Electronic Supplementary Information

Core-Shell Tecto Dendrimers Formed *via* Host-Guest Supramolecular Assembly as a pH-Responsive Intelligent Carrier for Enhanced Anticancer Drug Delivery

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Experimental

Materials

Generation 3 (G3) and generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers were purchased from Dendritech (Midland, MI). 1H-Benzimidazol-2-ylacetic acid (BM-COOH) was supplied from Shanghai Bi De Pharmaceutical Technology Co., Ltd. (Shanghai, China). 1-Adamantaneacetic acid (Ad-COOH), β-cyclodextrin (β-CD), and N, N-carbonyldiimidazole (CDI) were purchased from J&K Scientific Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) was from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Agarose was from Biowest (Nuaille, France). Doxorubicin (DOX) was from Beijing Huafeng Pharmaceutical Co., Ltd. (Beijing, China). HeLa cells were obtained from Institute of Biochemistry and Cell Biology (the Chinese Academy of Science, Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA). Penicillin and streptomycin were from Gino Biomedical Technology Co., Ltd. (Hangzhou, China). Cell Counting Kit-8 (CCK-8) was from 7Sea Biotech Co., Ltd. (Shanghai, China). 4', 6-Diamidino-2-phenylindole (DAPI) was acquired from BestBio Biotechnology Co., Ltd. (Shanghai, China). Regenerated cellulose dialysis membranes were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). The 3-dimensional (3D) Petri Dish was from Microtissues (Providence, RI).

Synthesis of G3.NHAc-BM and G5.NHAc-CD Dendrimers

G3.NH_2-BM was synthesized *via* EDC coupling of the surface amine group of G3.NH_2 dendrimers with the carboxyl group of BM. Briefly, EDC (32.21 mg) and BM-COOH (1.97 mg) were co-dissolved in DMSO (10 mL) under vigorous magnetic stirring at room temperature for 30 min, and then N-hydroxysuccinimide (NHS) (19.34 mg) was added to the mixture under stirring at room temperature for 3 h. The activated BM was dropwise added to a solution of G3 dendrimers (50 mg) in DMSO (10 mL) under vigorous stirring at room temperature for 3 days. The reaction mixture was dialyzed against

phosphate buffered saline (PBS, three times, 2 L) and water (six times, 2 L) for 3 days using a dialysis membrane with a molecular weight cut-off (MWCO) of 1000 to remove the excess reactants, followed by lyophilization to obtain the G3.NH_2-BM product. For comparison, G3.NH_2-Ad dendrimers were also prepared according to the procedures reported in the literature.[1]

To synthesize G5.NHAc-CD dendrimers, β-CD was first modified onto the surface of G5.NH_2 dendrimers according to a procedure described in our previous report.[2] Briefly, a DMSO solution (5 mL) containing β-CD (38.9 mg) was mixed with a DMSO solution (5 mL) of CDI (34.04 mg) under vigorous stirring at room temperature for 6 h. Then, G5.NH_2 dendrimers (30 mg) dissolved in DMSO (10 mL) was dropwise added to the above mixture solution under vigorous stirring at room temperature for 3 days. Afterwards, the reaction mixture was dialyzed against PBS (three times, 2 L) and water (six times, 2 L) for 3 days using a dialysis membrane with an MWCO of 10000. A further lyophilization process gave rise to a white power of the product of G5.NH_2-CD, which was stored at -20 ℃ for further use.

After that, the remaining amines of the G3.NH_2-BM dendrimers were converted to acetyl groups by reacting with acetic anhydride according to our previous work.[3] In brief, trimethylamine (84 μL) was added to an aqueous solution of the raw product of G3.NH_2-BM (20 mg) under magnetic stirring for 30 min. Then, acetic anhydride (47.6 μL) was added to the above mixture solution while stirring, and the mixture was allowed to react for 24 h. The crude product was extensively dialyzed against PBS (three times, 2 L) and water (six times, 2 L) for 3 days to remove the excess of reactants and byproducts, followed by lyophilization to get the G3.NHAc-BM product. Similarly, G3.NHAc-Ad and G5.NHAc-CD were also prepared.

