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Ultrasound-sensitive siRNA-loaded nanobubbles formed by hetero-assembly of polymeric micelles and liposomes and their therapeutic effect in gliomas

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ABSTRACT

Ultrasound (US)-sensitive nanobubble (NB) which may utilize the physical power of US exposure to improve delivery efficiency to target cells is emerging as one of the most promising nanocarriers for drug delivery. On the basis of successfully fabricating NBs with the ability of passively accumulating in tumor tissue, in this study we synthesized a US-sensitive NB bearing siRNA (siRNA-NB) for tumor therapy *via* a hetero-assembling strategy using the siRNA-complexed polymeric micelles and gas-cored liposomes. The US exposure-aided siRNA transfection effectively enhanced the gene silencing effect of siRNA-NBs both *in vitro* and *in vivo*, which resulted in much elevated level of cancer cell apoptosis. Consequently, significantly improved therapeutic effect was achieved in a nude mouse glioma model, using siRNA-NBs bearing siRNA to target the anti-apoptosis gene sirtuin 2 (SIRT2). These results show that, with the aid of US exposure, the US-sensitive siRNA-NB may be an ideal delivery vector to mediate highly effective RNA interference for tumor treatment.

1. Instruction

The growing number of studies about RNA-based therapeutics have shown the great potential of RNA interference (RNAi) in treating a variety of diseases [1–3], including cancer [4]. Despite the great potentials, cancer therapy with small interfering RNA (siRNA) is still facing several challenges. One major obstacle is the *in vivo* delivery of siRNA into cancer cells [4], due to the widely existing RNAase which may degrade siRNA and also the fact that the negatively charged siRNA is unable to penetrate through cell membranes [5]. Although different types of delivery vectors including viruses and cationic lipids [6] or polymers [7,8] have been applied to improve the siNRA delivery efficiency, still none of them is ideal. The second challenge is the tumor-targeted delivery of siRNA. Nanoparticles bearing siRNA can be modified with targeting ligands such as glycans, folates, peptides or antibodies to direct specific cell binding [9] and to improve the efficiency of siRNA delivery into cancer cells [10]. However, most of the cell receptors mediating such interaction express not only in cancer cells but also in the normal ones. Therefore, a more efficient tumor-targeting strategy for siRNA delivery is still urgently needed nowadays.

Ultrasonography is a widely used imaging technology, which is playing a vital role in clinical imaging diagnosis of many types of diseases. Development of US contrast agents (UCA) is now leading to a revolution both in the diagnostic [11] and therapeutic ultrasound (US). It has been found that MBs with various shells (polymers or phospholipids) and gas cores may possess desirable contrast enhancement abilities. On the other hand, MBs are known

Abbreviations: US, ultrasound; MBs, microbubbles; NBs, nanobubbles; siRNA-NBs, siRNA-loaded nanobubbles; UTMD, ultrasound targeted microbubble destruction; SIRT2, sirtuin 2; siRNA, small interfering RNA; SIRT2 siRNA, siRNA targeting SIRT2 gene; SCR, scrambled siRNA; cy3-SCR, cy3-labeled SCR; SIRT2-NBs, SIRT2 siRNA-loaded nanobubbles; SCR-NBs, SCR-loaded nanobubbles; mPEG-b-PLLys, poly(ethylene glycol)-b-poly(L-Lysine); N/P ratio, molar ratio of PEI-PCL nitrogen to siRNA phosphate; P/P_1 ratio, molar ratio of the phosphate groups in siRNA to that in gas-cored liposomes; siRNA-NBs US (+) or siRNA-NBs US (−), cy3-labeled SCR-loaded nanobubbles with or without US exposure; SIRT2-NBs US (+) or SIRT2-NBs US (−), SIRT2 siRNA-loaded nanobubbles with or without US exposure; SCR-NBs US (+), SCR-loaded nanobubbles with US exposure.

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as the cavitation nuclei that will destruct during low-frequency US exposure [12]. Cavitation induced by ultrasound targeted microbubble destruction (UTMD) is able to dig holes of about 300 nm in diameter on the cell membranes with a half-life of 20–50 ms [13], which is called "sonoporation", a phenomenon utilizable to increase the cell uptake of administered drugs and genes [14]. However, the intrinsic weakness of MBs limits their application in tumor therapy. In particular, the large-size of MBs prevent them from passing through the endothelial gaps of tumor blood vessels [15], and without MBs surrounding the tumor cells, UTMD cannot be applied to increase the permeability of cancer cell membrane for enhanced cell uptake of siRNA nanomedicine. Therefore, development of nanobubbles (NBs) which are able to cross the endothelial gaps and penetrate into the interstitial space of tumor cells more easily is of great importance nowadays.

