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.

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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 siRNA 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.

Ultrasoundography 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...
as the cavitation nuclei that will destroy during low-frequency US exposure [12]. Cavitation induced by ultrasound targeted micro-bubble 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 utilized 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/polyethyleneimine (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 = 5k Da) was purchased from Sigma–Aldrich. 4-Toluene sulfonyl chloride (TsCl) was purchased from Sinopharm Chemical Reagent Co. Ltd., China. Dibutylmethylamine (DMF) and HBr/ CH3OH were purchased from Sigma–Aldrich and used as received. The macroinitiator 4-methyl-sub-ε-amino poly(ethylene glycol) (mPEG-NH2) was prepared from mPEG-OH as reported [29]. Benzylxoycarbonyl-l-lysine N-carboxyl anhydride (CBLLys-NCA) was synthesized from l-Lysine HCl (Sinopharm Chemical Reagent Co., Ltd., China) as reported [30]. The resulting photopolymerizable MBs composed of CBLLys-NCA, mPEG-OH, and Pluronic F-68 were washed with ethyl ether at least 4 times. After rotary evaporation of solvents, the residue was dissolved separately in PBS. The two solutions were mixed by vigorous pipetting, 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.

2.2. 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-PDLLys) was synthesized by ring-opening polymerization of benzoxycarbonyl-l-lysine N-carboxy 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-PDLLys (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-PDLLys 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-PDLLys) 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/NANOS 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-PDLLys, gel electrophoresis was performed on a Bio-Rad Sub-Cell electrophoresis cell (Bio-Rad Laboratories, Inc., US) and images were obtained on a DNZ Bio-Imaging Systems (DNZ Bio-Imaging Systems Ltd, Israel). For the test, micelles were induced at various N/P ratios (0.5, 1.0, 2, 4, 8) and in a final volume of 6 x agarose gel loading dye mixture (i.e. 10 µl) for 30 min. siRNA and mPEG-b-PDLLys were dissolved separately in PBS. The two solutions were mixed by vigorous pipetting, kept at room temperature for 30 min, loaded onto the 0.5% agarose gels with ethidium bromide (EB, 0.1 µg/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 DPCC, 1 mg DSPE and 1 mg DPPA) were dissolved in 4 mL of chloroform and transferred into a 50 mL crimp-top vial to a thin phospholipid film by natural evaporation. The film was then hydrated with 4 mL of hydronat 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 then centrifuged at 20,000 rpm for 30 min, the supernatant was discarded and the bottom was replaced with C6F6 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...
the suspension by low-speed centrifugation (50 × g, 5 min) and discarded. Centrifuga-
tion of the suspension was performed in a 15 mL centrifuge tube at a higher speed (805 × g, 30 min). After removing the bottom liquid layer, the gas-voided li-
posomes were collected, re-suspended in 4 mL phosphate-buffered saline (PBS), and
stored at 4 °C.
The liposome concentration was determined using a hemacytometer. The
fluorescent compound Dil was used to detect the formation of gas-cored liposomes.
A drop of Dil-loading sample was transferred to a hemacytometer and observed
using a Carl Zeiss Avio-1 inverted fluorescence microscope. Three pairs of images
(one fluorescent and one bright field image of each field) at different fields were
acquired (400 × ). The liposomes were shown in the bright field images. The number of liposomes was then counted using WCIF Image software (v1.37; National In-
stitutes of Health, Bethesda, MA). Finally, the particle concentration (numbers/mL) was calculated using the same cell counting method. All measurements were carried
out in triplicate.

2.5. Preparation of gas-cored siRNA-NBs

1 × 10^11 of gas-cored liposomes and different amounts of siRNA micelles were re-
suspended in 600 μL PBS and mixed by vigourous pipetting, and then the mixture was
kept at room temperature for 30 min to allow siRNA-NB formation (Fig. 1). The
amount of siRNA loaded in the NBs was determined based upon the designed P/Pi
ratios, which were calculated as the number of phosphate groups in siRNA to that in
gas-cored liposomes. The particle size and zeta potential were determined by the 90
Plus/BI-MAS equipment as previously described.

2.6. RNase protection assay

RNase ONE® ribonuclease (Promega, Madison, WI, USA) was used to determine the
ability of siRNA-NBs in protecting siRNA from enzymatic degradation. 0.5 μg of
naked SCR or SCR complexed in siRNA-NBs (N/P ratio = 5, P/Pi ratio = 0.2) was
incubated at 37 °C in 1.5 mL Eppendorf tubes which contain 0.1 unit of ribonuclease
dissolved in the reaction buffer consisting of 10 mM Tris–HCl (pH 7.5), 5 mM EDTA and
0.2 μM sodium acetate. After 60 min incubation, 10 μg of heparin (Sigma, St. Louis,
MO, USA) was added into each solution at room temperature for 60 min. The samples were then loaded onto the 0.0% agarose gel with EB (0.1 mg/mL), and ran
with TAE buffer at 100 V for 40 min to examine the structural integrity of siRNA.
0.5 μg of SCR without enzymatic digestion was also run the same electrophoresis as a
control.

