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Supporting Information

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A pH-Responsive Drug-Delivery Platform Based on Glycol Chitosan–Coated Liposomes

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Material and Methods

Materials

Glycol chitosan was purchased from Wako Chemicals (Richmond, VA). Hydrogenated soy phosphatidylcholine (HSPC), cholesterol (Chol) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (DSPE-PEG2000-Carboxy) were obtained from Avanti Polar Lipids (Alabaster, AL). Doxorubicin hydrochloride (DOX) was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture medium (Dulbecco's Modified Eagle Medium), penicillin, streptomycin and heat-inactivated fetal bovine serum (FBS) were purchased from Gibco Life Technologies, Inc. (Grand Island, NY, USA). All other chemicals were used as received.

Preparation of nanometer-sized liposomes

Liposomes were prepared through the film hydration method followed by extrusion as previously described.[1] Briefly, HSPC: Chol: DSPE-PEG2000-Carboxy (50:45:5, molar ratio) were mixed in chloroform in a glass vial. The total amount of HSPC was 10 mg. The solvent was then removed using a direct stream of nitrogen until a thin film was formed, which was subsequently dried overnight in a vacuum desiccator. The lipid film was re-hydrated by adding an aqueous solution (10 mM PBS, pH 7.4) and incubating in a 60 °C water bath for 30 min, then sonicating for another 30 min at the same temperature. The obtained suspension was further subjected to 6 freeze-thaw cycles in liquid nitrogen and warm water, followed by extrusion 21 times through two stacked 100 nm Nuclepore polycarbonate filters using a stainless steel extruder (Avanti Polar Lipids).

Preparation of glycol chitosan conjugated liposomes

Glycol chitosan (30-100 kDa) was degraded and purified starting from commercially available ~400 kDa glycol chitosan (Wako Chemicals, Richmond, VA, USA) according to the previously published procedure.[2, 3]

The conjugation was performed through a two-step EDC/sulfo-NHS coupling reaction. Briefly, the first EDC activation reaction of liposome solution containing carbonyl groups was performed in MES buffer (0.1M MES, 0.5 M NaCl) at pH 5.5 by buffer-exchange with PD-10 Desalting Columns (GE Healthcare). Calculated amount of EDC · HCl and sulfo-NHS (2 molar equiv of carbonyl groups) were then added to the liposome solution, and carbonyl groups were activated at 25 °C on a rocker table for 0.5 h. After changing the pH to 7.4 immediately with phosphate-buffered saline (PBS, 0.01 M) by using PD-10 Desalting Columns, the glycol-chitosan solution (PBS, pH = 7.4, 1 molar equiv of carbonyl groups) was mixed with the above Sulfo-NHS activated liposome solution and then the solution was left to react 15 h on a rocker table at 25 °C. The excess reagent can be removed by a 100 kD Amicon Pro-Purification System (EMD Millipore, Billerica, Massachusettes) through six 20-minute cycles of centrifugation at 3,000 rcf at 4 °C.

Preparation of DOX loaded liposomes and GC-liposomes

The DOX-liposomes were prepared according to an active transmembrane pH gradient method as previously described.[4] Briefly, the lipids with the above mentioned composition HSPC: Chol: DSPE-PEG_{2000}- Carboxy (50:45:5, molar ratio) were mixed in chloroform, dried into a thin film and then hydrated in 300 mM aqueous ammonium sulfate solution (1 mL). After incubation in a 60 °C water bath for 30 min and sonication for another 30 min, this resulting dispersion was subjected to 6 freeze-thaw cycles. Afterwards, the liposomal solution was extruded 21 times through two stacked polycarbonate extrusion membranes (100 nm pore-size) that are maintained at 60 °C in a mini-extruder (Avanti Polar Lipids). The excess ammonium sulfate outside of liposome was removed by Sephadex G-25 (10 mL) gel-filtration chromatography pre-equilibrated with 150 mM NaCl solution. To prepare the doxorubicin (DOX) loaded liposomes, the Doxorubicin hydrochloride (DOX, 0.2 equiv of the total lipid content in PBS buffer) was added to the collected liposome solution followed by incubation at 50 °C for 24 h. The unloaded DOX was then removed by Sephadex G-25 (10 mL) gel-filtration chromatography. The as prepared DOX-loaded liposomes were stored at 4 °C until use.

The DOX loaded GC-liposome was prepared by conjugation of glycol-chitosan to the surface of DOX loaded liposome, according to the above-mentioned EDC/sulfo-NHS method.
The loading efficiency of DOX was determined by lysis of the liposomes with 5% Triton X-100 and measurement of the UV absorbance intensity at 480 nm for DOX, according to the standard calibration curve of free DOX.