Previous studies have revealed that NBs with diameters of 300–700 nm are promising contrast agents for extravascular ultrasonic imaging [16]. The phenomenon that NBs permeate the vasculature (pore size cutoff between 380 and 780 nm [17,18]) and remain in tumor tissue after intravenous injection is called passive tumor targeting [19]. Passive tumor targeting of NBs results in a high local bubble concentration in the surrounding of tumor cells, which may enhance cell permeability upon US exposure. Just like MBs, at the exposure of low-frequency US, cavitation and sonoporation can also be detected for NBs [20]. Subsequently, the therapeutic compounds such as siRNA may enter tumor cells near NBs. In contrast, damage in cells of normal tissue without passive NB accumulation is much lower [21]. The characteristics including passive targeting and cavitation-inducing ability make NBs a highly desirable non-viral vector for siRNA delivery.

Another major drawback in UTMD-assisted nucleic acid delivery is that nucleic acids or nucleic acid complexes are simply mixed with MBs/NBs in the formulations in most studies reported thus far [22]. For instance, cancer cells were incubated with the nucleic acids/polyethylenimine (PEI) micelles while separate MBs and US exposure were applied to enhance transfection of the nucleic acids [23,24]. However, when low-frequency US exposure is applied and cavitation appears, only the nucleic acids surrounding MBs can be efficiently delivered to the cytoplasm by the jet produced from the MBs collapse. To overcome the drawback, fabrication of siRNA-loaded NBs was recently reported in order to substantially improve the cell transfection efficiency with the aid of MB/NB-based UTMD. In these studies, cationic polymers were introduced to the surfaces of MBs/NBs for complexing nucleic acids [25,26]. Unfortunately, nucleic acids were exposed on the particle surface without sufficient protection from the *in vivo* RNase degradation in this case.

Herein, we report on a different type of siRNA-NB developed *via* a nano-particulate hetero-assembly of the siRNA-loaded polymeric micelles and liposomes, and expect that the unique complex structure may enable not only high siRNA transfection efficiency under UTMD but also an effective siRNA protection. The complex structure was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM). *In vitro* and *in vivo* studies were carried out to confirm the US sensitivity, siRNA delivery ability and therapeutic effect against cancer. In light of the recent discovery that sirtuin 2 (SIRT2) was an important anti-apoptosis gene especially over-expressed in gliomas [27,28], siRNA targeting SIRT2 was chosen for assessing the siRNA transfection of siRNA-NBs in rat C6 glioma.

2. Material and methods

2.1. Materials

Monomethoxy poly(ethylene glycol) (mPEG-OH, Mn = 2k Da) was purchased from Sigma–Aldrich. 4-Toluene sulfonyl chloride (TsCl) was purchased from Sinopharm Chemical Reagent Co Ltd., China. Dimethylformamide (DMF) and HBr/CH_3COOH were purchased from Sigma–Aldrich and used as received. The macroinitiator α-methyl-ω-amino poly(ethylene glycol) (mPEG-NH2) was prepared from mPEG-OH as reported [29]. Benzyloxycarbonyl-L-Lysine N-carboxylic anhydride (CBLLys-NCA) was synthesized from L-Lysine.HCl (Sinopharm Chemical Reagent Co., Ltd., China) as reported [30]. The following phospholipids in powdery form (Avanti Polar Lipids Inc. USA) were used in the fabrication of NBs without further purification: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; Mw: 734.05), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE; Mw: 748.06) and 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA; Mw: 670.88). Pluronic F-68 and glycerol were purchased from Sigma–Aldrich. Octafluoropropane (C_3F_8) gas was purchased from the R&D Center for Specialty Gases at the Research Institute of Physical & Chemical Engineering of Nuclear Industry (Tianjin, China). The fluorescent probe Hoechst 33342 was purchased from Beyotime (Haimen, China).