2.7. Transmission electron microscopy (TEM)

TEM was performed using a Hitachi model H-7650 TEM operated at 80 kV. Samples for TEM analysis were prepared by depositing a drop of the sample (10 μL)
onto carbon-coated copper grids and dried at room temperature. For the negative
counterstaining of samples, a small drop of phosphotungstic acid (PTA) solution (1 wt % in
water, pH value was adjusted to that of the sample solution) was added to the copper
grid, and then blotted with filter paper after 30 s. The grid was dried overnight in a
desicicator prior to TEM observation.

2.8. Stability and US sensitivity tests

To compare the stability of siRNA-NBs with that of gas-cored liposomes, in vitro
contrast enhanced US imaging experiments were carried out by using a custom-
made 2% (w/v) agarose mold as designed in previous study [19]. 1 mL of siRNA-
NBs or gas-cored liposomes with a bubble concentration of 1 × 10^11 bubbles/mL
was added to the sample wells for the test. A broadband 15LS-8 high-frequency
linear transducer of the clinical US scanner system (Acuson Sequoia 512, Siemens,
USA) with the transmitted power of ~18 dB was used. The contrast pulse sequencing (CPS) gain was 0 dB, and the focal zone was placed at a depth of 1.5 cm at the center
of the sample well. Three ultrasonic images were recorded for each samples every
10 min for the offline gray-scale intensity examinations (by the use of the Image
software). The quantitative gray-scale ultrasonic intensity of each image was defined as

\[ \frac{I(t) - I(0)}{I(0)} \]

Another pair of siRNA-NBs and gas-cored liposomes samples (3 × 10^10 bubbles/
ml) was placed in the sample wells for the US sensitivity tests. The samples were
exposed to low-frequency US (1 MHz, 1.0 W/cm²) for 1 min by using a self-made
transducer US system (contour of UltraSonic Imaging, Chongqing Medical Uni-
versity, Chongqing, China). Contrast enhanced US images (as above) were recorded
before and after the low-frequency US exposure using the 15LS-8 transducer in
CPS mode.

2.9. Cell transfection

Rat C6 glioma cells were cultured in RPMI-1640 medium supplemented with
10% FBS ( Gibco, Carlsbad, CA) and incubated at 37 °C with 5% CO2. In the trans-
feciton study, the cells were seeded in 12-well plates at 300,000 cells per well
performed at 37 °C in 1.5 mL of complete medium and the cells were incubated for another 48 h.

2.10. Transfection efficiency measurements

2.10.1. Flow cytometry analysis

C6 glioma cells at a density of 5 × 10^4 cells per well were cultured in 12-well
plates and transfected with naked cy3-SCR (control), cy3-SCR loaded in siRNA-
NBs with US exposure (siRNA-NBs US (−)) or without US exposure (siRNA-NBs US
(−)). Analysis of cy3-SCR content was conducted by flow cytometry FACSCalibur
(BO, USA), 24 h after transfection, cells were trypsinized and collected, washed with
PBS, and re-suspended in 0.5 mL of PBS. Cells without transfection were used
as a control for background calibration. Data were analyzed using the WinMDI software
(Version 2.9, Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA).

2.10.2. Confocal laser scanning microscopy

The intracellular distribution of cy3-SCR was detected by confocal laser scanning
microscopy (CLSM). Cells were transfected with cy3-SCR labeled siRNA-NBs
(1 × 10^6 per dish) and incubated overnight. 4 h after cy3-SCR transfection as mentioned in the
transfection section, cells were washed three times with PBS and stained with
cy3-SCR (Beyotime Biotech, China) for 15 min to identify the siRNA location inside cells. Cells were observed on a fluorescence microscope (Olympus, Japan).
Cy3 and Hoechst 33342 were excited at 514 and 352 nm. The emission wavelengths of cy3
and Hoechst 33342 are 455 and 595 nm, respectively.

