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Citation:
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DOI:10.1109/ULTSYM.2018.8580082

This is the accepted version of the article published in Proceedings-IEEE Ultrasonics Symposium 2018

The effect of sonication on extravasation and distribution of nanoparticles and dextrans in tumor tissue imaged by multiphoton microscopy

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Abstract— **Ultrasound (US) and systemic administration of microbubbles (MBs) have been shown to improve the delivery of drugs and nanoparticles (NPs) to tumor tissue. A better understanding of the mechanisms is crucial for effective delivery of NPs and drugs.**

To elucidate the kinetics of extravasation events, and relate it to the vessel diameter and flow, we have performed real-time intravital multiphoton microscopy during US sonication of tumors growing in dorsal window chambers. We studied the effects of four different mechanical index (MI) levels (0.2 to 0.8) while injecting SonoVue® or in-house made MBs with NPs in the shell (NPMB). We found that high MI of 0.8 induced a violent extravasation of both NPs and dextrans. Using lower MIs (0.2-0.6), less extravasation was detected and it occurred in vessels with larger diameters compared to sonication at MI 0.8. The rate of extravasation of both NPs and dextrans, and the displacement of NPs and dextrans from the vessel into the tumor tissue, correlated with MI. The observed extravasation events happened within milliseconds to minutes after the US exposure started. Moreover, we have observed a change of blood flow rate and direction with all the MIs tested.

Keywords—Ultrasound, microbubbles, real time imaging, multiphoton, blood vessel opening, cavitation, blood flow.

I. Introduction

Intravenous delivery of therapeutic agents to a tumor in patients, in optimal quantities with a limited exposure to normal tissue, is challenging. The administered agents cause severe side effects due to accumulation in healthy tissue. However, if the therapeutic agents are encapsulated in nanoparticles (NP), the distribution of the therapeutic agents and amount of drug reaching the tumor may be improved via the enhanced permeability and retention (EPR) effect [1]. However, improved efficacy has not been demonstrated in the clinic. Ultrasound (US) and systemic administration of microbubbles (MBs) have been shown to improve the delivery of drugs and NPs to tumor tissue by manipulating the tumor microenvironment in preclinical studies. Although the exact mechanisms underlying US and MB-mediated drug delivery are still not fully understood, the enhancement of drug accumulation may be due to thermal and/or non-thermal effects. The non-thermal effects are mechanical effects such as cavitation and acoustic radiation force, which are considered the most important mechanisms for therapeutic applications. There are various bioeffects associated with cavitation that enhance the permeability of the vasculature and improve the therapeutic response, but these effects can also cause vascular damage. Therefore, the behavior of MBs in blood vessels has to be well controlled by adjusting the US parameters in order to avoid permanently damaging the blood vessels. Therefore, to further enhance our understanding of how US and MBs induce vascular permeability and trans-vascular transport, direct observation of the behavior of the MBs in real time is necessary. To achieve this, we grew tumors in skin flap dorsal window chambers that allows us to simultaneously apply US and image the vasculature by intravital multiphoton microscopy. We compared two different MBs - in-house made MBs stabilized by polymeric NPs (NPMB) [2] and SonoVue® co-administered with the same polymeric NPs.

II. Material and Methods

A. Tumors, animal model, nanoparticles and microbubbles

The Research Council of Norway (Project number: 240316) funds this research. Additional funding from the Central Norway Regional Health Authority is much appreciated

