Electronic supplementary information

Acidity-triggered surface charge neutralization and aggregation of functionalized nanoparticles for promoted tumor uptake

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Experimental Section

Materials. Poly(lactic-co-glycolic acid) (PLGA) (LA/GA:85/15, I.V. 0.42 dl/g, carboxylic acid terminated) was acquired from Green Square (Taiwan). D-α-Tocopheryl polyethylene glycol 1000 succinate (TPGS), N,N′-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP) were supplied by Aldrich and used as received. N-Acetyl histidine was purchased from TCI. Doxorubicin hydrochloride (DOX) was obtained from Seedchem. Deuterium solvents used in ^{1}H-NMR measurements were obtained from Cambridge Isotope. Deionized water was produced from Milli-Q Synthesis (18 MΩ, Millipore). All other chemicals were reagent grade and used as received.

Conjugation of TPGS with N-acetyl histidine. TPGS (3.0 g, 2.0 mmol), N-acetyl-histidine (1.97 g, 10.0 mmol), DCC (3.30 g, 16.0 mmol) and DMAP (0.24 g, 2.0 mmol) were dissolved in anhydrous DMSO (10.0 mL). The reaction was carried

out under stirring at 40 ^{o}C for 48 h, followed by repeated filtrations to remove the byproduct, dicyclohexylcarbodiurea. The solution was then dialyzed (Cellu Sep MWCO 1000) against deionized water for 3 days to eliminate the residual reactants and DMSO. The final product was collected by freeze drying. The synthetic route of N-acetyl histidine-conjugated TPGS (NAcHis-TPGS) is illustrated in Fig. S1. The composition of NAcHis-TPGS was determined by ^{1}H-NMR using DMSO-d_6 as the solvent.

Preparation of DOX-free and DOX-Loaded Nanoparticles. The nanoprecipitation approach was employed to prepare DOX-loaded PLGA nanoparticles coated with either NAcHis-TPGS or TPGS. DOX in salt form was stirred with excess triethylamine (4-fold molar concentration with respect to DOX molecules) in dry DMSO in order to attain hydrophobic DOX (in free base form). NAcHis-TPGS (1.6 mg) in DMSO (0.1 mL) was added into phosphate buffer solution (3.5 mL) of pH 7.4. In addition, 2.0 mg PLGA and 0.4 mg DOX were dissolved in 0.4 mL DMSO and then added dropwise into the NAcHis-TPGS-containing phosphate buffer solution under stirring. The mixed solution was gently stirred at 30 ^{o}C for 30 min, followed by an equilibration period of 30 min. After repeated dialysis (Cellu Sep MWCO 12000-14000) against phosphate buffer solution (pH 7.4) at 4 ^{o}C to remove DMSO and unloaded DOX, the DOX-loaded NAcHis-TPGS/PLGA nanoparticles (denoted as DOX-loaded NHTPNs) used in this work were attained. For comparison, the DOX-loaded TPGS/PLGA nanoparticles (DOX-loaded TPNs) were prepared in a similar manner, using TPGS instead of NAcHis-TPGS.

Nanoparticles Characterization. The particle size, size distribution and zeta potential of DOX-free and DOX-loaded nanoparticles in buffer solutions of different pH values were measured by a Malvern ZetaSizer Nano Series instrument (He-Ne laser 4 mW, λ = 633 nm). The experimental results shown herein represent an average

of at least triplicate measurements. The morphology of the nanoparticles was examined by transmission electron microscopy (TEM) (HT7700, Hitachi, Japan). TEM sample was prepared by placing a few drops of the drug-loaded nanoparticle solution on a 300-mesh copper grid covered with carbon and then negatively stained with uranyl acetate solution (5.0 wt%) for 40 s. The sample was dried at 25 ^{o}C for 3 days before TEM examination.

Determination of Encapsulated DOX Level. DOX-loaded nanoparticles obtained from the lyophilization of the aqueous sample dispersion (0.5 mL) were dissolved in DMSO. The amount of DOX loaded in the nanoparticles was quantitatively determined by the fluorescence spectrometer (F-7500, Hitachi, Japan) with the excitation wavelength at 485 nm and emission wavelength at 560 nm. The calibration curve was established by the fluorescence intensity of DOX with various concentrations in the DMSO solution. The drug loading efficiency (%) is defined herein as (the weight of DOX in nanoparticles/weight of DOX in feed) × 100 and the loading content (wt%) as (the weight of DOX in nanoparticles/weight of DOX-loaded nanoparticles) × 100.