2.11. Molecular biology assays

2.11.1. Real-time PCR assay for mRNA level of SIRT2 genes

Molecular biology assays were carried out using experimental grouping as fol-
lows: SIRT2-NBs US (+) group, SIRT2-NBs US (−) group and SCR-NBs US (+) control
group. Total RNA was harvested from cell using the Trizol Reagent Kit (Invitrogen,
Karlsruhe, Germany). The first strand cdNA were synthesized using PrimeScript
RT reagent Kit (Takara Biotechnology, Japan). The mRNA expression of SIRT2 was
quantified using the 2^-ΔΔCt analytical method in triplicate using a StepOnePlus real-
time PCR System (ABI, USA). The glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) was used as the internal normalization standard. The primer sequences were shown in
Table S3. Real-time PCR was performed in a 20 μL reaction mixture containing
primers, FastStart Universal SYBR Green master (ROX) reagent (Roche Applied Sci-
ceine, Mannheim, Germany) and 2 μL cdNA sample.

2.11.2. Western blot analysis

48 h after siRNA transfection, total protein were extracted and quantified using
cytoBuster TM protein extraction reagent (Merck, Darmstadt, Germany) and bioc-
hemonic acid protein assay kit (Invitrogen, Carlsbad, CA, USA) separately. Protein
samples (20 μg) were separated on 10% sodium dodecyl sulfate polyacrylamine gel
electrophoresis (SDS-PAGE) and then transferred to polyvinyldione difluoride
(PVDF) membranes. After incubation with the blocking buffer (5% skim milk), the
membranes were incubated overnight at 4 °C with mouse antibody against SIRT2
(1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit antibody
against cleaved caspase-3 (1:500 dilution; Cell Signaling Technology, Danvers, USA).
Simultaneously, the membranes were incubated with rabbit antibody against tubulin (1:500 dilution; Cell Signaling Technology, Danvers, USA) as an internal standard for normalizing protein expressions. Horseradish peroxidase (HRP)-conjugated donkey anti-mouse or donkey anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used to amplify the signal. Protein signals were detected using chemiluminescence system (New Life Science Products, Boston, MA, USA).

2.12. Cancer cell apoptosis and cytotoxicity assays

Cells were seeded at a density of $2.5 \times 10^4$ per well on coverslips in 6-well plates, incubated overnight at 37°C, and transfected by SIRT2 siRNA as described already. 48 h after cell incubation, terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) assay was performed using an in situ cell death detection kit-POD (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer’s protocol. In brief, cells on the coverslips were fixed for 1 h with freshly prepared 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in PBS (pH 7.4) at room temperature. After incubating with blocking solution (0.3% H2O2 in methanol) for 10 min at room temperature and subsequently in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, permeabilized cells on each slide was incubated with 50 µL of TUNEL reaction mixture (with terminal deoxynucleotidyl transferase, TdT Enzyme) in a humidified chamber for 60 min at 37°C to catalyze polymerization of fluorescein-labeled nucleotides to free 3’-OH DNA ends. Then each slide was incubated with 50 µL of converter POD [anti-fluorescein antibody conjugated with peroxidase (POD)] for 30 min at 37°C to finish the conjugation of the DNA strand breaks with POD. Finally, the diaminobenzidine (DAB) substrate reacted with the POD-labeled cells to generate an insoluble brown DAB signal while shaded blue-green to greenish tan signifying non-apoptotic cells. The samples were analyzed under light microscope and documented on film for further quantitative analysis using ImageJ software. Determination of nuclear condensation was also carried out to detect the apoptosis of C6 glioma cells (see details in the Supplementary information).

Further quantitative analysis of cell apoptosis and necrosis with flow cytometry was performed using the Annexin-V-FLUOS Staining Kit (Roche Diagnostics, Mannheim, Germany), in which Annexin V-FITC labeled the early and late apoptotic cells while PI labeled the necrotic and late apoptotic cells. 48 h after the SIRT2-NB treatment, cells were trypsinized and collected. After being washed twice with PBS, the cells were labeled with Annexin V-FITC and PI according to the manufacturer’s protocols prior to fluorescence analysis on a flow cytometer (FACSCalibur, BD, USA).

MTT assay was used to evaluate the cytotoxicity after SIRT2 siRNA transfection into C6 glioma cells. All experiments were conducted in triplicate. Cells were seeded into 96-well plates at a density of 5000 cells per well and cultured overnight in a humidified atmosphere with 5% CO2. SIRT2 siRNA transfection was carried out as described above. After 48 h incubation, the medium was replaced with 100 µL of fresh medium containing 10 µL of MTT (Sigma, St. Louis, MO, USA) solution (5 µg/mL in PBS), and the cells were subsequently incubated for 4 h. The supernatant in each well was discarded and 100 µL of dimethyl sulfoxide was added to dissolve the substrate for 30 min. After gentle agitation for 5 min, the absorbance at 494 nm in each well was recorded on an Infinite F200 Multimode plate reader (Tecan, Männedorf, Switzerland).

