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Electronic Supplementary Information

A PEGylated megamer-based microRNA delivery system activatable by stepwise microenvironment stimulation

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Materials and Methods

Materials

Methoxy polyethylene glycol (PEG) with average MW of 5000 Da, N,N-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), and dimethyl sulfoxide (DMSO) was obtained from Aladdin (Shanghai, China). Carboxy-dimethylmaleic anhydride (CDM) was were purchased from TCI (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and 3'-dithiodipropionic acid-di(N-succinimidyl ester) (DSP) were purchased from Sigma-Aldrich (St. Louis, MO). Ethylenediamine-cored and amine-terminated second-generation (G2) polyamidoamine (PAMAM) dendrimers (MW 3256 Da) dissolved in methanol were purchased from Dendritech (Midland, MI). 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), Alexa Fluor® 647 conjugate of wheat germ agglutinin (WGA), and Pierce™ dialysis membranes were purchased from Thermo Fisher Scientific (Waltham, MA). Fluorescein-5,6-isothiocyanate (FITC)-labeled miR-122, Cyanine5 (Cy5)-labeled miR-122, and unmodified miR-122 (sence 5'-UGGAGUGUGACAAUGGUGUUUG-3', anti-sence 5'-AACACCAUUGUCACACUCCA UU-3') were synthesized by GenePharma (Shanghai, China). G2 dendrimer solutions were distilled to remove methanol and obtained as white gels. All the other chemicals were used as received without further purification.

Cells lines and animals

The HepG2 and Huh-7 cells (human liver cancer cells, from Shanghai Institute for Biological Science, China) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Waltham, MA) in a humidified atmosphere containing 5% CO_2 at 37 °C. BALB/c nude mice (female, 6 weeks old) was purchased from HFK Bioscience (Beijing, China). All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and all procedures were approved by Animal Care Committee of Zhejiang University School of Medicine. The xenograft tumor model was generated by injection of 1×10^6 Huh-7 cells suspended in 100 μL PBS per mouse subcutaneously to the subaxillary site.

Synthesis of BOMB

DSP-crosslinked G2 PAMAM dendrimers (GSSG) was synthesized according to the method previously described[1]. To a PBS buffer solution (pH = 7.4) containing G2 PAMAM dendrimer, a DMSO solution containing a certain amount of DSP were added dropwise. The reaction mixture was stirred at room temperature overnight, followed by extensive dialysis (Spectrum, MWCO 1000 Da) against PBS buffer and double-distilled water. The product was lyophilized and obtained as a white gel. The product was characterized by ^{1}H NMR (400 MHz, D_2O): δ 2.50-2.32 ppm, (m, -NCH_2**CH_2**$CONH$-); 2.65-2.54 ppm, (m, -$CONHCH_2$**CH_2**N- in G2 and -CO**CH_2**CH_2S-); 2.85-2.74 ppm, (m, -N**CH_2**CH_2CONH-); 3.35-3.22 ppm, (m, -$CONH$**CH_2**CH_2N-); 3.06 ppm, (t, J = 0.02 Hz, -$CONH$**CH_2**$CH_2NH_3^+$); 3.44 ppm, (t, J = 0.02 Hz, $CONH$**CH_2**$CH_2NH_3^+$); 2.91 ppm, (t, J = 0.02 Hz-$COCH_2$**CH_2**S-). IR (neat, cm^{-1}): 3449 (s, ν(,-NH_2 of G_2)), 1640 (s, ν(C=O)).

$Dlink_m$-conjugated PEG (PEG-$Dlink_m$) was prepared according to a previously reported method.[2] CDM (0.276 g, 1.5 mmol) was dissolved in 10 mL of dry dichloromethane, then oxalyl chloride (0.378 g, 3 mmol) and a catalytic amount of DMF (40 μL) were added. The solution was stirred for 15 min at 0 °C, and was then transferred to room temperature for further reaction for 1 h. After vacuum drying, the chloride acetylated CDM was reacted with 0.2 mmol of PEG in 10 mL of dry dichloromethane with 30 μL of pyridine as the catalyst. The reaction was performed at 25 °C for 3 h and terminated by adding the same volume of saturated ammonium chloride aqueous solution. The organic phase was separated, collected, dried, and precipitated into anhydrous diethyl ether at 0 °C twice. The obtained product was eventually dried to obtain a brown powder with a typical yield rate of ca. 87%. The product was characterized by ^{1}H NMR (400 MHz, D_2O) as shown in Figure S1a: δ 4.30-4.15 ppm, (m, -COO**CH_2**-); 3.49-3.45 ppm, (m,-**OCH_3** overlapped with -**CH_2CH_2**O- of PEG); 2.80-2.49 ppm, (m, -**CH_2CH_2**COO-); 2.10-1.95 ppm, (m, -**CH_3** of $Dlink_m$). IR (neat, cm^{-1}): 3448 (s, ν(O-H of residual PEG)), 2889 (s, ν(C-H), 1647 (s, ν(C=O) of $Dlink_m$), 1112 (s, ν(C-O)).

