Supporting Information

Noninvasive targeted transcranial neuromodulation via focused ultrasound gated drug release from nanoemulsions

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**Supplemental Materials and Methods**

*Nanoparticle formulation and characterization*

Adapting methods of prior reports of phase change nanoparticles\textsuperscript{1,2}, micelles of polymer (50 mg; polyethylene glycol-\textit{b}-polycaprolactone, PEG-PCL; MW 2000:2000 Da) and propofol (5 mg) were made by dissolving each into 1 mL of anhydrous tetrohydrofuran (THF), then adding 1 mL of PBS, mixing, and then vacuum evaporation of the THF overnight. Micelles were then diluted 3:10 in PBS and perfluoropentane (PFP) was added to a net 1:4 polymer:PFP (w/v) ratio. To emulsify the PFP, the mixture was sonicated in 1 mL volumes with an immersion micro-tip sonicator operating at 20 kHz center frequency (Model VCX500, Sonics and Materials Inc.; Newton, CT) operated at 30\% maximum amplitude for 30 sec. Free polymer and propofol were then removed via centrifugation at 5,000 rcf for 5 min, then removal of the supernatant, and then resuspension in fresh PBS. Centrifugation/resuspension was completed twice with the final resuspension at 700 \(\mu\)L. Then mixture was then mixed with an equivalent volume of hexane to extract residual free propofol, and 650 \(\mu\)L of the aqueous phase was collected and diluted with an additional 350 \(\mu\)L PBS. Particle size was determined with nanoparticle tracking analysis via NanoSight (Malvern Instruments, Worcestershire, UK). For \textit{in vivo} animal experiments, the above process was completed using sterile technique in cell culture hoods, with sterile reagents. For biodistribution experiments, 1 mg of a custom hydrophobic infrared fluorescent dye (IR800, LICOR Biosciences, Lincoln, NE) was included in the original micelle mixture (50:1 polymer:dye ratio w/w).

To evaluate the drug content in the nanoparticles, 100 \(\mu\)L of the nanoparticles were added to 900 \(\mu\)L of dimethyl sulfoxide (DMSO) to dissolve the polymer, PFP, and drug. The sample was then evaluated for fluorescence at 280 ex/310 em on a BioTek Synergy 2 plate reader (BioTek;
Winooshi, VT), and quantified for propofol using a standard curve of propofol in DMSO. This concentration of drug/mL of nanoparticles was then normalized to the amount of drug initially added to the PFP/micelle mixture to derive the percent encapsulation efficiency.

To test particle release efficacy *in vitro*, the particles were sonicated by loading into a custom designed chamber and sonicated using a focused ultrasound transducer (1 MHz center frequency; RK-300, FUS Instruments, Toronto, CA) with 10, 50, 100, or 150 ms bursts at 0.5 Hz burst frequency for 2 min (60 bursts) at 0.5, 1.0, or 1.5 MPa estimated peak *in situ* pressure. Samples were loaded as 200 µL with a layer of 100 µL of hexane placed on top of the aqueous phase to simulate the lipophilic sink of the brain parenchyma. Following FUS, 50 µL of the hexane phase was removed without disturbing the aqueous layer, and this was diluted by 100 µL hexane. The propofol concentration was quantified by UV fluorescence at 280 ex/310 em and compared to a standard curve of propofol in hexane.

**Animals**

All procedures included in this study were approved by the Johns Hopkins IACUC. Male Fischer 344 rats (150-200 gm weight; Charles River Laboratories, Wilmington, MA) were used throughout these experiments.

**Biodistribution**

For biodistribution experiments, propofol-loaded particles doped with an infrared fluorescent dye with maximum excitation of 770 nm and emission at 800 nm (LICOR Biosciences; Lincoln, NE) were prepared as described above under sterile conditions. These particles were administered intravenously via a 24 g tail vein catheter to rats (N=4) in a total volume of 1 ml. Timed retro-orbital blood samples were acquired in capillary tubes at 10 min, 20 min, 30 min, 40
min, 2 hr, 4 hr, and 8 hr. and split into two volumes. Whole blood sample fluorescence was assessed using a LICOR Pearl Impulse Imager (LICOR Biosciences; Lincoln, NE) and quantification was completed using regions of interest of the same size across samples, drawn to be within the capillary tube. As second volume of each sample was centrifuged in a microcentrifuge for a total of 10 min. The serum fraction from these samples were then collected and their fluorescence was quantified similar to the whole-blood samples. After the 24 h blood sample timepoint, animals were euthanized while under isoflurane anesthesia via cervical dislocation, and the major non-bowel organs were harvested. Organ fluorescence was also assessed via the LICOR Pearl Impulse Imager and quantified using regions of interest of the same size drawn to be within the image of each organ.