2.13. In vivo studies

All experiments and animal care were approved by the Institutional Animal Care and Use Committee of the Sun Yat-sen University. Rat C6 glioma cells were implanted into the BALB/c nude mice (4–6 weeks, 18–23 g) to grow tumor xenografts. Briefly, the mice were anesthetized with 10% chloral hydrate (400 mg/kg) and placed in a stereotactic frame. The cells ($1 \times 10^6$) were re-suspended in 0.1 mL of PBS and subcutaneously injected in the right back.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Illustrative principle of siRNA delivery to tumor tissue and tumor cells using siRNA-NBs and low-frequency US exposure. (A) The siRNA-NBs complexes were small enough for accumulating in tumor tissues after intravenous injection. (B) With the assistance of low-frequency US, the permeability of endothelium and cancer cell membranes was enhanced simultaneously due to the activated cavitation of siRNA-NBs. The released siRNA micelles were delivered into cancer cell directly by the enhanced permeability of cancer cell membranes, which was known as “sonoporation”. In addition, after low-frequency US exposure, the cationic siRNA copolymer micelles, which were effectively accumulated in tumor tissue by cavitation, would get into the cancer cells further by the endocytosis of the cells. Abbreviations: US, ultrasound; siRNA-NBs, siRNA-loaded nanobubbles.
The mice bearing tumor were randomly divided into four groups (n = 6 for all groups): SIRT2-NBs with US exposure group (SIRT2-NBs US (+)), SIRT2-NBs without US exposure group (SIRT2-NBs US (-)), SCR-NBs with US exposure group (SCR-NBs US (+)) and PBS control group. The siRNA-NBs containing 8 μg of SIRT2 siRNA or SCR were prepared at a total volume of 100 μL. After the samples were injected into tail vein, the tumors were exposed to low-frequency US at two time points of 15 min and 30 min with the frequency of 1 MHz, intensity of 2.0 W/cm² and duty cycle of 50% for 1 min. The procedure was repeated every 2 days from day 1 to day 19.

The tumor volume was monitored by caliper measurements and calculated by the following equation: Volume = 0.5 × L × W², in which “L” and “W” represent the length and the width of tumor. Survival rate was analyzed by a log-rank test based on the Kaplan–Meier survival analysis using SPSS statistical software.

2.14. Histology and immunohistochemistry

The mice were sacrificed and then tumors were collected and fixed with freshly prepared 10% PBS buffered formalin for 24 h. Hematoxylin- eosin (H&E) staining of tumor tissue sections (3 μm) was performed after deparaffinization. Three paraffin sections of each tumor were used for H&E.

Immunohistochemistry study was carried out as follows: tumor sections were deparaffinized with xylene and alcohol, washed with PBS for three times, and incubated in 10 ms citrate-buffer (pH 7.4) for 10 min at 90 °C. The sections were then treated with 0.3% hydrogen peroxide in methanol at 4 °C for 30 min to inactivate the endogenous peroxidases. After being blocked with 10% normal horse serum, 5% bovine serum albumin (BSA) and 0.5% Triton X-100 for 1 h at room temperature, the tissues were then incubated in the presence of mouse primary antibody against SIRT2 (1:50 dilution in 5% BSA) and rabbit polyclonal primary antibodies for cleaved caspase-3 (1:500 dilution in BSA) for 1 h at 37 °C, washed with PBS for three times, and further incubated for 1 h with horseradish conjugated donkey anti-rabbit IgG secondary antibodies (DAKO Corporation, Carpinteria, CA). The immuno-reactivity on the tissue sections was visualized using the peroxidase substrate DAB. Finally, the nuclei were stained by hematoxylin.

2.15. In situ TUNEL assay

In situ TUNEL assay was carried out on the excised tumor tissues using a Fragment Assay DNA Fragment detection kit according to the manufacturer’s protocol (EMD chemicals Inc, Darmstadt, Germany). In brief, tumor tissue sections were deparaffinized with xylene and alcohol, washed with PBS for three times, and incubated in 10 ms citrate-buffer (pH 7.4) for 10 min at 90 °C. The sections were then treated with 0.3% hydrogen peroxide in methanol at 4 °C for 30 min to inactivate the endogenous peroxidases. After being blocked with 10% normal horse serum, 5% bovine serum albumin (BSA) and 0.5% Triton X-100 for 1 h at room temperature, the tissues were then incubated in the presence of mouse primary antibody against SIRT2 (1:50 dilution in 5% BSA) and rabbit polyclonal primary antibodies for cleaved caspase-3 (1:500 dilution in BSA) for 1 h at 37 °C, washed with PBS for three times, and further incubated for 1 h with horseradish conjugated donkey anti-rabbit IgG secondary antibodies (DAKO Corporation, Carpinteria, CA). The immuno-reactivity on the tissue sections was visualized using the peroxidase substrate DAB. Finally, the nuclei were stained by hematoxylin.