PEG-$Dlink_m$-GSSG (BOMB) was synthesized by a ring-opening reaction of PEG-$Dlink_m$ with GSSG. GSSG (325.4 mg, 1.0 eqv) and PEG-$Dlink_m$ (500 mg, 1.0 eqv) were dissolved in 10 mL DMSO and stirred at room temperature in a dark place. After 2 h, the mixture was added to ultrapure water to obtain nanoparticles and further purified by ultrafiltration (MWCO = 14,000 Da) to remove impurities. The product was lyophilized and obtained as a white solid

with a yield of 82%. The product was characterized by ^{1}H NMR (400 MHz, D_2O) as shown in Figure S1a: δ 4.30-4.15 ppm, (m, -COO**C**$\mathbf{H_2}$-); 3.70 ppm, (s, -C$\mathbf{H_2}$C$\mathbf{H_2}$O- of PEG); 3.55-2.30 ppm, (m, -$\mathbf{CH_3}$ of PEG, -$\mathbf{CH_2CH_2}$COO- of $Dlink_m$ and GSSG protons); 2.00-1.80 ppm, (m, -$\mathbf{CH_3}$ of $Dlink_m$). IR (neat, cm^{-1}): 3446 (m, *v*(N-H) of G2), 2887 (s, *v*(C-H), 1640 (s, *v*(C=O)), 1112 (s, *v*(C-O)).

Determination of miR-122 loading capacity by gel electrophoresis assay

Gel electrophoresis was carried out in TAE running buffer (40×10^{-3} M Tris-acetate, 1×10^{-3} M EDTA) with a voltage of 80 V and 200 mA for 20 min in a Sub-Cell system (Bio-Rad Lab, Hercules, CA). miR-122 bands were visualized and photographed by an UV transilluminator and BioDco-It imaging system (UVP, Upland, CA).

Characterization of BOMB

The structures of BOMB and its components were characterized by proton nuclear magnetic resonance (^{1}H NMR). The ^{1}H NMR and NOESY spectra were recorded on a Varian 400 MHz NMR spectrometer at 22 °C. Chemical shifts were reported in parts per million (ppm) on the δ scale, and were referenced to residual protonated solvent peaks (δ = 4.79 ppm for residual peak of D_2O). All the spectra were evaluated with MestReNova 6.2 (Mestrelab Research, Santiago de Compostela, Spain). FT-IR spectra were measured from films of pure samples in the form of KBr discs on a Thermo Nicolet Nexus 5700 FT-IR spectrometer. For each spectrum, 128 scans were collected at resolution of 4 cm^{-1} over the range 400–4000 cm^{-1}.

Preparation of the BOMB/miRNA nanoparticles (BOMB/miR-122)

miR-122 was dissolved in DEPC H_2O and was then diluted to 0.1 μg/μL. Equivalent volume of BOMB solution was added dropwise to RNA solution according to the predetermined weight ratios. The minimum weight ratios to condemn the miRNA cargos were determined by Agarose gel electrophoresis retardation. A weight ratio tenfold greater than the minimum weight ratio, as conventionally used in previous cationic polymer studies[3], was applied to further experiments. The BOMB/miR-122 complex mixture solution was vortexed and incubated for 30 minutes at room temperature before further use.