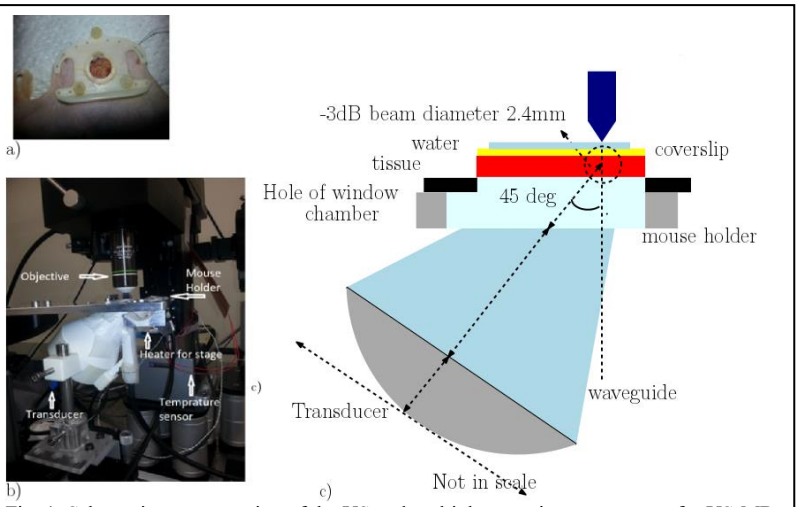

Fig. 1. Schematic representation of the US and multiphoton microscope setup for US-MB mediated drug delivery in a skin flap dorsal window chamber. a) Dorsal window chamber, b) The setup, and c) US and objective/light beams alignment (not in scale).

Human osteosarcoma cells (OHS) [3] (50 µl injection with 1.5 x 10^6 OHS cells) were grown as a xenograft in male BALB/c nude mice (weight 23-30g, n=21) in dorsal skinfold window chambers (Fig. 1a) [4, 5]. Tumors were allowed to grow for two weeks before treatment.

In-house self-assembled NP-stabilized MBs and the commercial phospholipid shelled SonoVue® MBs were used. Briefly, poly(ethyl butyl cyanoacetate) (PEBCA) NPs were synthesized by miniemulsion polymerization and contained the dye NR668 [2]. The PEBCA NPs were used to make NP-stabilized microbubbles by mixing with casein and perfluoropropane gas using an ultraturrax at 10000 rpm for 2 minutes [2]. Before each sonication 30 µl (4 mg/ml) of 2MDa FITC-dextrans was injected to visualize the blood vessels. A bolus injection of 50 µl of MB (2-5E8 MB/mL) was administered through the tail vein. When SonoVue® was used, 30 µl (20 mg/ml) of free PEBCA NPs were injected in addition.

B. Ultrasound setup, real-time multiphoton imaging and image analysis

A schematic representation of the experimental setup is depicted in Fig. 1b and Fig. 1c. A focused single element transducer with 1 MHz center frequency was used. The tumor was sonicated for 5 minutes with 10 ms pulse length and a pulse repetition frequency of 0.5 Hz. Peak negative pressure amplitudes measured in water, of 0.2, 0.4, 0.6 and 0.8 MPa were applied. The treatment and imaging schedule are shown in Fig. 2a. The multiphoton microscope (In vivo SliceScope from Scientifica) was equipped with a 20x water dipping objective (numerical aperture of 1.0) with a focal distance of 2 mm. Images were acquired in resonant scanning mode, at 31 fps (512 x 512 pixels) with a field of view of 400 µm x 400 µm. The filters in front of the two GaAsp detectors used were long pass 590 nm and band pass 525/50 nm for the observation of NPs and FITC-dextrans, respectively. The excitation wavelength was 790 nm (laser used: MaiTai DeepSee from Spectra-Physics). US exposure was done at two different positions in each window chamber (Fig. 2b).