2.16. Statistical analysis

The data were statistically analyzed using one-factor analysis of variance (SPSS software, version 13.0, SPSS Inc.). All data are expressed as the mean ± the standard error of the mean. A P value < 0.05 was considered statistically significant. All statistical tests were two-tailed.

3. Results

3.1. Characterization of siRNA-NBs

siRNA-NBs were prepared by the hetero-assembly of the positively charged siRNA micelles and negatively charged liposomes with a gas core (Fig. 1). The copolymer mPEG-b-PLLys were characterized as shown in Supplementary information (Fig. S1–S5). The ability of mPEG-b-PLLys to complex siRNA molecules by electrostatic interaction to form the cationic micelles was investigated by agarose gel retardation. The N/P ratio played an important role in the complexion of siRNA and mPEG-b-PLLys. The polymer started to form micelle with siRNA at low N/P ratio, and siRNA was fully neutralized at the N/P ratio of about 4 and 5 as revealed by the complete retardation of siRNA motion in agarose gel (Fig. 3A).

DLS measurements indicated that the zeta potential of siRNA micelles increased when the N/P ratio was higher. Zeta potential of the siRNA micelles first increased significantly from −5.18 mV to +22.65 mV along with N/P ratio increasing from 0.1 to 6, but then leveled off at even higher N/P ratios. On the other hand, the particle size reached the highest value (242 nm) at the N/P ratio of 0.5, and then decreased rapidly as the N/P ratio further increased. When the N/P ratio was higher than 6, the particle size kept relatively stable at about 60−65 nm (Fig. 3B). At the N/P ratio of 5, the siRNA micelle showed a high zeta potential (~21.97 mV) and a small diameter (66.5 nm), which were considered appropriate for further siRNA-NB preparation.

The average diameter of the gas-cored liposomes was 375.1 ± 8.9 nm as detected by DLS measurement. The liposomes then underwent electropositive coating of siRNA micelles to form the siRNA-NBs. As the P/Pᵣ ratio increased, although the particle sizes varied at a relatively narrow range between 327.0 nm and...
393.0 nm along with micelle adhering the liposomes, the zeta potential increased much more obviously from $-18.36$ mV to about $+15.0$ mV due to the neutralization of the negatively charged liposomes by the positively charged siRNA micelles (Fig. 3C). Considering that weak positive charge favors not only the adhesion of siRNA-NBs to the surface of cancer cells but also a low cytotoxicity, siRNA-NBs prepared at the P/P$_1$ ratio of 0.2 (zeta potential: $+9.82$ mV) was chosen for biological studies.

As shown in Fig. 3D, siRNA complexed in siRNA-NBs can be effectively protected from enzymatic degradation. After 60 min incubation with ribonuclease, even though most of the naked SCR was degraded, the structural integrity of SCR complexed in siRNA-NBs appeared still unchanged.

3.2. TEM images

Morphology and size distribution of the gas-cored liposomes and siRNA-NBs were measured by TEM as well. Both the liposomes (Fig. 4A) and siRNA-NBs (Fig. 4B) appeared spherical and non-aggregated, and showed particle size distribution ranging from 250 to 500 nm, which are similar to that detected by DLS. At high magnification, TEM observations clearly indicated that, unlike the liposomes which have fairly smooth surface (Fig. 4C), the siRNA-NBs possessed much more uneven surface (Fig. 4D). siRNA micelles (about 50–70 nm) adhering the surface of liposomes via electrostatic interaction were clearly shown. It was noted that both the liposomes and siRNA-NBs showed fractures on the particle surfaces, most likely due to the shrinkage of hollow bubbles during vacuum dry process.

3.3. The stability and US sensitivity of siRNA-NBs

Representative contrast enhanced US images at every 20 min US exposure times were shown in Fig. 5A. Results showed that the gray-scale intensities of siRNA-NBs and gas-cored liposomes did not change much up to 50 min. After 50 min, the signal intensities of both samples began to decrease. However, the gray-scale intensity of gas-cored liposomes dropped much more significantly than that of the siRNA-NBs (Fig. 5A, B), indicating that siRNA-NBs were structurally more stable than gas-cored liposomes. After low-frequency US applied to the sample wells, contrast enhanced US imaging was carried out. Gray-scale intensity of siRNA-NBs diminished immediately as that of the gas-cored liposomes (Fig. 5C).