Preparation of pH-Responsive Core–Shell Tecto Dendrimers (CSTDs)

To ensure that all of the β-CD moieties conjugated onto each G5 dendrimer were combined with G3.NHAc-BM dendrimers, the molar ratio of G5.NHAc-CD and G3.NHAc-BM dendrimers was controlled at 1 : 9. Typically, G5.NHAc-CD and G3.NHAc-BM dendrimers were respectively

dissolved in 5 mL of PBS (pH = 7.4), and mixed under stirring for 24 h at room temperature. The mixture was then dialyzed against water (MWCO = 10000) for 12 h to remove the free G3.NHAc-BM dendrimers and the product was obtained after lyophilization. For comparison, G5.NHAc-CD/Ad-G3.NHAc CSTDs were also synthesized according to a procedure described in our previous report.[1] Briefly, a 1: 9 molar ratio of G5.NHAc-CD and G3.NHAc-Ad units was used. G3.NHAc-Ad in 5 mL of water (4 mg mL^{-1}) was dropwise added to a water solution (5 mL) containing G5.NHAc-CD (2 mg mL^{-1}) under stirring for 24 h at room temperature. The mixture was dialyzed against PBS (three times, 2 L) and water (six times, 2 L) for 3 days using a dialysis membrane with an MWCO of 8000~14000. Finally, a lyophilization process was applied to yield the G5.NHAc-CD/Ad-G3.NHAc CSTDs.

Loading of DOX within the CSTDs

The DOX loading within the G5.NHAc-CD/BM-G3.NHAc and G5.NHAc-CD/Ad-G3.NHAc CSTDs was carried out according to a method described in our previous work.[4] Briefly, G5.NHAc-CD/BM-G3.NHAc (15 mg) or G5.NHAc-CD/Ad-G3.NHAc (15 mg) CSTDs were dissolved in 1.5 mL water. DOX with 15 mol equiv. of the corresponding dendrimers was dissolved in 300 μL methanol, and neutralized by addition of 5 μL triethylamine. The DOX solution was then added into an aqueous dendrimer solution under vigorous stirring overnight to allow the evaporation of the methanol solvent. Then, the G5.NHAc-CD/BM-G3.NHAc/DOX or G5.NHAc-CD/Ad-G3.NHAc/DOX mixture solution was centrifuged (7500 rpm for 10 min) to remove the precipitate, which is associated with the noncomplexed free DOX. The supernatant solution was lyophilized to obtain the G5.NHAc-CD/BM-G3.NHAc/DOX or G5.NHAc-CD/Ad-G3.NHAc/DOX complexes. The loading of DOX within the dendrimers was quantified by subtracting the free DOX amount in the collected precipitate from the initial DOX amount. Free DOX was quantified *via* UV-vis spectroscopy measurement at a wavelength of 481 nm according to our previous work.[4]

Characterization Techniques

One- and two-dimensional (rotating frame overhauser effect spectroscopy, ROESY; nuclear

overhauser effect spectroscopy, NOESY) NMR spectra were recorded using Bruker AV600 nuclear magnetic resonance spectrometer (Fällanden, Switzerland). Samples were dissolved into D_2O before NMR measurements. UV-vis spectra were acquired by a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Waltham, MA). Samples were dissolved in water or PBS before measurements. Hydrodynamic size and zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS model ZEN3600 system (Worcestershire, UK) equipped with a standard 633 nm laser. Atomic force microscopy (AFM) was performed using a Molecular Force Probe 3D (MFP-3D) AFM (Asylum Research, Santa Barbara, CA). AFM samples were prepared by dropping a diluted sample suspension on a silicon wafer and nitrogen-dried before measurements. Fourier transform infrared (FTIR) spectra were collected on a Nexus 670 spectrometer (Thermo Nicolet Corporation, Madison, WI). Fluorescence spectra were collected using a QuantMaster-40 fluorescence spectrophotometer (Protein Technologies, Inc., Tucson, AZ).

In Vitro Release Kinetic Study

Taking the DOX release from the G5.NHAc-CD/BM-G3.NHAc/DOX complexes as an example, the complexes (2 mg mL^{-1}) was dispersed in 1 mL of PBS (pH 7.4, 0.2 M) or acetate buffer (pH 5.4, 0.1 M), placed in a dialysis bag with an MWCO of 5000, and dialyzed against 9 mL of the corresponding buffer medium. The experiment was done in triplicate. All the samples were incubated in a vapor bathing constant temperature vibrator at 37 °C. At each specific time point, 1 mL of buffer medium was taken out from the outer phase of the corresponding buffer medium and the concentration of the released DOX was quantified by UV-vis spectroscopy. The volume of the outer phase buffer medium was maintained constant by replenishing 1 mL of the corresponding buffer solution. The DOX release from the G5.NHAc-CD/Ad-G3.NHAc/DOX complexes was performed under similar experimental conditions. Free DOX was also released similarly and used as control. In all cases, an equal amount of DOX from different complexes was used to ensure reasonable comparison among different groups.