Characterization of DOX-loaded liposomal formulations

Hydrodynamic diameter (Dh) and polydispersity index (PDI) of the liposomes were determined by the dynamic light scattering (DLS) techniques using a Malvern Zetasizer Nano-ZS instrument ((Malvern Instruments, Worcestershire, UK)) equipped with a 633 nm He-Ne laser at a scattering angle of 173° at 25 °C. For zeta-potential pH titrations, 10 mM HEPES buffer solution was prepared with pH values ranging from 6.00 to 7.75 in 0.25 unit increments. Each nanoparticle formulation was diluted to a final concentration of 100 μg liposomes/mL in the buffer at each pH, and mean nanoparticle zeta-potential was measured using a Zetasizer Nano-ZS.

Fourier transform infrared (FTIR) spectra were recorded on a Nicolet iS5 FT-IR Spectrometer. Cryogenic Transmission Electron Microscopy (Cryo-TEM) was performed at the University of Pennsylvania in the Nanoscale Characterization Facility (Philadelphia, PA). Lacey formvar/carbon grids (Ted Pella) were rinsed in chloroform to remove the formvar template. The resulting grids carbon coated with a Quorum Q150T ES carbon coater (Quorum Technologies, United Kingdom). Grids were cleaned with hydrogen/oxygen plasma for 15 seconds using the Solarus Advanced Plasma System 950 (Gatan, Pleasanton, CA). A 2 μl drop of nanoparticles in PBS was deposited onto the grid and added to a Gatan Cp3 cryoplunger (Gatan, Pleasanton, CA). The samples were blotted by hand and plunged into liquid ethane. Grids were transferred to a Gatan CT3500TR cryoholder (Gatan, Pleasanton, CA) and immediately inserted into a JEOL 2100 HRTEM (JEOL, Tokyo, Japan) operating at 200 keV. Micrographs were imaged with an Orius SC200 digital camera.

Cell culture

The human fibrosarcoma cell line HT1080 (ATCC) was cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), supplemented with 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C with 5% CO_2.

For *in vitro* different pH studies, cell culture medium was supplemented with 25 mM HEPES buffer.

Fluorescence microscopy

The cellular uptake behavior of DOX-liposomes or GC-DOX-liposomes was determined by fluorescence microscope toward HT1080 cells. The cells were seeded in 12-well plates at a density of 5×10^5 cells per well in 1 mL of DMEM and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. After removing culture medium, cells were incubated with DOX loaded (GC-) liposomes at an equivalent DOX concentration of 10 μg mL^{-1} containing DMEM of pH 7.4 or 6.5, respectively. After incubation for 2 h at 37 °C, cells were washed three times with cold PBS. All microscopy images were further acquired with an Olympus IX81 motorized inverted fluorescence microscope equipped with a back-illuminated EMCCD camera (Andor), an X-cite 120 excitation source (EXFO), and Sutter excitation and emission filter wheels.

Flow cytometry

HT1080 cells were seeded in six-well plates (2×10^5 cells/well) and cultured for 24 h. The original medium was replaced with Dox loaded (GC-) liposomes at an equivalent DOX concentration of 10 μg/mL in DMEM of pH 7.4 or 6.5, respectively. After incubation for 2 h at 37 °C, the cells were washed three times with cold PBS and processed with trypsin. The harvested cells were suspended in PBS and centrifuged at 1000 rpm for 5 min. The supernatants were discarded and the cell pellets were washed with PBS two more cycles. Then the cells were resuspended in 0.4 mL PBS. Flow cytometry analysis was performed by a BD LSR II Flow Cytometer. Flow cytometry data were analyzed using FlowJo software (TreeStar Inc., San Francisco, CA).

***In vitro* cytotoxicity**

The cytotoxicities of DOX, DOX-liposomes and GC-DOX-liposomes were evaluated with MTT assay against HT1080 cells. Briefly, the cells harvested in a logarithmic growth phase were seeded in 96-well plates at ~1×10^4 cells per well in 100 μL complete DMEM, and incubated at 37 °C in 5% CO_2 atmosphere for 24 h. After removing culture medium, DOX-loaded (GC) liposomes diluted in complete DMEM (100 μL) at pH 7.4 or 6.5 were added to cell wells with various concentrations from 40 μg/mL to 0.0128 μg/mL. After 4 h treatment, the medium was replaced by fresh DMEM at pH 7.4 and further incubated for 20 h. The culture medium was then removed and the cells were washed with PBS three times. Then 200

μL of DMEM and 20 μL of 5 mg/mL MTT assays stock solution in PBS were added. After incubating the cells for 4 h, the medium containing unreacted MTT was removed carefully. The obtained blue formazan crystals were dissolved in 200 μL per well DMSO. The absorbance of the solution was measured on a Tecan plate reader (Tecan) at 490 nm. Cell viability (%) was calculated based on the following equation: $(A_{sample}/A_{control}) \times 100\%$, where A_{sample} and $A_{control}$ denote as absorbencies of the sample well and control well, respectively.