3.4. siRNA transfection efficiency of siRNA-NBs with the aid of US exposure

Quantitative flow cytometry showed that the siRNA transfection of siRNA-NBs with the aid of US exposure was highly effective. Cells
treated with siRNA-NBs carrying cy3-SCR under US exposure were 50.3 ± 2.5% cy3-positive, which was significantly higher than the values for other groups (5.0 ± 0.3% for US (−) group, 1.3 ± 0.2% for control group). Therefore, US exposure has brought about 10 folds higher transfection efficiency in C6 glioma cells (Fig. 6).

C6 glioma cell uptake of siRNA-NBs (N/P = 5; P/P1 = 0.2) under low-frequency US exposure was evaluated using CLSM. Cy3-SCR was used to visualize the siRNA distribution for the assay. 4 h after the cells were incubated in the presence of siRNA-NBs carrying cy3-SCR with the aid of 1 min US exposure, the cell uptake and intracellular siRNA distribution of cy3-SCR were determined by CLSM observation. As shown in Fig. 7, under US exposure, siRNA was much more efficiently transferred into cells. Compared to the non-US and control groups, much more intensified red fluorescence was detected in the US exposure group. Moreover, siRNA was mostly observed in the cytoplasm and periphery of nuclei.

3.5. Suppression of SIRT2 gene expression

In comparison with the negative control group, the SIRT2 mRNA level decreased in C6 glioma cells treated with SIRT2 siRNA-NBs no matter US exposure was applied or not (Fig. 8A). Moreover, US exposure played a key role in further decreasing the SIRT2 mRNA level. The SIRT2-NBs US (+) group showed a 57.3% decrease in the SIRT2 mRNA level, which is significantly higher than that for the SIRT2-NBs US (−) group (only 16.9% decrease).

The expression of SIRT2 and activation of caspase-3 at the protein level were further determined by western blotting (Fig. 8B–D). The SIRT2 protein was most effectively depressed in the SIRT2-NBs US (+) group, resulting in the highest level of caspase-3 activation, i.e. the highest expression of the cleaved caspase-3 protein.

3.6. Cell apoptosis and growth inhibition induced by SIRT2 gene silencing

As shown in the TUNEL assay, C6 glioma cells treated with SIRT2 siRNA-NBs under US exposure showed significantly higher apoptotic level in comparison with other groups (Fig. 9A–C).

![Fig. 5. Stability of the siRNA-NBs and gas-cored liposomes analyzed by contrast enhanced US imaging in vitro (A). Compared with gas-cored liposomes, the gray-scale intensities of siRNA-NBs complexes declined more slowly, confirming strengthened stability (B). Low-frequency US exposure induced similarly immediate diminution in gray-scale intensity for both the siRNA-NBs and gas-cored liposomes (C), indicating that siRNA micelle adhering to gas-cored liposome surfaces did not change the US sensitivity of gas-cored liposomes. Abbreviations: siRNA-NBs, siRNA-loaded nanobubbles; US, ultrasound.](https://example.com/fig5.png)

![Fig. 6. Quantitative analysis of cy3-positive C6 glioma cells by flow cytometry after cell incubation at various conditions (n = 3, **P < 0.001 compared with siRNA-NBs US (+)). Cell incubation time: 24 h. Abbreviations: siRNA-NBs US (+) or siRNA-NBs US (−), cy3-labeled SCR-loaded nanobubbles with or without low-frequency US exposure.](https://example.com/fig6.png)
Further quantitative analysis of apoptotic cells using flow cytometry indicated that cells of the SIRT2-NBs US (+) group reached the highest apoptosis level of 33.5 ± 2.1%. By contrast, cells of the SIRT2-NBs US (-) and SCR-NBs US (+) groups only showed cell apoptosis ratios of 4.2 ± 0.5% and 1.9 ± 0.2%, respectively (Fig. 9D–F). In addition, SIRT2-NBs US (+) group exhibited a higher level of nuclear condensation compared with the other groups (Fig. S6).

MTT assay was conducted to detect the cell growth inhibition induced by SIRT2 siRNA transfection. The C6 glioma cells transfected with siRNA-NBs under US exposure had much lower viability (50.3 ± 2.0%) compared to the siRNA-NBs US (-) group (93.0 ± 1.9%) and SCR-NBs US (+) group (100.0 ± 2.4%), as shown in Fig. 10.

