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Drug-loaded bubbles with matched focused ultrasound excitation for concurrent blood–brain barrier opening and brain-tumor drug delivery

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ABSTRACT

Focused ultrasound (FUS) with microbubbles has been used to achieve local blood–brain barrier opening (BBB opening) and increase the penetration of therapeutic drugs into brain tumors. However, inertial cavitation of microbubbles during FUS-induced BBB opening causes intracerebral hemorrhaging (ICH), leading to acute and chronic brain injury and limiting the efficiency of drug delivery. Here we investigated whether induction of drug (1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU)-loaded bubbles (BCNU bubbles) to oscillate at their resonant frequency would reduce inertial cavitation during BBB opening, thereby eliminating ICH and enhancing drug delivery in a rat brain model. FUS was tested at 1 and 10 MHz, over a wide range of pressure (mechanical index ranging from 0.16 to 1.42) in the presence of BCNU bubbles. Excitation of BCNU bubbles by resonance frequency-matched FUS (10 MHz) resulted in predominantly stable cavitation and significantly reduced the occurrence of potential hazards of exposure to biological tissues during the BBB opening process. In addition, the drug release process could be monitored by acoustic emission obtained from ultrasound imaging. In tumor-bearing animals, BCNU bubbles with FUS showed significant control of tumor progression and improved maximum survival from 26 to 35 days. This study provides useful advancements toward the goal of successfully translating FUS theranostic bubble-enhanced brain drug delivery into clinical use.

1. Introduction

Glioblastoma multiforme (GBM) is one of the most deadly and common forms of malignant brain tumor in humans [1,2]. GBM patients typically receive surgical resection coupled with radiotherapy and chemotherapy, yet tumor recurrence remains high, with only modest improvements in survival. The prognosis of GBM patients is poor, with a median survival of 12–15 months and only 3–5% of patients surviving for more than 3 years [3–5]. In addition, most tumors recur locally within 2 cm of the original lesion [6,7].

The chemotherapeutical drug 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU, carmustine) is widely used to treat GBM. It acts as an alkylating agent to induce interstrand cross-links in RNA, DNA and proteins, thereby improving patient survival [8]. However, like other chemotherapeutic agents, the clinical effectiveness of BCNU is severely limited by its short half-life (less than 15 min in vivo [9]) after intravenous (i.v.) systemic administration. Besides the short half-life, the blood–brain barrier (BBB) and blood–tumor barrier (BTB) also present major obstacles to delivery of most therapeutic agents as they block their penetration into the tumor [10]. These barriers have necessitated routine treatment with very high doses of drugs in an attempt to deliver the required amount to the tumor, despite serious complications such as myelo-suppression, pulmonary fibrosis, and damage of hepatic and renal function [11].

Local, transient and reversible opening of the BBB has recently been achieved by transcranial focused ultrasound (FUS) in the presence of microbubbles, and the feasibility of using FUS-BBB opening to promote the delivery of therapeutic agents, including drugs, antibodies and genes, from the vasculature into the brain has been confirmed [12–14]. Microbubble-enhanced FUS has also been verified as an excellent method to enhance brain drug delivery for the treatment of glioma [15,16]. We recently reported the design of novel therapeutic bubbles capable of serving dual func-

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tions by acting both as catalysts for FUS-BBB opening and as drug carriers to directly treat brain tumors. This concept was implemented by the successful fabrication of size-controlled, high-payload drug (1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU)-carrying bubbles (BCNU bubbles), which allowed concurrent BBB opening and local delivery of BCNU for glioma treatment [17].

Acoustic cavitation plays a critical role in microbubble-enhanced FUS-BBB opening. Previous studies reported that FUS-BBB opening involves both inertial and stable cavitation of commercially available bubbles [18]. However, BBB opening was found to be accompanied by mild capillary abnormalities and erythrocyte extravasation [19,20]. Most recent studies have used similar FUS exposure conditions, which inevitably cause inertial cavitation of commercially available, polydispersed microbubbles [21,22]. With these microbubbles, the small exposure threshold difference between BBB opening and inertial cavitation makes it challenging to separate stable and inertial cavitation [18]. We recently developed submicron-sized, in-house manufactured bubbles with the unique feature of predominantly inducing stable cavitation upon application of matched-frequency FUS exposure [23]. By avoiding the triggering of inertial cavitation, brain damage (mainly erythrocyte extravasations) was indeed significantly decreased, making the treatment much safer. However, stable-cavitation-dominant FUS-BBB opening (i.e. durable FUS-BBB opening triggered mainly by stable cavitation) for enhanced central nervous system (CNS) chemotherapeutic agent delivery, particularly for glioma treatment, has not been confirmed under these exposure conditions.

The purpose of this study was to investigate the feasibility of using drug-loaded bubbles for concurrent stable-cavitation-dominant FUS-BBB opening and triggering of local release of chemotherapeutic drug for glioma treatment. Specifically, we hypothesized that: (i) resonance causing stable cavitation of drug-loaded bubbles would be sufficient to trigger chemotherapeutic drug release and benefit glioma treatment; and (ii) under stable cavitation, the potential hazard of inertial cavitation could be largely eliminated to increase the safety of the treatment. The efficiency of in vivo drug delivery was determined by measuring the amount of BCNU delivered into brain tissues, and histological examinations were conducted to confirm the safety of the procedure. Finally, the feasibility and treatment efficacy of this drug delivery system were evaluated in an animal glioma model.

