Mechanistic insights and visualization of the ultrasound-triggered size conversion of microbubbles to nanobubbles with two-photon intravital microscopy

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Background, Motivation and Objective

We recently reported development of high payload porphyrin-encapsulated multimodal microbubbles (pMBs), and their ultrasound (US)-triggered conversion to nanobubbles (NBs) with actively increased accumulation in targeted tumor tissue (Huynh et al, *Nature* 2015). This platform holds significant implications for nanomedicine delivery, optical and US imaging, and photo/sonodynamic therapeutic applications, but remains limited by a lack of mechanistic and biophysical understanding.

Statement of Contribution/Methods

Mechanistic insights on the conversion process were made in vessel phantoms. Agent behavior was assessed in response to 1 MHz pulses (2.5 ms length, 4 s PRP) at 0.15-1 MPa at concentrations of 10^7-10^9 mL^{-1} via cavitation detection, size measurements, and absorbance / fluorescence spectroscopy. Direct dynamic visualization and investigation of US-stimulated fluorescent bubble behavior in functional tumor-affected microcirculation *in vivo* was then performed with a ring transducer (PZT-4, thickness mode, 1.18 MHz) and PVDF receiver integrated in a two-photon window chamber setting. This enabled simultaneous acoustic stimulation and detection, fluorescent monitoring of biophysical interactions, and tracking of spatiotemporal extravasation of daughter nanostructures. Motivated by our recent work suggesting pNB potential for imaging and therapeutics (Pellow et al, *PMB* 2018), we then explored the cavitation potential of daughter nanostructures *in vivo*.

Results/Discussion

Results indicate that conversion is not primarily due to shrinkage, but rather a complex process involving gas loss and shell shedding during stable oscillations to form 5x more nanoscale supramolecular structures that contain gas. These results are complemented by intravital imaging to study vascular effects during sonication, primarily changes in permeability for the actively enhanced extravasation and uptake of *in situ* generated nanostructures. Simultaneous acoustic monitoring demonstrates stable cavitation during conversion and suggests the presence of NBs. Mechanistic and biophysical interaction insights in real-time contribute to an improved understanding of not only this delivery platform, but also these multimodal MB and NB agents, progressing toward their utility for imaging and therapeutic applications both within and beyond the vasculature.

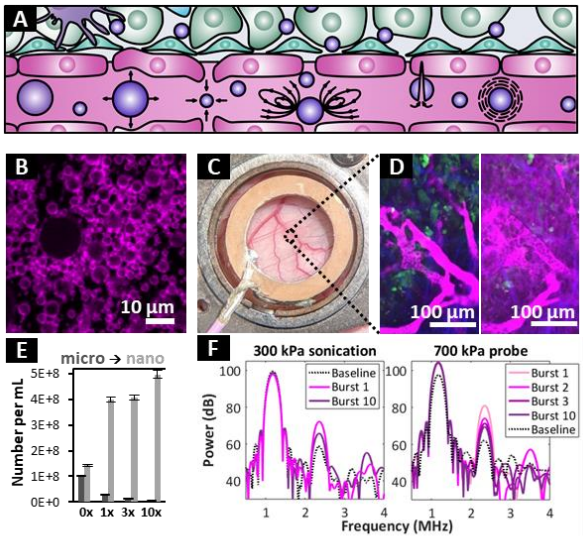

Figure 1. (A) Concept sketch of MB interactions with the vasculature, and conversion to extravasating daughter nanostructures. **(B)** Image of fluorescent MBs which are injected into functional tumor-affected microvasculature in a dorsal window chamber shown in **(C)**. A custom ring transducer is set in the window for concurrent two-photon fluorescence microscopy and acoustic stimulation. **(D)** Sample images (magenta: fluorescent bubbles, green: GFP-FaDu tumor cells, blue: collagen) demonstrating pre- and post- US-triggered vascular permeabilization and size conversion. **(E)** Binned coulter measurements indicating a 5x increase in nanostructures formed from MBs with US exposure. **(F)** Acoustic monitoring during initial sonication demonstrating stable cavitation during conversion, and a subsequent 700 kPa probing pulse sent 30 minutes later (long after MB dissolution) to investigate the possible presence of extravascular nanobubbles. Here, the probe demonstrates harmonic signals above baseline that decrease with subsequent bursts, consistent with the presence of NBs capable of undergoing ultrasound-mediated destruction.