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Microbubble volume: A definitive dose parameter in blood-brain barrier opening by focused ultrasound

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***Abstract*—Microbubble-assisted focused ultrasound (MB+FUS) is currently being developed to improve drug delivery through the blood-brain-barrier (BBB). However, microbubble dosing has posed challenges for treatment efficiency and safety due to differences in size distribution, as well as the prevalent practice of re-purposing FDA-approved ultrasound contrast agents (UCAs) for therapeutic use. Here, we explore a novel method of establishing microbubble dose, even retroactively, in MB+FUS BBB disruption (BBBD) studies. Specifically, we controlled microbubble size and concentration using a previously established centrifugal size-isolation technique, and adjusted the microbubble volume dose (MVD; 1-40 µL/kg) of two discrete monodisperse formulations (26-µm and 6-µm diameter) for MB+FUS BBBD in adult male Sprague-Dawley rats. Serial multi-site treatment was explored in the right and then left striata to determine the effects of microbubble pharmacokinetics. Near-infrared fluorescence microscopy of extravasated Evans Blue dye, our permeabilization indicator, was summed across brain slices after treatment to establish relative BBB opening. A linear trend between MVD and dye extravasation was observed for both treatment sites. Our results indicated that MVD, not microbubble size, determined the extent of MB+FUS BBBD. This result collapses previous microbubble dosing parameters of size distribution and concentration to one parameter: microbubble volume dose, which facilitates comparison of previous studies, as well as the planning of future MB+FUS BBBD studies. Additionally, a preliminary study of MB+FUS-facilitated gene delivery, expression, and immune response in neural tissue was conducted with dsAAV1-CMV-GFP.**

Keywords—blood brain barrier, disruption, permeabilization, microbubbles, ultrasound, FUS, drug delivery, sonoporation

I. Introduction

Despite the burgeoning supply of promising neurotherapeutic agents, the highly vascularized brain presents a mechano-chemical obstacle to parenchymal drug delivery in the form of the blood-brain barrier (BBB). Current surgical methods of defeating the BBB are highly invasive, and non-invasive methods, such as detergents, pose concerns related to safety and control. Microbubble-assisted focused ultrasound (MB+FUS) BBB disruption (BBBD) enables non-invasive targeted drug delivery to the brain parenchyma by transiently opening the BBB in millimeter-scale volumes. FUS drives MBs to oscillate in the targeted volume, effecting nanoscale disruptions in the vascular wall and enabling passage of drug molecules. Previous studies have shown that the magnitude of this transient permeabilization is significantly affected by microbubble size [1], [2].

The reliance on commercial ultrasound contrast agents, which are highly variable and polydisperse in size, has sparked debates regarding microbubble dosing [3]-[5], and hampered efforts to compare results and determine thresholds for MB+FUS BBBD safety and efficacy. Here, we present on the development and validation of a novel dosing scheme, microbubble volume dose (MVD), which can be applied to any microbubble formulation.

II. Methods and Materials

A. Materials and sonoporation

Adult Sprague-Dawley male rats (300-400 g) were obtained from Charles River Laboratories, and size-isolated (2- and 6-µm; Fig. 1A) cationic lipid-coated microbubbles (MBs) (70 mol% 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 20 mol% 1,2-distearoyl-3-trimethylammonium-propane (DSTAP) and 10 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(polyethylene glycol)-2000] (DSPE-PEG-2000) were obtained from Advanced Microbubbles Laboratories, LLC (Boulder, CO, USA). Anesthesia of rats was initiated and maintained with isofluorane (0.5-5%). Rats were placed in stereotactic ear bars and placed on a heating pad for the remainder of the procedure. Evans Blue (4 mL/kg, 4% wt/vol) and microbubbles of varying concentrations were tail-vein injected into the rat immediately before FUS. FUS application at the right and left striata was achieved with a Therapy and Imaging Probe System (TIPS; Philips Healthcare, Andover, MA, USA; 1 MHz, 0.5 MI with skull attenuation) (Fig. 1B).

B. Fluorescence imaging

After the procedure, the brain was extracted and sectioned into 500-µm sections. Near-infrared fluorescence imaging of the sections was achieved with a LI-COR Odyssey (Lincoln, Nebraska, USA). Five slices were selected for fluorescence intensity analysis, centered on the slice with the highest

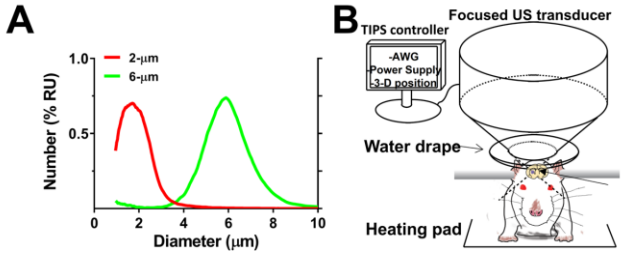

Fig. 1. A) Microbubble number-weighted size distribution. B) Diagram of transcranial sonoporation layout. Microbubble dose effect vs. (C) microbubble size and concentration and D) microbubble volume dose.

fluorescence intensity. Summed fluorescence intensity was then calculated across the region of interest (striatum) on both left and right sides of the brain [6].