Morphological study and measurement of particle size and surface charge

The hydrodynamic radius and the surface charge of polyplexes were measured on a Zetasizer Nano ZS (Malvern, Worcestershire, UK) by dynamic light scattering (DLS), with a laser light wavelength of 635 nm at a 90° scattering angle. Specifically, 20 μL of G2/miR-122, GSSG/miR-122, and BOMB/miR-122 polyplexes containing 1 μg of miR-122 were vortexed for 6 seconds followed by 30 min incubation at room temperature, and were then diluted to 1 mL with H_2O at the time of measurement. The particle size and ζ potential of miRNA-free G2, GSSG, and BOMB were directly measured in H_2O solution. miRNA-loaded BOMB and GSSG nanoparticles were observed using a Hitachi HT-7700 transmission electron microscope (Tokyo, Japan). To evaluate the acid sensitivity of polymers, G2, GSSG and BOMB were incubated in PBS (pH = 6.5) at 37°C with constant shaking, and the corresponding ζ potentials of above solutions were monitored and recorded at different time points.

Serum stability of BOMB system

For hemolytic studies, the red blood cell (RBC) suspension was dispersed in distilled water and PBS buffer. Distilled water was considered as 100% hemolytic, and PBS buffer as non-hemolytic as control. A series concentration of BOMB/miR-122 were added to a 2% w/v solution of freshly prepared sheep red blood cells in PBS buffer and incubated for 1 h at 37°C in a shaking water bath. The samples were centrifuged (1500 g for 10 min) and the supernatants were assayed spectrophotometrically for the presence of free hemoglobin (λ= 540 nm). As for the time-dependent size pattern of BOMB/miR-122 (100 μg/mL in PBS buffer and serum-containing solution) was measured at a predetermined time point.

Characterization of miRNA release behavior

BOMB/miR-122 was incubated at pH 6.5 or pH 7.4 with or without dithiothreitol (DTT) in DEPC H_2O in triplicate and incubated at 37 °C with gentle shaking (87 rpm). The cumulative release of miRNA was monitored and recorded at different time points. The incremental released amount of miRNA in the supernatant was measured using the Quant-iT™ RiboGreen

(Invitrogen, Carlsbad, CA) assay.[4, 5] Cumulative release (R%) data was fitted to post-treatment time (t) in hours by the following formula[6]:

$$R\% = R_{max} \times (1 - 2^{-\frac{t}{t_{1/2}}})$$

where R_{max} is the maximum cumulative release amount, and $t_{1/2}$ is the half-life of first-order release.

Demonstration of *in vitro* internalization

The Huh-7 and HepG2 cells were transferred to ultralow attachment 6-well plates and incubated in DMEM medium at pH 7.4. Naked FITC-labelled miR-122 (miR^{FITC+}), G2/miR^{FITC+}, GSSG/miR^{FITC+}, and BOMB/miR^{FITC+} pretreated with pH 6.5 or pH 7.4 PB buffer for 4 h were added to the media at a final concentration of 1 μg/well. After 4 h, the spheroids were harvested and washed with PBS three times and trypsinized into single cells. The cells were then washed with PBS for three times, resuspended in 200 μL of PBS, and subjected to flow cytometry (FACS) analyses on a BD FACS Calibur flow cytometer (BD Bioscience, Bedford, MA). For visualization study of cellular uptake, the cells were stained with Alexa Fluor® 647 WGA for 1 hour and washed with PBS. The prepared cells were then fixed with 4% paraformaldehyde for 10 minutes, and the nuclei were stained with DAPI after the removal of paraformaldehyde residues. The cells were imaged under a Radiance 2100 confocal laser scanning microscope (Bio-Rad, Hercules, CA) to visualize the fluorochromes with the following excitation (Ex) and emission (Em) wavelength: DAPI (Ex 350 nm, Em 470 nm), FITC-labeled miR-122 (Ex 490 nm, Em 525 nm), and Alexa Fluor® 647 WGA (Ex 650 nm, Em 665 nm).

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol Reagent (TaKaRa, Shiga, Japan). One microgram of total RNA was used as template to generate the cDNA with the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. Fold changes were calculated by the ΔΔCT method. Primer pairs were synthesized by Sangon

Biotech (Shanghai, China) as follows: forward GGAAGGCTGGAGTGTGACAATG, reverse GTGCAGGGTCCGAGGT.

In vivo distribution study

For *in vivo* fluorescence imaging, naked Cy5-labelled miR-122 (miR^{Cy5+}), GSSG/miR^{Cy5+}, or BOMB/miR^{Cy5+} was intravenously administered into mice *via* tail-vein injection at an equivalent dose of 1.0 mg miR^{Cy5+} per kg mouse body weight. At the predetermined time points, the mice were anesthetized with 2.5% isoflurane and imaged using the Xenogen IVIS Lumina system (Caliper Life Sciences, Alameda, CA). The results were then analyzed using Living Image 3.1 software (Caliper Life Sciences, Alameda, CA).