2. Materials and methods

2.1. Synthesis of submicron-sized BCNU-loaded bubbles

BCNU bubbles were synthesized with BCNU solution (Bristol-Myers Squibb, NY, USA), dipalmitoylphosphatidylcholine (Avanti Polar Lipids, AL, USA), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (Avanti Polar Lipids), and perfluoropropane (C_3F_8), as described previously [17]. BCNU bubble suspensions were fabricated by violent shaking for 45 s using an agitator. The hydrophobicity of the BCNU molecules allowed them to be embedded in the bubbles, where they attached to the phospholipid shell by hydrophobic interactions. We previously showed that these BCNU bubbles retained all the important physical characteristics of pure lipid bubbles for ultrasound-induced destruction [17]. Pure-lipid bubbles (unloaded bubbles) were prepared by the same method as BCNU bubbles for comparison.

2.2. Characterization of BCNU bubbles

2.2.1. Size distribution, bubble concentration and drug payload

The fabricated BCNU-loaded and control unloaded bubbles were counted and sized using a Coulter counter equipped with a 30 μm sensor orifice (Multisizer 3, Beckman Coulter, Miami FL, USA) for the 0.7–20 μm range. Smaller particles (<1 μm) were measured by dynamic light scattering (DLS; Nanosizer-S, Malvern, London, UK).

The structural features of BCNU bubbles were visualized with a cryogenic transmission electron microscope (Tecnai F20, Philips) operated at 200 kV. Samples (4 μl) were pipetted onto holey carbon film–covered 300-mesh copper grids (HC300-Cu, PELCO). The grids were blotted at 100% humidity and 4 °C for 3 s and plunge-frozen into liquid ethane cooled by liquid nitrogen using a Vitrobot (FEI, Hillsboro, OR). The grids were then stored under liquid nitrogen and transferred to the electron microscope using a cryostage. The samples in the holes of the carbon film were observed by transmission electron microscopy (TEM), using a microscope with a 70 μm objective aperture. The low dose condition for each exposure was ~20 e $Å^{-2}$. Images were collected at 5 k or 50 k magnification and 2–3 μm defocus, and were recorded with a 4 k × 4 k CCD camera (Gatan, USA). The resonance frequency of BCNU bubbles was estimated by the reported acoustic attenuation method [24].

The drug loading and encapsulation efficiency were determined by a reverse method using high-performance liquid chromatography (HPLC) with a UV detector (L-2400, Hitachi, Tokyo, Japan) [17]. The mobile phase for HPLC was performed with deionized water-to-methanol proportions of 40:60 (vol./vol.) at a flow rate of 2 ml min^{-1} and a detection wavelength of 237 nm. The BCNU was quantified at a retention time of 3.2 min.

2.2.2. In vitro drug leakage

The interactions between lipid and drug can be destroyed by electrolytes, producing dissociation and drug loss from drug-loaded bubbles [25]. We therefore determined the amount of leakage of BCNU from bubbles in PBS at 4 and 37 °C. The BCNU bubble suspensions were centrifuged to quantify the amount of BCNU leakage ($W_{leakage}$) by HPLC, as:

$$\text{BCNU leakage percentage } (\%) = W_{leakage}/W_{\text{BCNU bubble}} \times 100\% \quad (1)$$

2.3. In vitro FUS-induced BCNU release

BCNU bubbles were exposed to FUS in vitro and the efficiency of ultrasound-triggered release of BCNU was evaluated. Two exposure conditions from single-element FUS transducers were employed to trigger BCNU release. The first FUS transducer operated at 1 MHz (diameter = 25.4 mm focus length = 52.7 mm; V302, Panametrics, MA, USA) and the second at 10 MHz (diameter = 23.0 mm, focus length = 31.0 mm; SU-110, Sonic Concepts Inc., WA, USA). A function generator (WW2571, Tabor Electronics, Haifa, Israel) created sonication pulses which were amplified with a radio frequency (RF) power amplifier (150A100B, AR, PA, USA) to drive the transducer. A self-assembled external impedance matching circuit was used to match the electric impedance of the transducer with the output impedance of the amplifier. FUS sonication (10 MHz) was applied at 0.5, 1.5, 2.5, 3.5 and 4.5 MPa acoustic pressure (equivalent to a mechanical index (MI) ranging from 0.16 to 1.42) for 50 cycles, with a 10 Hz pulsed repetition frequency (PRF) and a sonication duration of 4 min. For comparison with unmatched FUS frequency exposure, 1 MHz FUS sonication was applied with the same parameters, except with acoustic pressure ranging from 0.3 to 1.5 MPa (equivalent to an MI of 0.3–1.5). Note that the pulse length was kept constant between 1 and 10 MHz FUS sonication.

The in vitro echogeneity and cavitation behavior of BCNU bubbles during FUS sonication were measured by B-mode imaging and

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