Cytotoxicity Assay

To investigate the feasibility of the drug delivery system for biomedical applications, we performed systematic evaluation of the *in vitro* cytotoxicity of the drug-free CSTDs and drug-loaded CSTDs *via* standard CCK-8 cell viability assay. HeLa cells were regularly cultured and passaged under normal cell culture conditions. To test the cytocompatibility of the drug-free CSTDs, HeLa cells were seeded in 96 well plates at the density of 1×10^4 cells per well with 100 μL of DMEM containing 10 % FBS, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin for each well. The cells were incubated at 5% CO_2 and 37 °C overnight to bring the cells to confluence. The next day, the medium in each well was replaced with fresh medium containing different drug-free CSTDs at different concentrations. After 24 h, the cells were washed three times with PBS and treated with 100 μL of DMEM containing 10% CCK-8 for each well for additional 2 h. A Thermo Scientific Multiskan MK3 ELISA reader (Waltham, MA) was used to record the absorbance of each well at 450 nm. To test the therapeutic efficacy of the drug-loaded CSTDs, the process was similar except the incubation of cells with fresh medium containing G5.NHAc-CD/BM-G3.NHAc/DOX or G5.NHAc-CD/Ad-G3.NHAc/DOX complexes with different DOX concentrations (0.25, 0.75, 0.5, 1, 2, 4, 6, and 8 μg mL^{-1}, respectively). For each sample, triplicated wells were tested and the data were presented as mean ± SD.

Flow Cytometric Analysis

To quantitatively confirm the cellular uptake of DOX within HeLa cells, the DOX fluorescence within the cells was monitored using a FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA). HeLa cells were seeded into a 12-well tissue culture plate (2×10^5 cells per well) for 24 h. Then, the cell medium of each well was replaced with fresh DMEM containing the G5.NHAc-CD/BM-G3.NHAc/DOX or G5.NHAc-CD/Ad-G3.NHAc/DOX complexes at different DOX concentrations,

and the cells were incubated for 4 h under regular culture conditions. After that, the medium in each well was discarded and the cells were washed with PBS for 3 times, trypsinized, centrifuged, and resuspended in 1 mL of PBS before flow cytometry analysis. For each sample, 1×10^4 cells were counted, and each measurement was repeated for 3 times. Cells treated with PBS were used as control.

Confocal Microscopic Observation

Confocal laser scanning microscopy (CLSM, Carl Zeiss LSM 700, Jena, Germany) was used to qualitatively confirm the intracellular uptake of DOX-loaded CSTDs. HeLa cells were seeded in 12-well plates at a density of 2×10^5 cells per well in 1.0 mL of complete DMEM containing 10% FBS, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin. The cells were regularly cultured overnight. After that, the medium was substituted with complete DMEM containing DOX-loaded CSTDs at a final DOX concentration of 2 μg mL^{-1}, and the cells were incubated at 37 °C for additional 4 h. Then, the culture medium was removed and cells were washed with PBS for three times, fixed with 4% paraformaldehyde for 30 min at room temperature, and stained with DAPI for 20 min before CLSM observation.

Penetration and Antitumor Therapy of 3D Tumor Spheroids

3D tumor spheroids composed of HeLa cells were constructed by a "hanging drop" technique as previously reported with some modifications.[5] Briefly, 500 μL of agarose solution (2%, w/v, sterile saline) was dropped into the mold in the 3D petri dish. Air bubbles were removed *via* pipet suction. After that, the solidified gels were separated from the mold, placed to each well of a 12-well plate, and equilibrated for more than 15 min with DMEM. Then, a cell suspension (190 μL) containing 5×10^5 HeLa cells was slowly added to each well of the 12-well plate, and after standing for 10 min, 2.5 mL of fresh cell culture medium was slowly added to each well to let the cells to aggregate and grow.