Determination of Bcl-9 and β-catenin expression by Western blotting

Huh-7 cells were seeded into 6-well plates at a density of 2×10^5 cells/well and incubated for 18 h. Cells were then treated with PBS, naked miR-122, GSSG/miR-122, or BOMB/miR-122 pretreated with pH 6.5 or pH 7.4 for 72h, washed with ice-cold PBS and harvested in 100 μL cell lysis buffer (Cell Signaling, Danvers, MA) containing protease inhibitors (Cell Signaling, Danvers, MA). For tumor tissue samples, we used to keep in liquid nitrogen, were lysed in ice-cold radioimmunoprecipitation buffer supplemented with phosphatase inhibitor cocktails (Cell Signaling, Danvers, MA), centrifuged and then protein was collected. The protein concentration of lysates was determined using the bicin choninic acid method (Thermo, Rockford, IL). Lysate samples (40 μg/lane) were separated using 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with Tris-buffered saline/0.1% Tween 20 (TBS/T) containing 5% bovine serum albumin (BSA) and incubated overnight at 4 °C with anti-Bcl-9, anti-β-catenin, or anti-GADPH. The membranes were washed three times with TBS/T, incubated with goat anti-mouse horseradish peroxidase cell antibody for 2 h at 4 °C, washed. The membranes were then washed and immunoreactive bands were developed using an enhanced chemiluminescence reagent (GE Healthcare, Piscataway, NJ) and visualized by autoradiography kit (Kodak; Rochester, NY).

***In vitro* therapeutic effect by cell viability assays**

Huh-7 cells were seeded in 96-well plates at 8000 cells/well in 200 μL of complete DMEM medium supplemented with 10% FBS and flowed to grow for 16 h. Subsequently, the cells were treated with BOMB, naked miR-122, GSSG/miR-122, or BOMB/miR-122 pretreated with pH 6.5 or pH 7.4 PB buffer for 4 h. After 72 h treatment, an MTT stock solution (0.5 mg/ml) was added to each well. After incubation for another 4 h, the medium was removed and the resulted Formazan crystal was dissolved in 100 μL of DMSO. The absorbance was measured at 570 nm using a Bio-Rad 550 microplate reader (Bio-Red, Hercules, CA). The relative cell viability (V%) related to control cells cultured in the media without the polymers was calculated by the following relationship: $([A]_{test} - [A]_{blank})/([A]_{control} - [A]_{blank}) \times 100$. The half half-maximal inhibitory concentration (IC_{50}) was determined by best-fit value in the following formula[6]:

$$V\% = \frac{100}{1+ ([miR-122]/IC_{50})^{p}}$$

where V% is the relative viability and [miR-122] is the dose (μg/mL) of miR-122 or equivalent dose of BOMB after N/P ratio conversion.

***In vivo* effect on tumor growth**

When the tumor volume was approximately 50 mm^3, the mice were randomly divided to different groups, and received treatment by intravenous injection of PBS, free miR-122, GSSG/miR-122, BOMB/miR-122, every other day at a dose of 40 μg miR-122 per mouse. The tumor volume was monitored every 2 days. The tumor volume was calculated by 0.5 × length × $width^2$.

Histology and immunohistochemistry studies

One day after the last treatment, mice were sacrificed and tumor tissues were fixed with 4% paraformaldehyde 24 h at 4°C, dehydrated in graded ethanol, embedded in paraffin, and then cut into 4-μm-thick sections. Tissue sections were stained with hematoxylin/eosin (H&E). The proliferation status of tumor cells was detected using primary antibody of Ki67 (1:25, Abcam, Cambridge, MA), then incubated with secondary antibody of a Rb IgG (H+L)/HRP

(ZSGB-BIO, Beijing, China). Paraffin-embedded tumor sections were obtained for immunohistochemical staining of Bcl-9.

Statistical analyses

Experiments were repeated at least in triplicate. Data are presented in term of mean ± s.d. Statistical analyses were performed using Prism 7 (GraphPad, La Jolla, CA) with the indicated analytical methods. The flow cytometry data were analyzed using FlowJo 10 (TreeStar, Ashland, OR). Best-fit values and 95% CIs were calculated using indicated nonlinear regression. $p < 0.05$ was considered significant.