In Vitro Drug Release. The release performance of DOX from drug-loaded nanoparticles was assessed by the dialysis technique. The DOX-loaded nanoparticle dispersion (1.0 mL) was subjected to dialysis (Cellu Sep MWCO 12000-14000) against the buffer solutions of pH 7.4 and 6.0 (20 mL, ionic strength 0.15 M) at 37 ^{o}C, respectively. At prescribed time intervals, 0.65 mL of dialysate (pH 6.0 or 7.4) was withdrawn for analysis and replaced with an equal volume of fresh medium. The amount of released DOX was determined by fluorescence measurements using the pertinent calibration curve of DOX with different concentrations in the aqueous solution of either pH 6.0 or 7.4.

In Vitro Cellular Uptake. Free DOX, DOX-loaded NHTPNs and TPNs were

dispersed in DMEM of different pH values (7.4, 6.5 and 6.0), respectively, to a DOX concentration of 20 μM. Afterward, HeLa cells seeded at a density of 3×10^5 cells per well in 6-well culture plates were treated with the above solutions at 37 ^oC for 2 h. After being wash three times with PBS, cells were lysed by addition of DMSO (0.65 mL). Intracellular drug amount was quantitatively analyzed by the fluorescence measurement using the pertinent calibration curve of DOX with different concentrations in DMSO. For the microscopic observation, HeLa cells (3×10^5 cells/well) seeded onto 22 mm round glass coverslips in 6-well culture plates were incubated with free DOX, DOX-loaded NHTPNs and TPNs (DOX concentration 20 μM), respectively. After being washed twice with PBS and immobilized with 4 % formaldehyde, the nuclei of the treated cells were stained with Hoechst 33258 for 10 min. The intracellular distribution of DOX was examined on a Nikon ECLIPSE Ti-U inverted microscope (Japan) equipped with a Hoechst set (Ex. 360 nm and Em. 461 nm) and a DOX set (Ex. 488 nm and Em. 590 nm)

In Vitro Cytotoxicity. HeLa cells (1×10^4 cells/well) were seeded in a 96-well plate containing DMEM (100 μL) with 10% FBS and 1% penicillin and incubated at 37 ^oC for 24 h. The medium was then replaced by 100 μL of fresh medium (pH 7.4, 6.5 or 6.0) containing either free DOX, DOX-loaded NHTPNs or DOX-loaded TPNs at varying DOX concentrations or drug-free nanoparticles and cells were incubated for additional 24 h. After being washed twice with PBS and addition of 100 μL fresh DMEM, the cells were re-incubated for 24 h. MTT (5.0 mg/mL, 5.0 μL) was then added into each well, followed by incubation at 37 ^oC for 4 h. After discarding the culture medium, DMSO was added to dissolve the precipitate and the resulting solution was measured for absorbance at 570 nm using a SpectraMax M5 microplate reader.

In Vivo Imaging and Biodistribution. IR780, a hydrophobic near-infrared (NIR) fluorescence dye, was encapsulated into the DOX-loaded NHTPNs and TPNs for NIR fluorescence imaging to evaluate tumor accumulation and biodistribution of these nanoparticles. Male BALB/cAnN.Cg-Foxnlnu/CrlNarl mice (6~8 week old) were purchased from the National Laboratory Animal Center (Taiwan). Animals received care in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals. The procedures were approved by the Administrative Committee on Animal Research of the National Tsing Hua University (Taiwan). To establish tumor model, 3.5×10^6 HeLa cells were subcutaneously injected into the right thighs of mice. When tumor volume of the mice reached 80-100 mm^3, the tumor-bearing mice were treated by tail vein injection with PBS, DOX/IR780-loaded NHTPNs and TPNs at an IR780 dosage of 10.9 μg/kg. The fluorescence signals of IR780 (Ex. 780 nm and Em. 804 nm) at 3, 8 and 24 post-injection were collected on the IVIS. The treated mice were then sacrificed at 48 h post-injection and the major organs harvested for individual organ imaging by IVIS. Furthermore, the tumor was then harvested and stored for cryo-section. The primary antibody, rat anti-mouse CD31, and the secondary antibody, Alexa Fluor 488® goat anti-rat, were used for the IHC identifications of tumor neovessel in the cryo-sections. The cell nuclei were stained with DAPI. All stained tumor sections were examined by Nikon ECLIPSE Ti-U inverted microscope with the fluorescence channels for DAPI, Alexa Fluor 488 and DOX.