Tumor model establishment

Approximately 6-week-old female nu/nu nude mice (Charles River Laboratory, Charles River, MS, USA) were maintained in accordance with the Insitutional Animal Care and Use Committee of the University of Pennsylvania. Mice were anesthetized using isoflurane, and T6-17 cells were injected subcutaneously into the back left flank (2×10^6 cells in 0.1 mL of PBS). The tumor nodules were allowed to grow to a desirable volume before use.

***In vivo* imaging of DOX loaded liposomes and GC-liposomes**

Six nu/nu nude mice bearing T6-17 tumor nodules were randomly divided into two groups and injected with liposomes and GC-liposomes into their tail veins, respectively (with an equivalent DOX dose of 5 mg/kg body weight). After 24 h, three mice from each group were sacrificed. The organs (heart, liver, spleen, lung, kidney and tumor) of the carcasses were excised, followed by washing the surface with fresh water three times. Then these organs were imaged by the IVIS 200 imaging system (Xenogen). All images were processed with the IVIS software.

***In vivo* antitumor efficacy**

The tumor model was established as described above. When the tumor nodules grew to ca. 50-150mm^3, tumor-bearing nu/nu nude mice were randomly assigned to 4 groups with 6 mice in each group and were intravenously injected three times via tail vein injection at a dose of 5 mg DOX/kg body weight on day 0, 2, and 4, respectively.

Tumor length (major axis of the tumor) and width (minor axis of the tumor) were measured with calipers. The weight and tumor volume of each mouse were measured every two days over a period of 16 days. The tumor volume was calculated using the following equation: Tumor volume (V)= (length *$width^2$)/2. The curve of tumor growth was plotted using the average relative tumor volume within each experimental group at the set time points.

Hematoxylin and Eosin (H&E)

Processing, staining, and evaluation of the tumor were carried out by the Cancer Histology Core within the Perelman School of Medicine at the University of Pennsylvania.

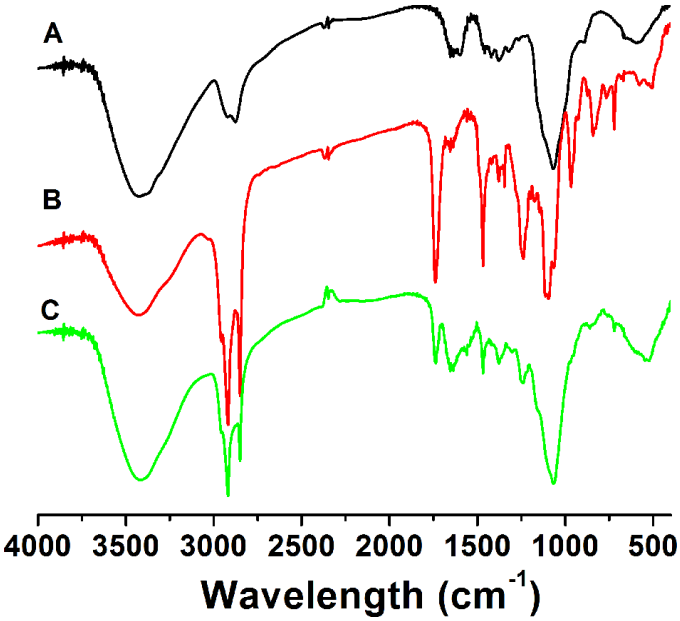

Figure S1. FTIR characterization of glycol chitosan (A), liposomes (B) and GC-liposomes (C).