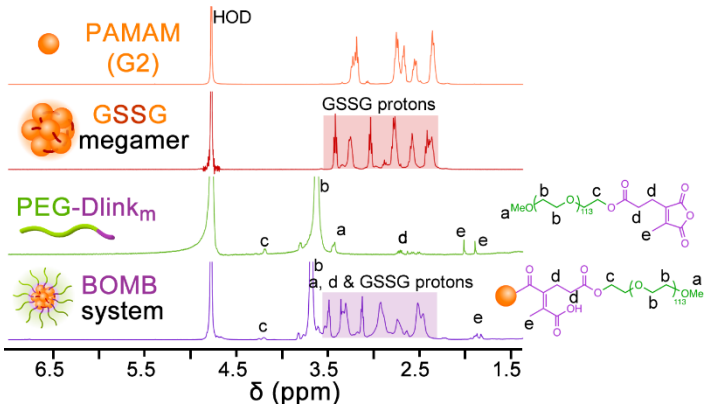

Figure S1a. ^{1}H NMR spectra of second-generation (G2) polyamidoamine (PAMAM) dendrimer, GSSG megamer, PEG-Dlink$_m$ conjugate, and BOMB system measured by an AVANCE III 400 MHz spectrometer at 22 °C.

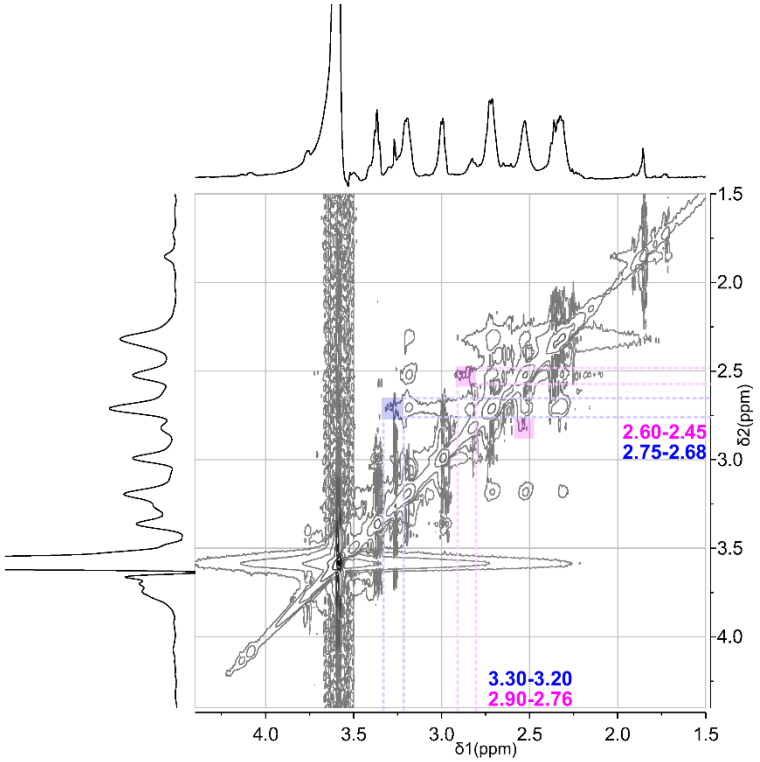

Figure S1b. Analysis of NOESY spectrum of the BOMB complex. The peak correlation of PEG-Dlink$_m$ conjugate cavities (s, 3.28 ppm) and G2 moiety (m, 2.75-2.68 ppm) is highlight in blue; αCH_2 of the disulfide cavities (m, 2.90-2.76 ppm) and G2 moiety(m, 2.75-2.68 ppm) is highlight in red.

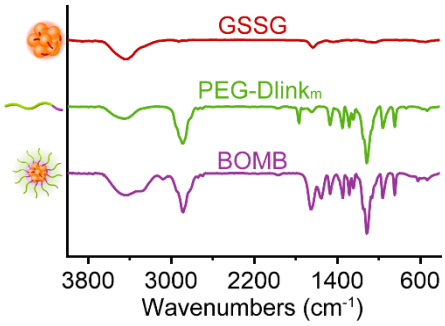

Figure S2. FT-IR spectra of GSSG, PEG-Dlink$_m$, and BOMB complexes. Results of infrared (IR) spectroscopy supported the concurrent presence of absorbance peaks of PEG-Dlink$_m$ conjugates at 1788 cm^{-1} (carbonyl groups in Dlink$_m$) and 1211 cm^{-1} (C-O bonds in PEG) and GSSG megamer at 3446 cm^{-1} (amino groups) in BOMB system.