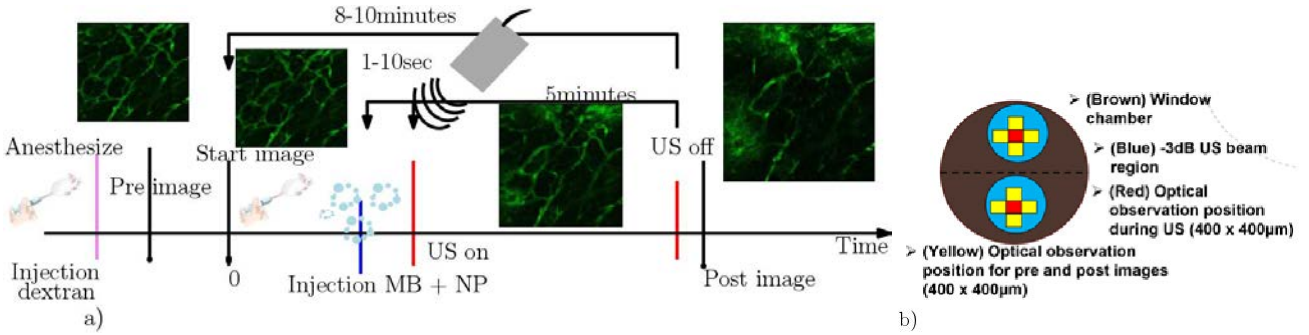

Fig. 2. a) The treatment and imaging schedule, b) the two treatment and imaging areas where pre, during US and post images were acquired. After 2MDa FITC-dextrans were injected, multiphoton pre-scans were–acquired, video scanning started and NPMBs or SonoVue MBs and NPs, were injected. Then, the tumor was sonicated using US. At the end, post scans were acquired.

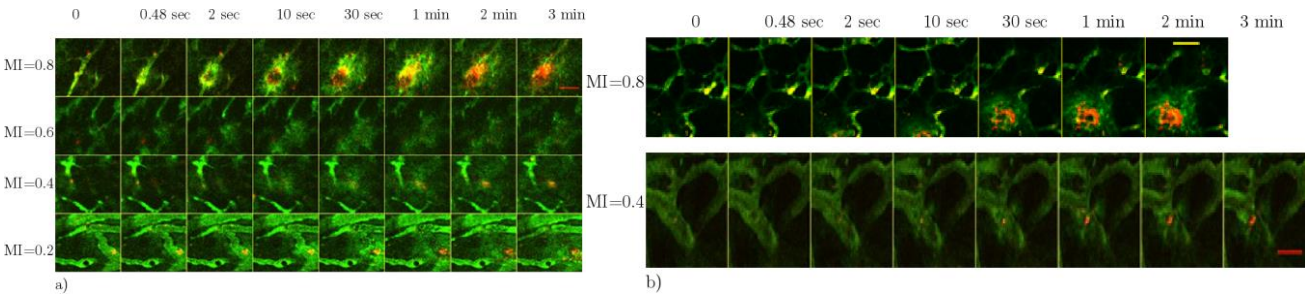

Fig. 3. Extravasation and penetration of NPs (red) and dextrans (green) as function of time after opening of the blood vessel by the US-MB interactions a) for NPMBs with MI 0.8, 0.6, 0.4 and 0.2, b) from SonoVue with MI 0.8 and 0.4. The MI is displayed on the left and the time after opening of the blood vessel is displayed at the top. Zero time corresponds to the time immediately before opening of the vessel. Scale bar=50µm.

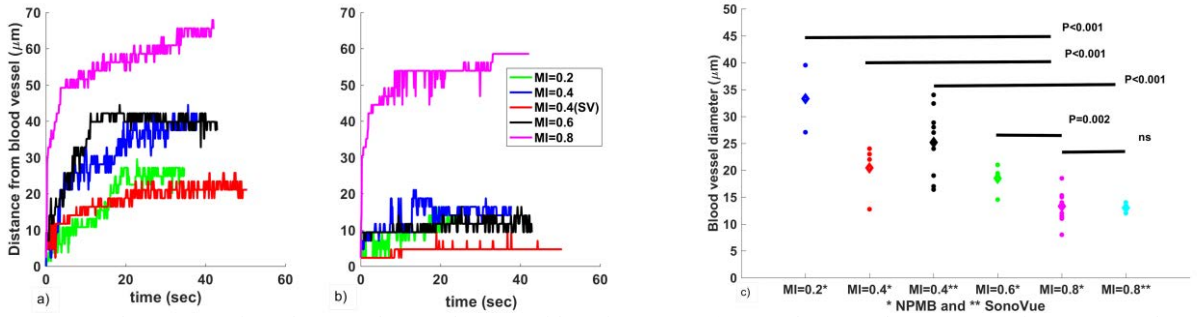

Fig. 4. Extravascular extravasation of NPs and FITC-dextrans with various MIs. (a) FITC-dextrans. (b) NPs. Zero time corresponds to the time immediately before opening of the blood vessel. (c) Blood vessel diameter. Each ○ represents one blood vessel where extravasation was observed, and ◊ represents the mean.