**Seizure model, EEG acquisition and analysis**

Rats were weighed and administered ketamine/xylazine (85/13 mg/kg) intraperitoneally (IP) for anesthesia. A 24 g tail vein cannula was placed. The dorsal fur was removed via electrical clipper and then a chemical depilatory (Veet, RB Inc, purchased through Amazon). This skin was then washed with saline and isopropanol. Three subdermal silver EEG electrodes (1 recording, 1 reference, and 1 ground) were placed overlying the parietal cortex with bregma as a reference. The silver electrodes (IVES EEG; Model # SWE-L25 – MA, USA) were implanted and subdermally fixed with minimal adhesive.

The animal was placed supine on the bed of a focused ultrasound transducer (1 MHz center frequency; RK300, FUS Instruments, Toronto, CA), with ultrasound gel used to couple the dorsal scalp to a Kapton membrane pad containing degassed water, which was itself coupled to the ultrasound transducer with degassed water. The head orientation and position was fixed with a vendor provided bite bar and nose cone integrated with the transducer bed, via which supplemental
oxygen was provided at 2 L/min. The sub-dermal electrodes were then connected to a tethered pre-amplifier and commutator. The lead wires were placed to ensure that they did not cross the central dorsal scalp to allow for ultrasound transmission. EEG recordings and synchronous video data were acquired using Sirenia Acquisition software (Pinnacle Technology Inc. Kansas, USA) according to previously established protocols\textsuperscript{3,4}. EEG files were recorded in the EDF format. Data were sampled at 400 Hz with pre-amplifier gain of 100 and bandpass filtered filters between 1-60 Hz to remove ambient noise.

Following acquisition of an EEG baseline of 5-10 min, animals were administered the chemoconvulsant pentyleenetetrazole (PTZ) 45 mg/kg IP\textsuperscript{5}. Animals were monitored via real-time EEG and visual inspection for evidence of convulsive and seizure activity. Repeat administration of 45 mg/kg IP doses of PTZ were administered until clear seizure activity was noted by both visual inspection (clear tonic-clonic limb twitching) and real-time EEG, within 5 min of the last PTZ dose. Animals required 2-4 doses of 45mg/kg PTZ to achieve this state in this study.

Animals were then administered the indicated sterile particles in 1 mL total volume intravenously as a slow bolus with a 100 µL sterile saline flush. After several minutes to allow for stabilization of the EEG trace following any handling-related seizure activity and post-ictal depression, at least 5 min of a new EEG baseline was acquired. Focused ultrasound was then applied with 1.0 MPa estimated peak in situ pressure (estimated as in O’Reilly et al.\textsuperscript{6}) in 50 ms bursts delivered every 1 sec for a total of 1 min (60 bursts) delivered to each of two points 2.5 mm to the left and right of midline, 15 mm caudal to the eyes, which translates to approximately 5 mm caudal to bregma. 10 min of EEG traces were then acquired. Then, if convulsive/seizure activity persisted, FUS was applied as above except with 1.5 MPa of estimated peak in situ pressure. Two animals that received propofol particles did not have appreciable seizure activity after the first FUS
application at 1.0 MPa and did not receive FUS at 1.5 MPa peak in situ pressure. After 10 min more of EEG trace acquisition, an adequate depth of anesthesia was confirmed and the animal was euthanized via perfusion fixation or cervical dislocation. Perfused animal brains were then harvested. Throughout this procedure, ketamine/xylazine anesthesia depth was confirmed via toe pinch, and if a visible toe pinch response was present then a repeat dose of the same amount of ketamine/xylazine was given. However, if seizure induction with PTZ had been completed, and the animal was evidently waking from anesthesia, the animal was excluded from further experimentation. An electrical artifact from unshielded components of the FUS system precluded EEG analysis during FUS applications.