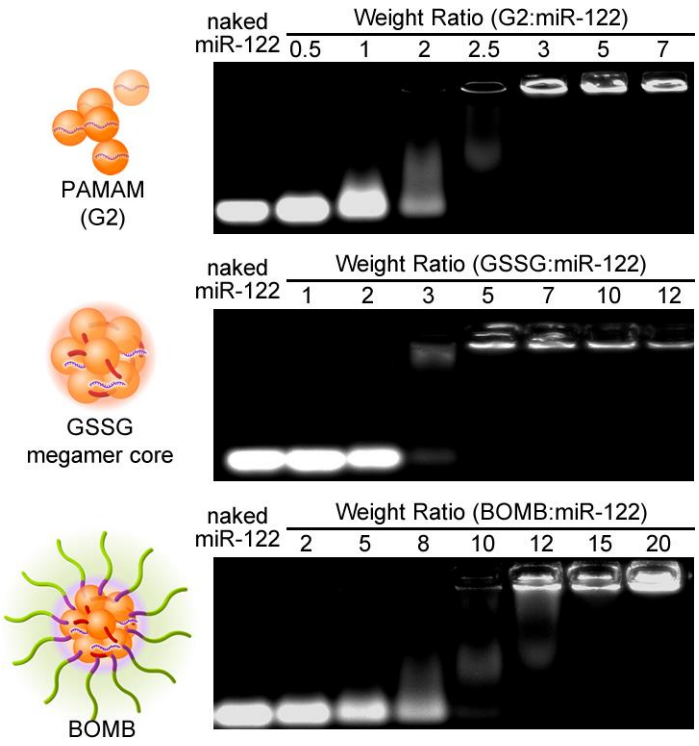

Figure S3. Agarose gel electrophoresis retardation of miR-122 complexed with G2 PAMAM dendrimer, GSSG megamer core, or BOMB at different weight ratios. Complete miRNA complexation by G2 PAMAM dendrimer, GSSG megamer, and BOMB occurred at the minimum weight ratios of 3:1, 5:1, and 15:1, respectively.

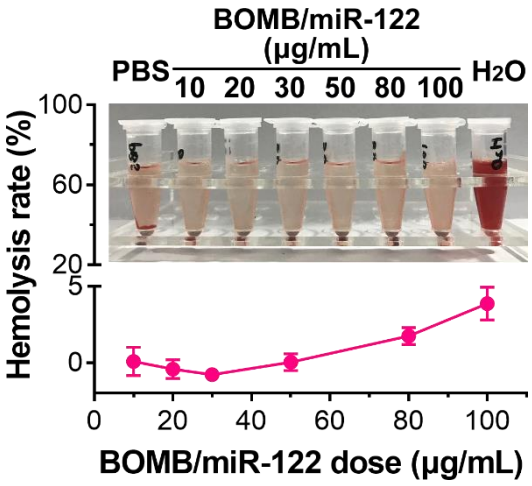

Figure S4a. Dose-dependent hemolysis rate of BOMB/miR-122 nanoparticles, and corresponding representative images. BOMB/miR-122 or PBS did not induce hemolysis of the red blood cells, while water resulted in severe hemolysis.

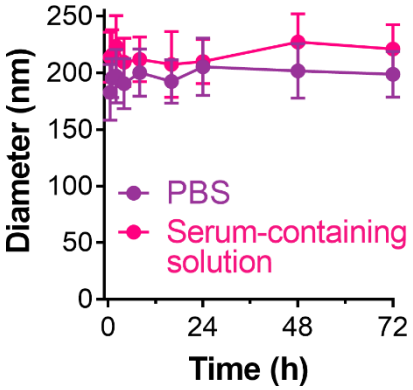

Figure S4b. Time-dependent size patterns of BOMB/miR-122 nanoparticles in PBS or serum-containing solution.