Images were analyzed in ImageJ and MATLAB software. First, the images were median filtered (3x3) and segmented. Vascular masks were created using the first frame of the image. Intravascular and extravascular fluorescence intensities of NPs and dextrans were determined from the average intensity in pixels located within or outside of the vascular mask, respectively, with background subtraction. To reduce noise, video frame averaging of three consecutive images was performed. In videos where extensive extravasation was observed, a circle with the radius of the blood vessel and concentric circles spaced by 3 pixels (2.23μm) starting from the blood vessel were drawn. The normalized signal intensity (with respect to the maximum intensity of the whole image) of NPs and dextrans in the blood vessel and in the different annuli over both time and distance were computed. Then, the rates of extravasation of the NPs and dextrans were estimated. In addition, the average diameter of the blood vessel where extravasation was observed was computed from the pre-images, and the rate of flow of the NPs was calculated by tracking distances moved between frames.

III. Result and Discustions

Imaging the vasculature by intravital multiphoton microscopy in real time during US sonication revealed extravasation of NPs and dextrans, which indicates the opening of the blood vessel. Moreover, change in flow rate and flow direction were observed, and occasionally even the blood flow stopped for short periods. Representative images

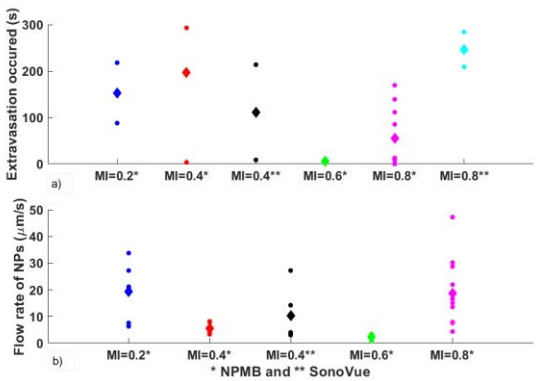

Fig.5. Time when extravasation occurred on the vessel (a), and flow rate of the nanoparticles (b). Each ○ represents one blood vessel where extravasation was observed in (a), and one NP tracked in (b). ◊ represents mean in both (a) and (b).

of extravasation of NPs (red) and dextrans (green) from vasculature and penetration into extracellular matrix as a function of time are shown in Fig. 3. Images of extravasation from sonications of MI 0.8, 0.6, 0.4 and 0.2 when injecting NPMBs, and MI of 0.8 and 0.4 when injecting SonoVue® and NPs are shown in Fig. 3a and Fig. 3b, respectively.

Combining US and SonoVue®, considerably fewer extravasation events were observed in the field of view during US exposure compared to US and NPMB. The difference in inducing extravasation is probably due to differences in the behavior of the two MBs. Size and concentration injected of the two MBs are quite similar, although the NPMB might have a broader size distribution [2] than SonoVue® and differences in amount of MB reaching the tumor tissue can be different. The circulation time, the shell and gas core of the two MBs are different. The circulation time of NPMBs is longer than for SonoVue®. The half-life of NPMBs in mice is approximately 2-3 times longer than for SonoVue®. Thus, the cavitation activity can persist longer for NPMBs compared to SonoVue® causing more microstreaming and microjets affecting the capillary walls. NPMBs have a NP/protein shell and perfluoropropane core, whereas SonoVue® has lipid shell and sulfur hexafluoride (SF_6) core. These two parameters are crucial for the behavior of the MBs. The NP/protein shell is thicker and stiffer compared to the lipid shell, which is soft and elastic. The importance of shell properties was demonstrated in a study comparing Optison MB with a shell of denaturated albumin and lipid-shell Definity, where Optison MB induced larger destructions of the blood-brain barrier than Definity [6].