3.7. Therapeutic effect of SIRT2 siRNA in vivo

Based on the in vitro results that SIRT2 siRNA delivered by siRNA-NBs with the aid of US exposure can effectively suppresses SIRT2 expression and enhance apoptosis in C6 glioma cells, the therapeutic effect of siRNA-NBs was further evaluated in C6 glioma-implanted nude mice. After the repeated treatments by SIRT2-NBs under low-frequency US exposure, tumor growth was effectively slowed down (Fig. 11A, B). Starting from day 12, tumor volume in the SIRT2-NBs US (+) group was much smaller than that in the other groups. In addition, no significant difference was detected between the SIRT2-NBs US (-) group and SCR-NBs US (+) group or PBS group (Fig. 11). The averaged tumor volumes were 1594.8 ± 139.8 mm³, 3280.8 ± 401.1 mm³ (P < 0.001), 3166.3 ± 381.7 mm³ (P < 0.001) and 3421.2 ± 336.4 mm³ (P < 0.001) at the time point of 28 days for the SIRT2-NBs US (+), SIRT2-NBs US (-), SCR-NBs US (+) and PBS groups, respectively. The time course survival rates of mice with different treatments were in accord with the results of tumor growth inhibition. Mice in the SIRT2-NBs US (-) group, SCR-NBs US (+) group and PBS group died from day 27, day 19 and day 21, respectively. In comparison, the SIRT2-NBs US (+) group demonstrated much better therapeutic effect. It was observed that in this group 83.3% of the mice survived longer than 35 days (Fig. 11C).

The histological changes of the tumors after treatments were compared using H&E staining. The H&E stained sections of tumor tissue from the SIRT2-NBs US (-) group, SCR-NBs US (+) group and PBS group appeared more hypercellular and showed obvious nuclear polymorphism (Fig. 12). However, tumor tissues from the SIRT2-NBs US (+) group were most hypocellular and exhibited the highest level of tumor apoptosis and necrosis, indicating the best therapeutic effect of SIRT2-NBs with the aid of US exposure.

Further evidences confirming the expression of the SIRT2 and cleaved caspase-3 proteins in tumor tissue were provided by in situ immunohistochemistry assay. The brown stains indicated the SIRT2 and cleaved caspase-3 proteins expressed in tumor tissues, and the cell nuclei were stained blue as a contrast. Tumor tissues from the SIRT2 siRNA-NBs US (+) group showed the lowest level of SIRT2 protein whereas the highest level of cleaved caspase-3 protein. However, the expression levels of SIRT2 and cleaved caspase-3 were completely reversed in tumor tissues of the SIRT2-NBs US (-), SCR-NBs US (+) and the PBS groups (Fig. 12). That is, relatively high level of SIRT2 protein was observed in the cytoplasm, and the cleaved caspase-3 protein was rare. These results demonstrated...
Fig. 8. Analysis of SIRT2 gene suppression of siRNA-NBs in C6 glioma cells. The suppression of SIRT2 gene in C6 glioma cells evaluated at mRNA level by real-time PCR (A). SIRT2 expression and caspase-3 activity analyzed at protein level by western blotting (B). The expression of SIRT2 protein was markedly suppressed in the SIRT2-NBs US (+) group (C), leading to the highest level of caspase-3 activation (D). (**P < 0.01, compared with SIRT2-NBs US (+) group, n = 3). Abbreviations: SIRT2-NBs US (+) or SIRT2-NBs US (−), SIRT2 siRNA-loaded nanobubbles with or without low-frequency US exposure.

Fig. 9. C6 glioma cell apoptosis assessed by TUNEL staining assay 48 h after the treatments, in the SIRT2-NBs US (+) group (A), SIRT2-NBs US (−) group (B) and control group (C). Magnified image of the red rectangular area marks a representative apoptotic cell, whereas the magnified green rectangular area shows a normal tumor cell. The percentage of apoptotic cells quantified by Annexin V-FITC and PI flow cytometry in the SIRT2-NBs US (+) group (D), SIRT2-NBs US (−) group (E) and control group (F). Q1: late stage apoptotic cells; Q2: necrotic cells; Q3: normal viable cells; Q4: early stage apoptotic cells. Abbreviations: SIRT2-NBs US (+) or SIRT2-NBs US (−), SIRT2 siRNA-loaded nanobubbles with or without US exposure; US, ultrasound. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
that the treatment using SIRT2 siRNA-NBs with the aid of US exposure were able to suppress the SIRT2 expression and to activate the caspase-3 gene very effectively.

Finally, studies of TUNEL assays were carried out to correlate the cell apoptosis with the inhibition of SIRT2 and up-regulation of cleaved caspase-3 in tumor tissues. Apparently, cell apoptosis was the most significant in the SIRT2-NBs US (+) therapeutic group among all groups (Fig. 12).