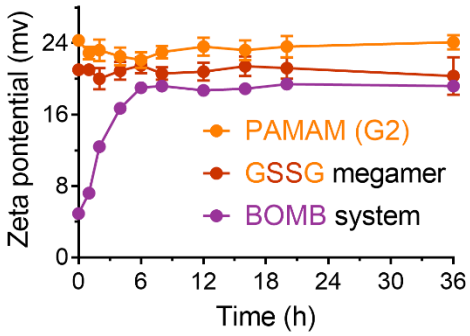

Figure S5. Time-dependent ζ potential change of G2 PAMAM dendrimer, GSSG megamer core, and BOMB at pH 6.5.

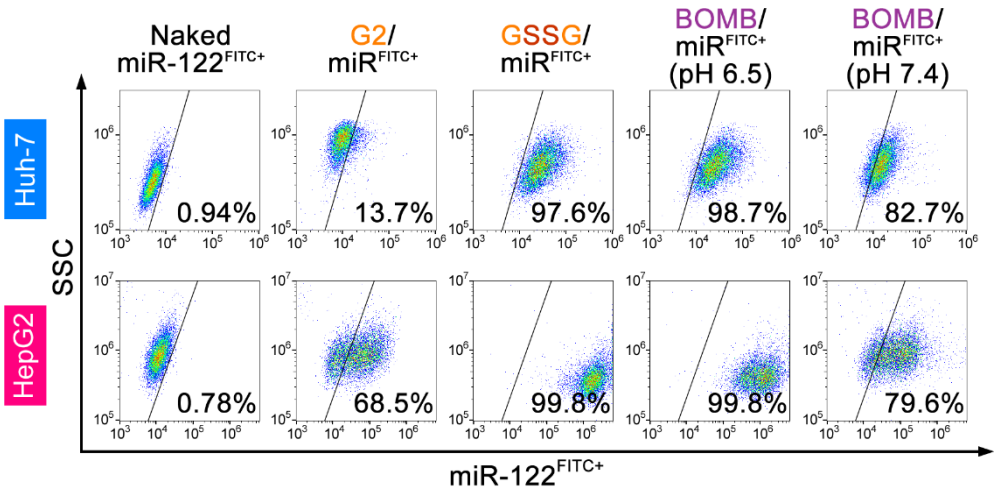

Figure S6a. Representative cellular uptake patterns of naked fluorescein (FITC) -labelled miR-122 (miR^{FITC+}), G2/miR^{FITC+}, GSSG/miR^{FITC+}, and BOMB/miR^{FITC+} at pH 6.5 or pH 7.4 in Huh-7 and HepG2 liver cancer cells by flow cytometry analysis. The miR^{FITC+}-internalized cells were characterized by positive FITC expression by the gated fluorescence level.

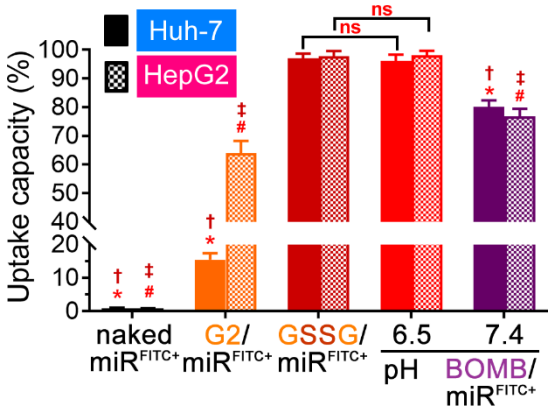

Figure S6b. Cellular uptake capacity of naked fluorescein (FITC) -labelled miR-122 (miR^{FITC+}), G2/miR^{FITC+}, GSSG/miR^{FITC+}, and BOMB/miR^{FITC+} pretreated at pH 6.5 or pH 7.4 in Huh-7 and HepG2 liver cancer cells. $^{*,\#}$ $p < 0.01$, *vs*. BOMB/miR^{FITC+} at pH6.5, †,‡ $p < 0.01$, *vs*. GSSG/miR^{FITC+}; Tukey test. ns, not significant.

