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Folate-conjugated gene-carrying microbubbles with focused ultrasound for concurrent blood-brain barrier opening and local gene delivery

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ABSTRACT

Previous studies have demonstrated that circulating DNA-encapsulated microbubbles (MBs) combined with focused ultrasound (FUS) can be used for local blood-brain barrier (BBB) opening and gene delivery. However, few studies focused on how to increase the efficiency of gene delivery to brain tumors after the released gene penetrating the BBB. Here, we proposed the use of folate-conjugated DNA-loaded cationic MBs (FCMBs). When combined with FUS as a trigger for BBB opening, FCMBs were converted into nanometer-sized vesicles that were transported to the brain parenchyma. The FCMBs can selectively aggregate around tumor cells that overexpressed the folate receptor, thus enhancing gene delivery via folate-stimulated endocytosis. Our results confirmed that FCMBs can carry DNA on the surface of the MB shell and have good targeting ability on C6 glioma cells. In addition, the optimized FUS parameters for FCMBs-enhanced gene delivery were confirmed by cell experiments (center frequency = 1 MHz; acoustic pressure = 700 kPa; pulse repetition frequency = 5 Hz; cycle number = 10000; exposure time = 1 min; FCMBs concentration = 4×10^7 MB/mL). *In vivo* data also indicated that FCMBs show better gene transfection efficiency than MBs without folate conjugation and the traditional approach of directly injecting the gene. This study described our novel development of multifunctional MBs for FUS-triggered gene delivery/therapy.

1. Introduction

Over the past few decades, numerous studies have reported on treatment outcomes from gene therapy for central nervous system diseases, such as neurodegenerative diseases and brain tumors (e.g., glioblastoma multiforme, GBM). Compared with chemotherapy, gene therapy genetically modifies or kills source cells, achieving tumor elimination without systemic toxicity [1,2]. However, the treatment efficiency of current gene therapies for brain tumors varies due to the protective structure of the cerebral capillaries, referred to as the blood-brain barrier (BBB) and blood-tumor barrier (BTB), which prevents foreign therapeutic agents from entering the tumor tissue. While the obstacle of the BBB and BTB could be bypassed via direct trans-cranial gene injection or by viral and non-viral gene carriers, limited gene diffusion area and low gene transfection efficiency are major issues with these techniques [3,4]. In addition, because glioma cells are typically surrounded by normal neural cells, a non-invasive, localized and targeting gene delivery platform needs to be developed to preferentially transfect brain tumor cells while sparing healthy tissues.

Microbubbles (MBs) are gas-filled microspheres consisting of a biocompatible shell and lipids, proteins or polymers that are

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typically utilized to enhance the contrast ability of ultrasound imaging. The physical structure of MBs makes them as suitable drug or gene carriers that can prolong the half-life of the therapeutic substances and also enable the triggered release of internalized agents at regions of interest via focused ultrasound (FUS) sonication [5,6]. Furthermore, the shell of the MBs can be conjugated with targeting markers, allowing selective aggregation of MBs to target cells for specific molecular theranostic purposes. The mechanical interactions (e.g., cavitation and radiation force) between FUS and MBs have been widely utilized to locally and temporally increase the permeability of BBB/BTB, allowing particles smaller than 187 nm to transvascularly enter the brain tissue [7]. Previous studies have demonstrated that the drug-loaded MBs can be converted into 5–500 nm drug-containing vesicles following FUS sonication. These vesicles cross the vascular and enter surrounding cells by acoustic microstreaming, thereby improving drug accumulation [8]. Although the transfection efficiency of DNA-loaded MBs with FUS has been confirmed, few studies have focused on developing a targeted gene delivery method to improve the delivery of therapeutic genes into brain tumor cells after penetrating the BBB/BTB.

DNA replication in malignant tumor cells requires a high consumption of folate [9]. In order to obtain sufficient folate, folate receptors (FR) are frequently overexpressed on the surfaces of cancer cells, including C6 glioma cells, but in the absence of normal tissues. The FR has a high affinity for folate (KD~10^{-10} M). After folate binding, the cell membrane surrounding the folate-FR complex invaginates into an endosome. Then, the endosome is acidified and the folate molecule is released into the cytosol. The physiological property of the FR makes it amenable for use in FR-targeted strategies for tumor diagnostics and therapeutics [10]. Several *in vitro* and *in vivo* studies have shown that proper conjugation of folate to the substance surfaces (e.g., drug or DNA) can enhance drug delivery to FR-positive tumors via folate-stimulated endocytosis [10].