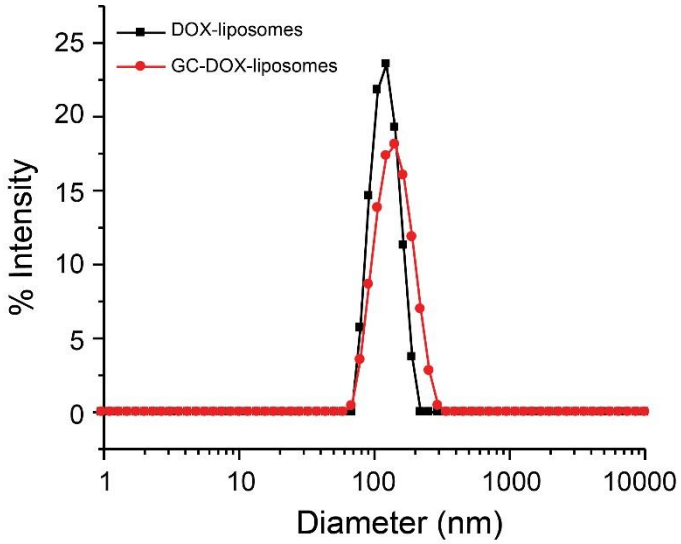

Figure S2. Intensity-weighted size distribution of DOX-loaded liposomes with and without GC conjugation as measured by dynamic light scattering (DLS).

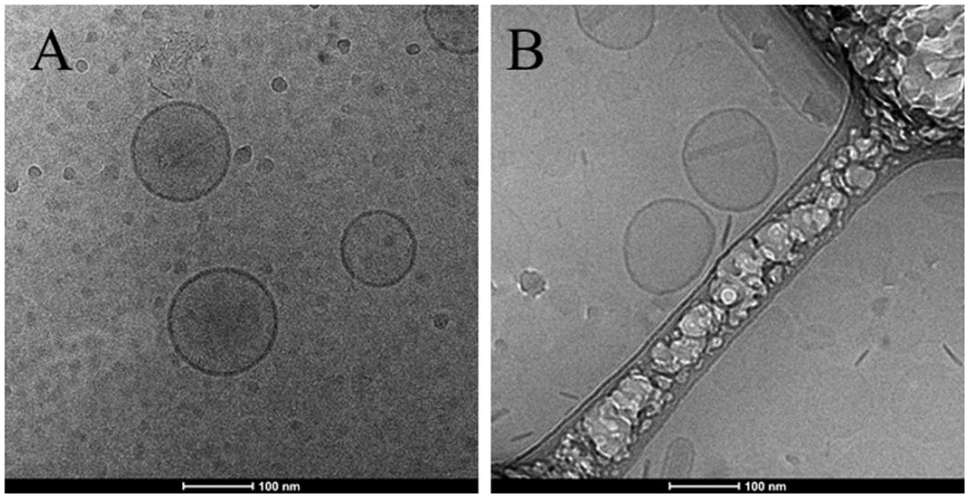

Figure S3. Cryo-TEM of DOX-liposomes (A) and GC-DOX-liposomes (B).

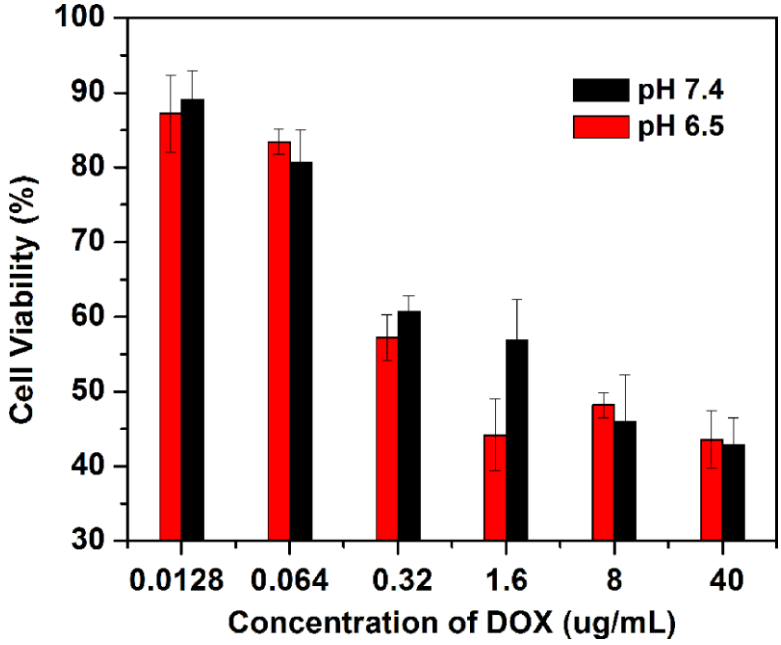

Figure S4. Cell viability of HT 1080 cells after incubation with DOX at various DOX concentrations for 4 h followed by 20 h in fresh medium at 37 °C. Data are presented as the average ± standard deviation (n=4).

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