C. Calculation of MVD

MVD was defined as the volume of encapsulated gas injected per unit weight of the subject (μL/kg), and was generated with the measured microbubble size distribution by first calculating the volume fraction:

$$\phi = \int_0^\infty c dv \quad (1)$$

where c is the concentration from the volume-weighted size distribution (# mcirobubbles/volume) and v is microbubble volume. The MVD is then calculated by taking the product of the volume fraction and the fluid volume dose:

$$MVD = \phi \cdot FVD \quad (2)$$

where FVD is the total volume of fluid (microbubbles and carrier fluid) injected into the subject (μL/kg).

D. Viral transduction and immune response

Delivery of dsAAV1-CMV-eGFP was conducted with the aforementioned ultrasound settings and 20 μL/kg of 6-μm microbubbles. PFA-fixed brain slices were stained and imaged one week after MB+FUS BBBD for EGFP fluorescence, NeuN, DAPI, and IBA1, an indicator of activated macrophages.

III. Results and Discussion

A. Effect of microbubble size and concentration

As previously suggested by studies utilizing size-isolated microbubble formulations [1], [2], an increase in microbubble size resulted in an increase in dye extravasation given equal concentrations. Additionally, a novel linear interaction between microbubble concentration and dye extravasation was observed in our study: increasing microbubble concentration resulted in a linear trend for both 2- and 6-μm microbubbles (R^2 = 0.78 and R^2 = 0.86, respectively; Fig. 2A).

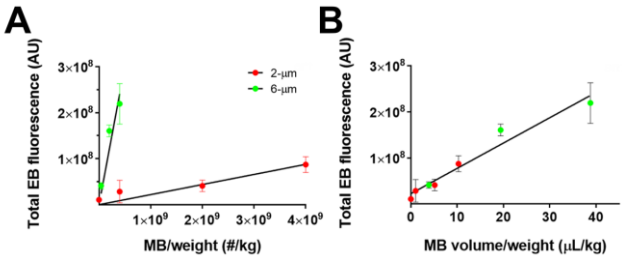

Fig. 2. Microbubble dose effect vs. (A) microbubble size and concentration and B) microbubble volume dose.

B. Effect of microbubble volume dose

Microbubble volume dose linearly correlated with extravasated Evans Blue on both the initial right striatum treatment region (R^2 = 0.90) and the left (R^2 = 0.68) (Fig. 2B). This result is in line with previous trends seen with image contrast persistence [7], and suggests that pharmacokinetics plays a significant role in MB+FUS BBBD.

C. Viral transduction and immune response

EGFP expression was observed two weeks after MB+FUS BBBD at 20 μL/kg of 6-μm microbubbles (Fig. 3A). NeuN and DAPI staining of neurons and nuclei demonstrated showed no noticeable qualitative loss in neuronal cell count (Fig. 3B). IBA1 staining revealed activated macrophage presence throughout the treatment site (Fig. 3B). Interestingly, petechiae observed immediately after MB+FUS were not observed in brains processed after the one-week timepoint (Fig. 3C and D).

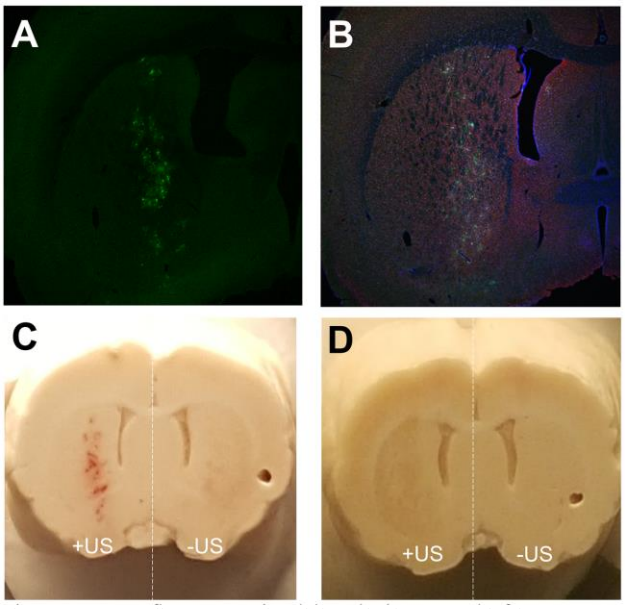

Fig. 3. A) eGFP fluorescence in adult rat brain one week after MB+FUS BBBD with dsAAV1-CMV-eGFP and 20μL/kg of 6-μm microbubbles. B) Fluorescent image of eGFP (green), DAPI (blue), NeuN (red), and IBA1 (gray). C) Petechiae resulting from 20μL/kg of 6-μm microbubbles immediately after MB+FUS BBBD, and (D) lack of petechiae one week after treatment. Note that hole on the –US side of the brain slice was generated in the course of histological analysis, and was not the result of the MB+FUS procedure.

It is important to note that transduction and inflammation were achieved with MVDs significantly higher than what was required to achieve BBBD. This suggests that an optimal therapeutic window exists between microbubble volume dose and immune response.

IV. Conclusion

In this study, we demonstrate a novel and precise measurement of BBBD using near-infrared fluorescence intensity

measurements of extravasated Evans Blue. Additionally, we show that the effect of varying microbubble concentration, and more importantly, volume—which takes into account both microbubble size and concentration—is predictably linear when compared with permeabilization. Finally, we achieve successful viral vector delivery using the dosimetry established in the study, and characterize the disappearance of petechiae and the persistence of inflammation at the treatment site.

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