Traditional targeting of MBs has been modified with special antibodies or ligands on the surface of the MB shell, which can bind to disease-associated molecular markers expressed on endothelial cells such as endothelial markers (VEGFR2) and intercellular adhesion molecule (ICAM-1) [11,12]. These vascular targeting pathways permit the selective attachment of MBs to the vessel of the tumor, thereby releasing the payload of MBs into the tumor and potentially affecting nearby normal tissues. However, an effective treatment for glioma must penetrate the BBB/BTB with few side effects to normal tissue. In this study, we constructed a DNA-loaded (luciferase plasmid) folate-inserted cationic MBs (termed FCMBs) for BBB/BTB opening and selective gene delivery (Fig. 1). In conjunction with FUS, the DNA-loaded FCMBs are broken down into DNA-containing vesicles that have increased BBB/BTB permeability. After crossing the BBB/BTB, the folate ligands on the vesicles of FCMBs aggregate at FR-overexpressing cancer cells and delivery gene to the tumor cells via endocytosis, increasing the efficiency of brain-gene delivery in a rat GBM model.

2. Materials and methods

2.1. Preparation of plasmid DNA

In this study, we used a plasmid encoding the red firefly luciferase gene (pFLuc, Thermo Fisher Scientific IL, USA) with expression driven by a cytomegalovirus promoter. pFLuc, a reporter gene, was used to evaluate FUS-mediated transfection efficiency *in vitro* and *in vivo* via bioluminescence imaging. pFLuc was extracted from *E. coli* DH5a by plasmid extraction kit (NucleoBond Xtra Maxi EF, Macherey-Nagel, Düren, Germany). The purity and concentration of the extracted DNA were determined by a spectrophotometer with an UV absorption wavelength at 260/280 nm (NanoDrop 2000, Thermo Fisher Scientific, IL, USA). The quality of purified pFLuc was analyzed by subsequent electrophoresis and enzyme restriction to ensure that the vesicles of DNA were in full agreement with the DNA map of the provider.

2.2. Preparation of FCMBs and pFLuc-loaded FCMBs

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, Avanti Polar Lipids, AL, USA), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP, Avanti Polar Lipids), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG 2K, Avanti Polar Lipids) (molar ratio of 31.5:3.9:1.8) were dissolved in chloroform. The chloroform was then removed via an evaporator (R-210, Büchi Labortechnik AG, Flawil, Switzerland). The folate-conjugated lipids were fabricated by direct covalent bonding of folate (Sigma-Aldrich, MO, USA) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG 2K-Amine, Avanti Polar Lipids) via amide bond formation (Fig. S1). A solution of folate was prepared in DMSO with a concentration of 4.4 mg/mL, activated with carbodiimide (molar ratio of 1:12.5) at 60 °C for 1 h. Then, the folate solution was mixed to the solution of DSPE-PEG 2K-Amine (1.6 mg/mL in PBS) in a molar ratio of 4:1 to prepare DSPE-PEG 2K-folate at 60 °C. The morphology of FCMBs could be imaged via bright field microscope images, while the green fluorescence image of FCMBs were observed by embedding 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (DSPE-NBD, Avanti Polar Lipids) into the lipid shell of FCMBs. The cationic property of DPTAP enables spontaneous attachment to the DNA by electrostatic interaction. The lipid film was then mixed with glycerol-PBS (5 μL/mL), DSPE-PEG 2K-folate and C_3F_8 gas. The solution was shaken in an agitator for 45 s to form FCMBs. Then, the unreacted lipids were removed from FCMBs via centrifugation (2 min, 2000 g). The CMBs (DSPE-PEG 2K, DPPC, and DPTAP, without folate) and normal MBs (DSPE-PEG 2K, and DPPC, without folate) were prepared to compare the characteristics of FCMBs. For preparation of pFLuc-loaded MBs, pFLuc was mixed with FCMBs and CMBs, gently rotated for 30 min, and then centrifuged at 2000 g for 1 min to separate unloaded pFLuc from well-conjugated pFLuc-FCMBs/pFLuc-CMBs.

2.3. Characteristics of DNA-loaded FCMBs

The size and concentration of FCMBs were measured by a coulter counter (Multisizer 3, Beckman Coulter Inc., CA, USA). The efficiency of conjugating folate onto FCMBs was measured by a spectrophotometer (Cary 50, Agilent, CA, USA) with UV absorption at 280 nm. The zeta-potential of FCMBs was analyzed by a dynamic light-scattering system (DLS, Nanosizer-S, Malvern, London, UK). The DNA loading efficiency of pFLuc-FCMBs was analyzed using a reverse method with the spectrophotometer. Serial amounts of pFLuc (5–120 μg) were added into 10^9 FCMBs. The unbound DNA was removed by centrifugation (2 min, 2000 g). The liquid phase was evaluated to determine the amount of residual DNA after pFLuc-FCMBs production. The DNA loading efficiency of pFLuc-FCMBs was estimated as follows:

$$\textit{DNA loading efficiency}(\%) = \frac{\textit{weight of pFLuc loaded on } 10^9 \textit{FCMBs}}{\textit{total weight of pFLuc added in } 10^9 \textit{FCMBs}} \times 100\%$$