Sonication at MI 0.8 induced more violent extravasation than at the lower MI, and the black spot seen in these videos (Fig. 3a and Fig. 3b) might be due to erythrocytes, which indicate blood vessel damage. The extravasation and penetration, as illustrated by the rate of transvascular transport, increased with increasing MI, which is shown in Fig. 4a and Fig. 4b for dextrans and NPs, respectively. The dextrans and the NPs extravasated faster and deeper into the interstitium for the higher MIs. Within 8s, the dextrans penetrated around 51, 30, 25 and 15 μm for MI of 0.8, 0.6, 0.4 and 0.2 respectively when NPMB was injected, whereas NPs penetrated similar to dextrans for MI of 0.8 and around 9 μm for the other MIs (Fig. 4a, Fig. 4b). At MI of 0.8, the maximum penetration of dextrans and NPs are 65 and 59 μm, respectively when NPMBs were injected. For the lower MIs, the NPs accumulated mostly inside the

blood vessels and the maximum penetration was around 14 µm. This could be explained by a smaller size of opening created in the blood vessel wall by the lower MIs. The difference in the rate of extravasation and penetration between NPs and dextrans could be due to their sizes. The diameter of the NPs are approximately 150 nm [2], while the diamter of 2MDa dextrans is reported to be approximately 60 nm [7]. Interestingly, the observed extravasation especially the more violent extravasation at MI 0.8, appeared immediately after a reduction or even a full stop in blood flow, and/or change in blood flow direction. Changes in flow rates and directions appeared for all MIs applied, but extravasation could not always been observed in the field of view, but could happen at positions outside field of view. The range in flow rates immediately after extravasation is given in Fig 5b. The changes in blood flow can be due to MBs causing obstruction of the blood flow and increasing the microvascular pressure which will facilitate extravasation [8].

When using lower MIs (0.2-0.4), the extravasation occurred in vessels with larger diameters compared to MI of 0.8 (Fig. 4c). Statistical analysis (two-tailed t-test) shows significant differences between MI of 0.8 and all the other MIs. After sonication with higher MI (0.8 and 0.6), extravasation was typically observed in vessels with diameters around 10-20 µm, whereas for the lower MIs (0.2 and 0.4), extravasation from vessels ranging from 20 to 40 µm in diameter was observed, as has been reported in other studies [9, 10]. It is known that the boundary conditions imposed by the vessel wall influence the resonance frequency and the oscillation of a MB when the vessel diameter gets small [11]. With an adequately rigid vessel, the effect will typically be a reduction in bubble resonance frequency compared to a free space situation. If the bubble resonance frequency falls below the sonication frequency in the smallest vessels (diameter 10-20 µm), a higher MI will typically be required to obtain the same effect from cavitation. This might explain why we do not observe extravasation in the smallest vessels except with the highest MI.

The observed extravasation events occurred at different time points and locations, and happened within milliseconds to minutes during the US exposure (Fig. 5a). For MI of 0.8, the extravasation occurred in the range of 32 ms to 179 s after US onset, while for the other MIs, the occurrence is from 3s to 240 s (Fig. 5a). The occurrence of cavitation events varies in locations within the US beam. This variation could be due to variations in MB distribution in tumor [11].

IV. Conclusion

In this study, a multiphoton microscope was used for real time, video rate imaging during US to investigate the effect of US and MBs in enhancing the permeability of the blood vessel. We found a violent extravasation of the agents with the higher MI with both NPMBs and SonoVue®. Moreover, we observed a change of blood flow rate and direction during US with both MBs, immediately before the extravasation. The observed extravasation events appeared within milliseconds to minutes after onset of US exposure and were confined within the US beam. These observations using intravital multiphoton microscope reveal new knowledge on the temporal and spatial extravasation of NPs and dextrans during US exposure, which is highly useful for optimizing such treatments.

Acknowledgment

The Authors thank to Spiros Kotopoulis for his assistance in designing the transducer holder, and Anne Rein Hatletveit (SINTEF) is thanked for producing NPs and NPMBs.

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