When the tumor spheroids reached an appropriate volume, the penetration capacity of the drug-loaded CSTDs and the therapeutic efficacy of the 3D tumor spheroids were evaluated. To study the penetration of G5.NHAc-CD/BM-G3.NHAc/DOX and G5.NHAc-CD/Ad-G3.NHAc/DOX complexes, the HeLa tumor spheroids were incubated with free DOX, G5.NHAc-CD/BM-G3.NHAc/DOX and G5.NHAc-CD/Ad-G3.NHAc/DOX complexes at a DOX concentration of 2 μg mL^{-1}. After 24 h of incubation, the tumor spheroids were washed three times carefully with PBS and observed using CLSM with Z-stack scanning. The DOX fluorescence intensity in each HeLa tumor spheroid sample was calculated by ImageJ Z-stack according to the literature.[6]

The antitumor therapy effect *in vitro* was investigated by incubating the HeLa tumor spheroids with PBS, free DOX, G5.NHAc-CD/BM-G3.NHAc/DOX and G5.NHAc-CD/Ad-G3.NHAc/DOX complexes, respectively for 7 days at equivalent DOX concentration (2 μg mL^{-1}). The tumor spheroids were monitored every other day by a phase contrast microscope for 7 days to check the volume change in different groups.

Statistical Analysis

One-way analysis of variance statistical method was performed to evaluate the significance of the experimental data. A value of 0.05 was selected as the significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Surface potentials of CSTDs dispersed in water. Data are provided as mean ± SD (n = 3).

Sample	Zeta potential (mV)
G5.NHAc-CD/Ad-G3.NHAc	11.3 ± 1.70
G5.NHAc-CD/BM-G3.NHAc	11.8 ± 1.55

Table S2. Hydrodynamic sizes of single dendrimers and CSTDs dispersed in water. Data are provided as mean ± SD (n = 3).

Sample	Hydrodynamic size (nm)	Polydispersity index (PDI)
G5.NHAc-CD/Ad-G3.NHAc	202.6 ± 23.94	0.356 ± 0.074
G5.NHAc-CD/BM-G3.NHAc	245.0 ± 11.20	0.248 ± 0.022
G5.NHAc-CD	174.8 ± 6.12	0.475 ± 0.075
G3.NHAc-BM	95.42 ± 20.32	0.373 ± 0.122

Table S3. The drug loading content (DLC) and the drug loading efficiency (DLE) of G5.NHAc-CD/Ad-G3.NHAc/DOX and G5.NHAc-CD/BM-G3.NHAc/DOX complexes.

Sample	Drug loading content (DLC)	Drug loading efficiency (DLE)
G5.NHAc-CD/Ad-G3.NHAc/DOX	4.93%	78.31%
G5.NHAc-CD/BM-G3.NHAc/DOX	5.16%	82.76 %

Table S4. Complexation Capacity of DOX with CSTDs

Sample	Number of DOX molecules per CSTD
G5.NHAc-CD/Ad-G3.NHAc	8.6
G5.NHAc-CD/BM-G3.NHAc	9.2

Table S5. The half maximal inhibitory concentrations (IC_{50}) of the G5.NHAc-CD/Ad-G3.NHAc/DOX complexes, G5.NHAc-CD/BM-G3.NHAc/DOX complexes, and free DOX.

Sample	IC_{50} (μg mL^{-1})
G5.NHAc-CD/Ad-G3.NHAc/DOX	2.22
G5.NHAc-CD/BM-G3.NHAc/DOX	1.13
Free DOX	0.70