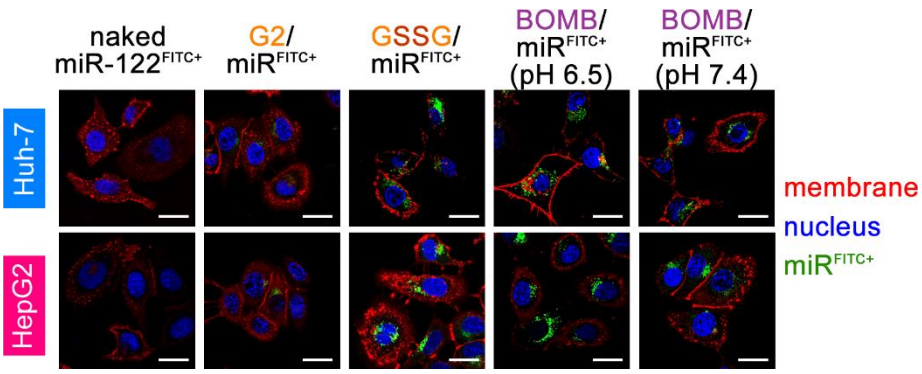

Figure S7. Representative laser-scanning confocal microscopy images of Huh-7 or HepG2 liver cancer cells incubated with naked fluorescein (FITC, green)-labelled miR-122 (miR^{FITC+}), G2/miR^{FITC+}, GSSG/miR^{FITC+}, and BOMB/miR^{FITC+} pretreated at pH 6.5 or pH 7.4. The cell membrane was stained with Alexa Fluor® 647 WGA (red), and the nuclei were stained with DAPI (blue). The bars represent 20 μm.

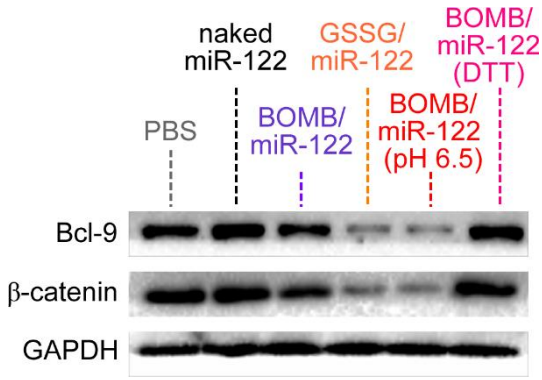

Figure S8. Expression of Bcl-9 and β-catenin in Huh-7 cells treated with PBS, naked miR-122, BOMB/miR-122, GSSG/miR-122, or BOMB/miR-122 pretreated at pH 6.5 or with DTT stimuli.

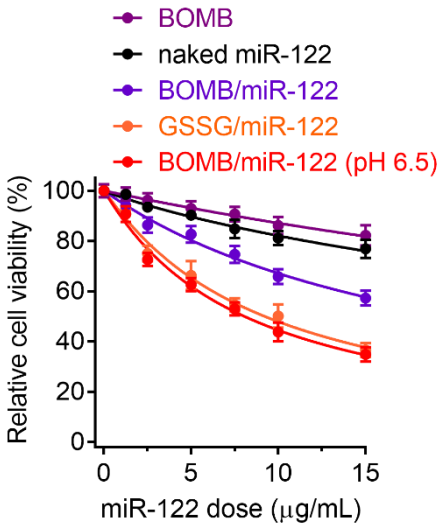

Figure S9. MTT cell viability assay towards Huh-7 cells after 72 h treatment with BOMB vector, naked miR-122, GSSG/miR-122, BOMB/miR-122, or BOMB/miR-122 pretreated at pH 6.5. The data was fitted in a dose-response model, yielding a best-fit half maximal inhibitory concentration (IC_{50}) of 20.4 μg/mL (17.7 – 24.6, 95% CI) miR-122 equivalent dose in cells treated with BOMB/miR-122. External stimulation with pH 6.5 pretreatment resulted in a significantly reduced IC_{50} of 8.1 μg/mL (7.5 – 8.7, 95%% CI) ($p < 0.01$, *vs*. BOMB/miR-122 with no stimuli, extra sum-of-squares F test), similar to GSSG treatment with a IC_{50} of 9.2 μg/mL (8.4 – 10.2, 95%% CI). Specifically, the BOMB vector itself only showed very slight toxicity even in large doses. Best-fitted lines were shown in corresponding colors.

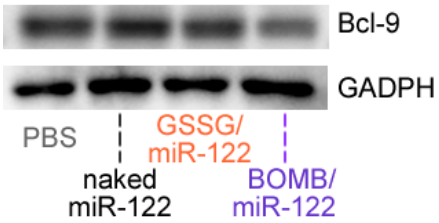

Figure S10. Expression of Bcl-9 in xenograft tumors treated with PBS, naked miR-122, GSSG/miR-122, or BOMB/miR-122.

Notes and references

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