4. Discussion

The combination of microbubbles (MBs) and low-frequency US exposure has shown great potential to improve the gene transfection efficiency and targeting ability of cationic polymer micelles [23,31]. However, several crucial problems still remained for the in vivo application of the US and MBs mediated siRNA therapy of cancers [32–34], and chief among them is the large particle size of MBs. Commercial US probes such as SonoVue®, Optison® possess micro-scale dimensions which limit their applications outside blood vessels, because these large particles are unable to penetrate the gaps between vascular endothelial cells. Although permeability of the vascular endothelium can be enhanced by low-frequency US cavitation, the membrane permeability of cancer parenchymal cells remains unchanged if MBs cannot approach these cells [35]. This means that it is still difficult to transport drugs into cancer cells. Therefore, creation of US-sensitive drug delivery agents having nano-dimensions (i.e. the NBs), which enable the accumulation of NBs around cancer cells for US cavitation leading to enhanced cell membrane permeability, is of great importance at present.

We recently developed a nano-scale US contrast agent with gas core (NBs, referred to as gas-cored liposomes here) for tumor US imaging. In vivo results showed that this agent could effectively accumulate in tumor tissue, which brought about the potential for tumor-targeted drug delivery [19]. In this study, to turn the NBs into a siRNA nanocarrier, siRNA was complexed with mPEG-b-PLLys to...
get a positively charged siRNA micelle, which was then loaded onto the negatively charged surfaces of gas-cored liposomes by electrostatic interaction. The results of the changes of gray-scale intensities before and after US exposure indicated that the siRNA-NBs remained good US sensitivity (Fig. 5), which implied a great potential of this multifunctional system in UTMD-assisted in vivo tumor therapy.

We assume that our siRNA-NBs may effectively potentiate the siRNA transfection in tumor (Fig. 2). When low-frequency US exposure was applied at the tumor area, cavitations appeared both in the capillary vessels and tumor tissue because of the presence of siRNA-NBs, resulting in permeability enhancement in both the vascular endothelium and cancer-cell membranes [31,36]. Consequently, siRNA-NBs were effectively delivered into tumor tissue first. Subsequently, the siRNA micelles adhering to the NBs were released in tumor tissue upon cavitation and were further driven into cytoplasm by acoustic streaming which had a sonoporation effect on the cancer parenchymal cell membrane. On the other hand, the positively charged surface of siRNA micelles may also facilitate their delivery into cancer cells via endocytosis. The above hypothesis was supported by the high siRNA transfection efficiency.

As a nano-scale siRNA carrier, the siRNA-NBs possessed tumor passive targeting ability resulted from the enhanced permeability and retention (EPR) effect [37]. However, as the particle size increased, the EPR effect of nanocarriers weakened as well, which is the underlying reason for the insufficient tumor targeting ability and low siRNA transfection efficiency of siRNA-NBs with relatively large sizes compared to the traditional nanocarriers. With the aid of US exposure, this drawback can be effectively avoided by the cavitation and sonoporation effects. Results obtained in the in vivo tumor treatment experiments supported the above notion. The US exposure group showed statistically better therapeutic effect than the non-US one. The combination of the siRNA-NB passive targeting ability and US exposure of local tumor area essentially improved the therapeutic effect of siRNA.

It is known that SIRT2 activity plays a key role in maintaining the survival of glioma cells since SIRT2 may inhibit the activation of caspase-3 and p53 which lead to the anti-apoptosis effect in cancer cells [28,38]. Molecular biological experiments in this study demonstrated that the ideal therapeutic effect of siRNA-NBs in C6 glioma treatment can be attributed to the application of low-frequency US exposure which greatly increased the delivery efficiency of SIRT2 siRNA into tumor tissue and cells.

5. Conclusion

A US-sensitive nanocarrier, siRNA-NBs bearing a gas core, which can effectively carry siRNA was developed for tumor therapy. Accumulation of the nano-structured siRNA-NBs in tumor tissues can be achieved via the EPR effect, which however would never occur for the traditional micro-dimensional MBs. With the aid of low-frequency US exposure, in tumor tissue siRNA micelles can be released from the siRNA-NBs and then effectively delivered into cancer cells. Since the large particle size and insufficient siRNA loading of MBs are two major challenges for the application for UTMD technique in tumor therapy at present, siRNA delivery with the US-sensitive siRNA-NBs appeared very meaningful. Both the in vitro and in vivo studies have indicated that, with the aid of US
cavitation and sonoporation effects, the US-sensitive siRNA-NBs may possess a great potential to achieve ideal therapeutic effect of RNA interfering in cancer treatment.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.02.067.

References


T. Yin et al. / Biomaterials 34 (2013) 4532–4543