk of thrombosis in the peritumoral region, producing favorable (e.g., by blocking drug dilution due to reduced blood flow) or undesired effects in the tumor treatment (e.g., by reducing drug delivery following vessel blockage). Future studies are warranted to evaluate the occurrence and extent of hemorrhage in orthotopic tumor models deeper in the body.

Several limitations of our study need to be acknowledged. First, while real-time US imaging was used to document MB destruction, it cannot sufficiently indicate the occurrence of inertial cavitation *in vivo* because the decrease of image intensity can result from several mechanisms, including deflation, dissolution, and/or inertial cavitation of MB [46]. Future work will incorporate real-time passive cavitation monitoring in our treatment platform to evaluate inertial cavitation *in vivo* and its correlation with the delivery outcomes. Second, the range of acoustic parameters tested for optimal drug delivery was restrained by the capabilities of our available US system. Therefore, the optimized parameter set found in our study may not be a universal optimum. Third, the delivery amount and penetration depth reported in this study were obtained in a well vascularized cancer model. In hypovascularized cancer with different morphological characteristics, these measurements may vary. Finally, the amount of free miR-122 released from PLGA-PEG-NP was not quantified because RT-PCR cannot differentiate between free or still encapsulated miRs, and future studies are warranted to assess downstream effects of released miR-122 in colon cancer following successful delivery via sonoporation.

In conclusion, our results suggest that FDA-approved PLGA-PEG-NP encapsulated with miR-122 can be successfully delivered into human colon cancer xenografts using an optimized US and MB mediated sonoporation platform. Guided by US imaging, this drug delivery approach is safe and spatially localized to the anatomical region under consideration. Our results pave the way towards future studies assessing the downstream effects of delivered drugs such as miR-122 in cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

5. Acknowledgements

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Figure 1.

Experiment setup for phantom studies. The cavitation initiation transducer was operated at 1.8MHz to induce microbubble cavitation. Detection of cavitation was performed both passively using a 10-MHz single element transducer as well as actively by using a 5-MHz ultrasound imaging transducer. The cavitation initiation and detection transducers were placed perpendicular to each other and co-focused at the cavitation chamber containing microbubbles.

Figure 2.

In vivo experimental setup and treatment timeline. The focal beam of the cavitation initiation transducer was electronically steered across 6 locations to cover the entire tumor volume. The high frequency imaging transducer was aimed at the target tumors at 45° angle to provide anatomical guidance before the treatment and to monitor the microbubble perfusion/destruction process during the treatment. Microbubble perfusion and ultrasound insonation were alternated in the 10-minute-length treatment windows.

Figure 3.

Inertial cavitation dose (ICD) measured in the tissue mimicking phantom by the 10-MHz passive cavitation detection transducer after varying a) pressure, b) pulse length, c) pulse repetition frequency (PRF), and d) microbubble concentration. Error bars = $\pm$ standard deviation (n=6 each). * $p < 0.001$.

Figure 4.

a) Representative images of microbubble solution in tissue mimicking phantom after exposure to ultrasound at different acoustic pressures. b) Hyperechoic flickering cluster (in the red dashed circle), likely caused by cavitating microbubbles, was seen at the focus of the transducer during the exposure to ultrasound at 6.9 MPa. The hyperechoic vertical lines (blue arrows) are artifacts from interferences of the cavitation pulses. c) The signal intensity in the focal region, marked as red box in panel (a), was plotted against the pressures. Note that significant acoustic shadowing was caused by the concentrated microbubble solution before ultrasound exposure. As microbubbles were destroyed by ultrasound, the shadow disappeared, and the phantom image below the cavitation chamber became visible. The focal region of the cavitation transducer appeared hypoechoic after ultrasound exposure, indicating destruction of microbubbles. The spatial extent of microbubble destruction was broadened with increasing pressures. Data are mean $\pm$ standard deviation ($n=4$ each). Images are displayed with a 40dB dynamic range. Other treatment parameters were fixed at pulse length = 5 cycles, PRF = 100Hz, and microbubble concentration = 1×10^8 /mL.

Figure 5.