For EEG analysis, using Sirenia Sleep software (Pinnacle Technology Inc. Kansas, USA), automated spectral analysis was completed with total and theta band (6-12 Hz) EEG power calculated in 10 s bins of the raw EEG trace. Power values greater than 10 S.D. beyond the mean trace values were considered artefactual outliers and removed from subsequent analysis. Each power timecourse was normalized by its average power within the three minutes prior to FUS application. Raw, not normalized total baseline power prior to FUS administration was 586.8 +/- 174.4 µV²/s (mean +/- s.e.m.) for propofol and 495.3 +/- 141.0 µV²/s for blank animals, with no statistical significance for this comparison. Normalized total power values over a 5-minute period prior to FUS application were 1.037 +/- 0.054 for propofol and 1.014 +/- 0.061 for blank animals, with no statistical significance for this comparison.

Ex vivo MRI

Fixed brains harvested following EEG/FUS experiments were scanned while submerged in fixative on a 17.6 T MRI (Bruker 750 MHz; Billerica, MA) in axial and coronal planes covering the whole brain using flip angle = 180, effective TE/TR=12.8/5000 ms, RARE factor=4, matrix =
128 x 128, FOV = 20 x 20 mm, slice thickness = 1 mm. All MRI studies were reviewed by a board-certified radiologist for evidence of parenchymal damage.

Histology

Following ex vivo MRI, fixed brains were transferred to a 15% sucrose solution for 3 days, then a 30% sucrose solution for 2 days and then flash frozen with dry ice and stored at -80 °C. Brains were then sectioned in the coronal plane at 40 µm thickness using a cryotome (Leica, Buffalo Grove, IL) over a 2 mm span centered at the expected FUS sonication site. Fixed frozen sections were mounted on Super Frost Plus glass slides (VWR, Radnor, PA). Slides were stained with Cresyl Violet and imaged under bright field and fluorescence on a MCID 7.0 Elite (InterFocus Imaging, Ltd., Cambridge, UK) at coordinates matching the in vivo FUS targets. All acquired sections were reviewed for evidence of damage within the parenchyma that would suggest FUS or particle mediated injury.

In vivo MRI and Serum Propofol Quantification

In a separate cohort, a tail vein catheter was placed, ketamine/xyazine anesthesia was induced, supplemental oxygen was provided, and propofol-loaded particles were administered intravenously. Using a 11.7T MRI (Bruker, N Billerica, MA), T2-weighted and T1-weighted scans were completed. Parameters for T2 scans were flip angle = 90, effective TE/TR=30/2500 ms, RARE factor=8, matrix = 128 x 128, FOV = 35 x 35 mm, slice thickness = 1 mm. Parameters for T1 scans were flip angle = 90, effective TE/TR=5.5/1500 ms, RARE factor=4, matrix = 256 x 256, FOV = 35 x 35 mm, slice thickness = 1 mm. Then sonication was delivered to the same targets as the EEG experiments using 1.0 MPa estimated in situ pressure, delivered in 50 ms bursts, 1 burst per focus at 1 Hz burst frequency for 60 sec. Magnevist (0.2 µl/gm; Bayer, Whippany, NJ) was
administered intravenously. The T1-weighted MRI was repeated post contrast administration to assess for BBB opening. All MRI images were reviewed by a board-certified radiologist for evidence of parenchymal damage.

To quantify residual propofol in the bloodstream of the rats after particle and FUS administration, timed retro-orbital blood samples were collected from rats into heparinized tubes 1 minute after particle injection, 1 minute after sonication, and 10 minutes after sonication. Blood was centrifuged at 5,000 rcf for 5 minutes to separate out the serum. Propofol concentration in serum was then quantified following the method reported in Cussonneau et. al. with some modification. Serum samples were mixed with an equal volume of a 600mg/L solution of thymol in acetonitrile and centrifuged at 2,000 rcf for 10 minutes. 100 µL of the supernatants were taken for HPLC. Separation and propofol quantification was performed using a Waters 600 HPLC System (Waters; Milford, MA). The stationary phase consisted of an HC-C18(2) (250 mm × 4.6 mm, 5 um) column (Agilent Technologies; Santa Clara, CA), and the mobile phase utilized a ratio of 65:35 (v/v) acetonitrile to water. Thymol was used as an internal standard. A 10 µL injection volume, 1 mL/min flow rate, and 25 minute separation time were used for sample separation.

Propofol standards were prepared by diluting propofol in serum and mixing with an equal volume of 600 mg/L thymol in acetonitrile. The standard curve was generated by plotting the peak area ratio of propofol to thymol as a function of propofol concentration at an absorbance wavelength of 270 nm. The propofol concentration in the bloodstream at the time points was quantified using the standard curve.
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