Two siRNA duplexes designed to target SIRT2 gene (SIRT2-1 and SIRT2-2) were purchased from Genpharm (Shanghai, China). They are 21nt long double stranded RNA oligos with dTdT overhang at 3′ end and have sequences as shown in Table S2. The siRNA cocktail containing SIRT2-1 and SIRT2-2 (equal molar ratio) was named as SIRT2 siRNA. The cy3-labeled scrambled siRNA (cy3-SCR) was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Synthesis of poly(ethylene glycol)-b-poly(L-Lysine) diblock copolymer (mPEG-b-PLLys)

Poly(ethylene glycol)-b-poly(Benzyloxycarbonyl-L-Lysine) diblock copolymer (mPEG-b-PCBLLys) was synthesized by ring-opening polymerization of benzyloxycarbonyl-L-Lysine N-carboxylic anhydride (CBLLys-NCA) using mPEG-NH2 as a macroinitiator. Under an argon atmosphere, a dry Schlenk flask equipped with a magnetic stirrer was charged with mPEG-NH2 (1.0 g, 0.5 mmol). CBLLys-NCA (8.4 g, 27.5 mmol) was dissolved in 20 mL of anhydrous DMF and then added into the flask under the protection of argon. The polymerization was performed at 35 °C for 3 days. Subsequently, the reaction mixture was precipitated into a large amount of cool diethyl ether, filtered, washed with diethyl ether, and finally dried in vacuum at room temperature, to get the copolymer as a white powder (7.6 g, 92%). The obtained copolymer mPEG-b-PCBLLys (2.0 g) was dissolved in TFA (5 mL) at 0 °C, and then HBr/acetic acid (33%, 2.0 mL) was added. After stirring at room temperature for 2 h, an excess amount of diethyl ether was added, and the precipitated polymer was washed with ethyl ether at least 4 times. After rotary evaporation of solvents, the residue was dried in vacuum at room temperature to yield a grey powdery product (1.0 g, 90%). Details for the characterizations of mPEG-b-PLLys were described in the supplementary information.

2.3. Preparation of siRNA micelles

0.5 μg siRNA and a designed amount of diblock copolymer (mPEG-b-PLLys) were dissolved separately in PBS. The two solutions were mixed by vigorous pipetting, and then the mixture was kept at room temperature for 30 min to allow formation of the polyplex micelle. The amount of polymer used to complex siRNA was determined based on the preset N/P ratios, which were calculated as the number of nitrogen atoms in the polymer over that of the phosphate groups in siRNA. The micelles thus obtained were characterized by dynamic light scattering (DLS). Measurements were performed at 25 °C on a 90 Plus/BI-MAS equipment (Brookhaven Instruments Corporation, USA). The data for particle size and zeta potential were collected on an autocorrelator with a detection angle of scattered light at 90° and 15°, respectively. For each sample, data obtained from five measurements were averaged to yield the mean particle size and zeta potential.

In order to assess the siRNA condensation ability of mPEG-b-PLLys, gel electrophoresis was performed on a Bio-Rad Sub-Cell electrophoresis cell (Bio-Rad Laboratories, Inc., US) and images were obtained on a DNR Bio-Imaging Systems (DNR Bio-Imaging Systems Ltd., Israel). For the test, micelles were induced at various N/P ratios (0, 0.5, 1, 2, 4, and 8) in a final volume of 6 × agarose gel loading dye mixture (i.e. 10 μL) for 30 min. siRNA and mPEG-b-PLLys were dissolved separately in PBS. The two solutions were mixed by vigorous pipetting, kept at room temperature for 30 min, loaded onto the 0.9% agarose gels with ethidium bromide (EB, 0.1 mg/mL), and ran with Tris-acetate (TAE) buffer at 100 V for 40 min. siRNA motion retardation were visualized by irradiation with UV light.

2.4. Preparation of gas-cored liposomes

Liposomes were prepared using a thin-film hydration-sonication method. Briefly, all phospholipids (18 mg DPPC, 1 mg DSPE and 1 mg DPPA) were dissolved in 4 mL of chloroform and transferred into a 9-cm culture dish to form a thin phospholipid film by natural evaporation. The film was then hydrated with 4 mL of hydration liquid consisting of 10% glycerol (v/v) and 2 mg/mL Pluronic F-68, maintained at 37 °C in a shaking incubator for 1 h to form liposomes. The liposomal suspension was transferred to a 50 mL centrifuge tube, and the air above the liquid was replaced with C_3F_8 gas using a 50 mL syringe equipped with a long and fine needle. Finally, the solution was sonicated using a VCS 130 PB ultrasonic processor (Sonics, USA) at 130 W for 5 min. Large bubbles were separated as a thin layer from