Representative contrast-enhanced ultrasound images of a human colon cancer xenograft during a 2-minute treatment cycle. Image signal increased during the perfusion period with microbubbles floating into the tumor, and then substantially decreased during sonoporation. Microbubble destruction occurred quickly after ultrasound application. The discrete drop of image signal intensity (see images at 70 sec, 80 sec, and 90 sec) corresponded to each steering of focal beam.

Figure 6.

a) Representative immunofluorescence images of human colon cancer xenografts treated with different ultrasound pressures show increased amount of SPN (*red*) delivered into the tumor tissue with increasing pressures compared to no ultrasound (control). Note, tumor vessels are stained in green and tumor cytoskeleton was stained in blue. Ratios of the total area of SPN to the total area of CD31 vessel staining (b) and to the total area of F-actin cytoskeleton staining (c) are also shown. Both ratios increased with increasing pressures. Data are mean $\pm$ standard deviation (n= 5 each).

Figure 7.

a) Representative maximum intensity confocal immunofluorescence image reconstructed from 3D confocal microscopy data sets of human colon cancer xenograft treated with ultrasound and microbubbles (6.9 MPa) shows extravasated SPN (*red*) compared to tumor vessels (*green*) in the imaged volume. Orthogonal slices indicated by the gray dashed lines further confirmed presence of SPN in tumor parenchyma (*blue*) and away from the vessel lumen (contoured in *green*). b) Box blot shows changing tumor penetration depth with various acoustic pressure levels. The central mark, upper edge, lower edge, and whiskers of each box indicate median, 75th, 25th percentile, and the range of 54 measurements made for each acoustic pressure level.

Figure 8.

a) Representative H&E images of human colon cancer xenografts treated with different acoustic pressures. Small areas of hemorrhage (as shown in the close-up view of the boxed area) was observed in some of the treated tumors. b) Box plot shows a slightly increasing trend towards higher hemorrhage at higher acoustic pressures. Each dot represents individual data points and the bars label the mean in each group. Note that there was already hemorrhage in non-treated tumors ($n = 5$ each).

Figure 9.

RT-PCR quantification of the fold change in miR-122 in tumors treated with control PLGA-PEG-NP (left, $n = 3$ each) or miR122 loaded PLGA-PEG-NP (right, $n = 7$ each). The miR-122 levels were expressed as fold changes relative to the endogenous levels of miR-122 in control animals with neither nanoparticle administration nor ultrasound and microbubble treatments. * $p = 0.004$. ** $p = 0.002$. *** $p = 0.001$.

Table 1

Experimental series in phantoms.

	Peak negative pressure (MPa)	Pulse length	PRF (Hz)	MB concentration (counts/mL)	Total numbers of pulses
Series 1	0, 0.8, 1.7, 2.5, 3.3, 3.9, 4.5, 5.4, 6.9	5 cycles (2.8 μ s)	100	1×10^8	200
Series 2	2.4	3, 5, 7, 10, 15 cycles (1.7, 2.8, 3.9, 5.6, 8.3 μ s)	100	1×10^8	200
Series 3	3	5 cycles (2.8 μ s)	10, 20, 50, 100	1×10^8	200
Series 4	3	5 cycles (2.8 μ s)	100	4×10^6 , 1×10^7 , 2×10^7 , 4×10^7 , 1×10^8 , 2×10^8	200

Table 2

In vivo experimental series. MiR-122 was only loaded in the third experimental group (miR-122 loaded PLGA-PEG-NP) and not in the others (SPN and control PLGA-PEG-NP).

Nanoparticle type	Total injected nanoparticles	Peak negative pressure (MPa)	Number of tumors	Number of animals
SPN	5×10^{11}	0 (control)	16	31
		1.7	5	
		2.5	5	
		3.9	5	
		5.4	5	
		6.9	5	
Control PLGA-PEG-NP	7.3×10^{11}	0 (control)	3	3
		5.4	3	
miR-122 loaded PLGA-PEG-NP	6.5×10^{11}	0 (control)	7	7
		5.4	7	

Table 3

Grading of Tissue Damage following Sonoporation

Grade	Definition
0	No hemorrhage
1	0 - 10 % of area with hemorrhage
2	11 - 25% of area with hemorrhage
3	26 - 50 % of area with hemorrhage
4	> 50 % of area with hemorrhage