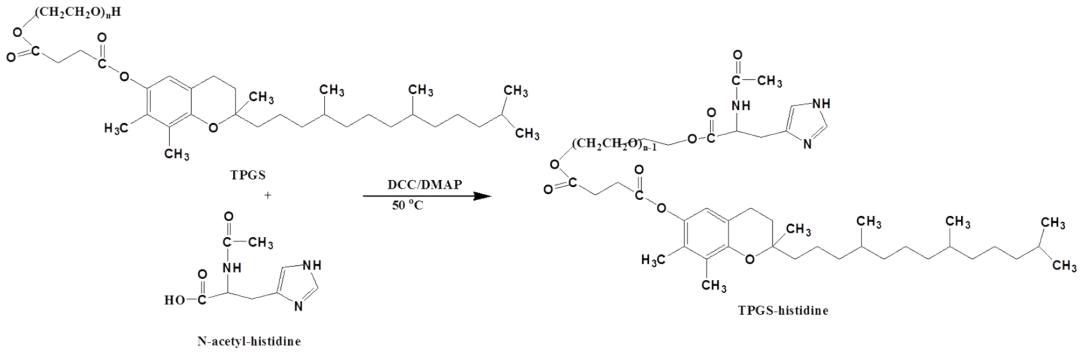

Fig. S1 Synthetic route and chemical structure of NAcHis-TPGS.

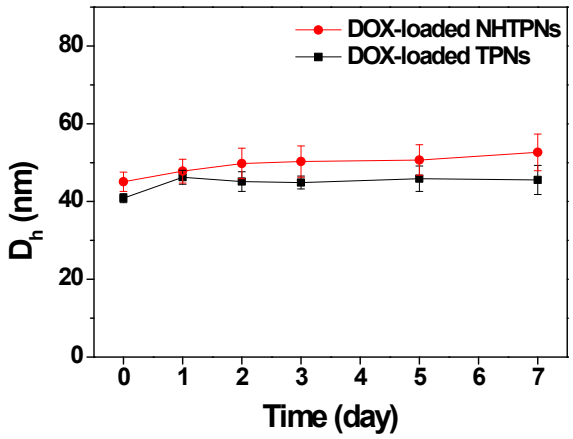

Fig. S2 Colloidal stability of DOX-loaded NHTPNs and TPNs in PBS (pH 7.4) over a time period of 7 days.

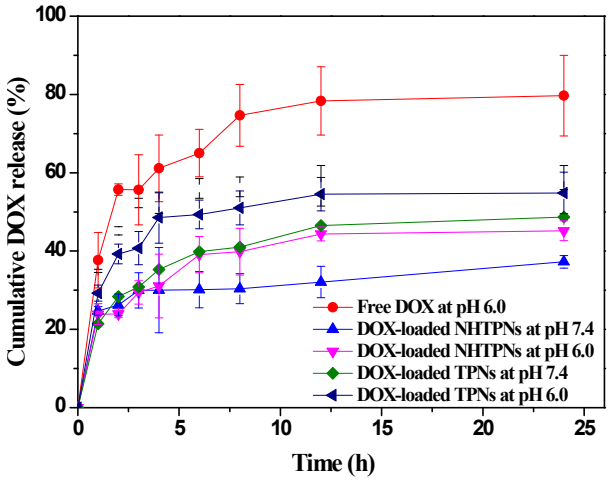

Fig. S3 Cumulative drug release profiles of DOX-loaded NHTPNs and TPNs in aqueous solutions of pH 7.4 and 6.0 at 37 °C, using dialysis technique. For comparison, dialysis of free DOX at pH 6.0 is also included