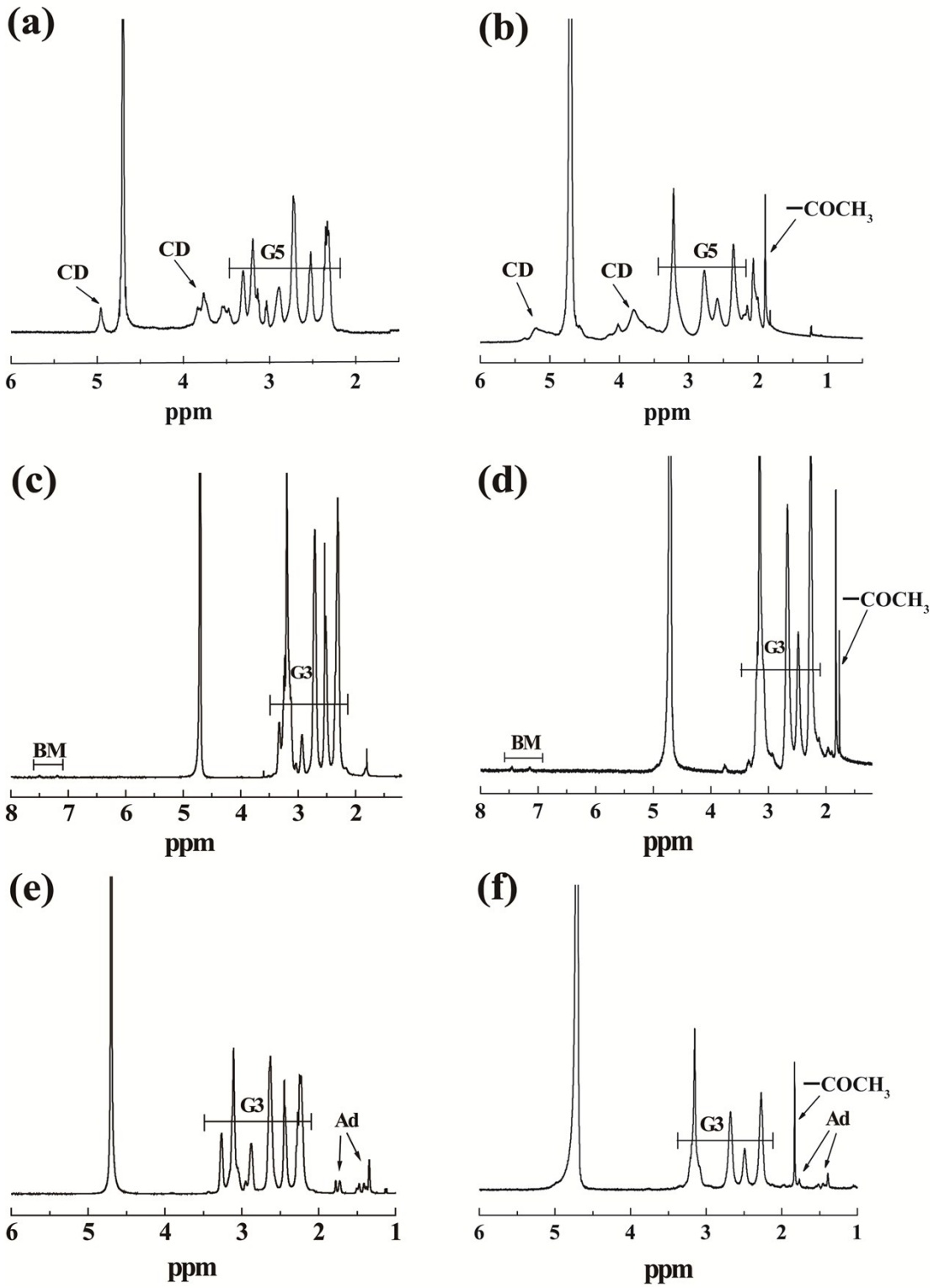

Figure S1. ^{1}H NMR spectra of G5.NH_2-CD (a), G5.NHAc-CD (b), G3.NH_2-BM (c), G3.NHAc-BM (d), G3.NH_2-Ad (e) and G3.NHAc-Ad (f) dendrimers dissolved in D_2O.

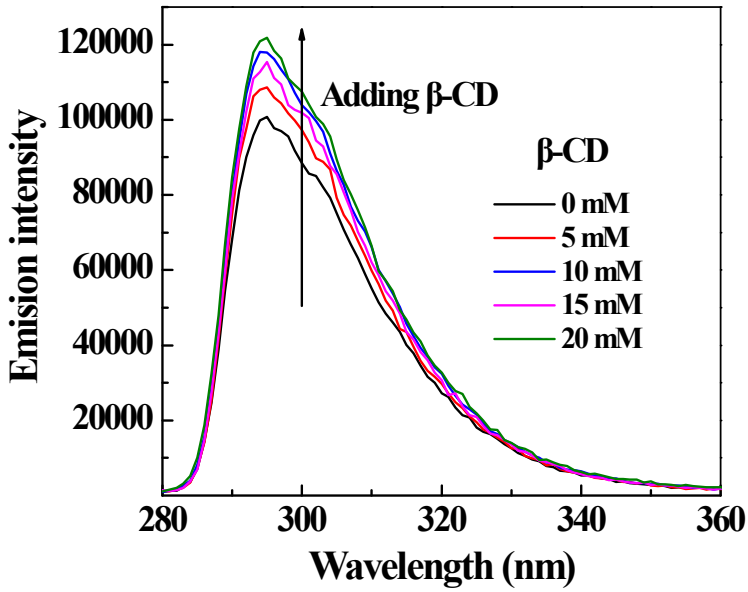

Figure S2. Fluorescence emission spectra of β-CD/BM mixture (the concentration of BM was fixed at 10 mM).

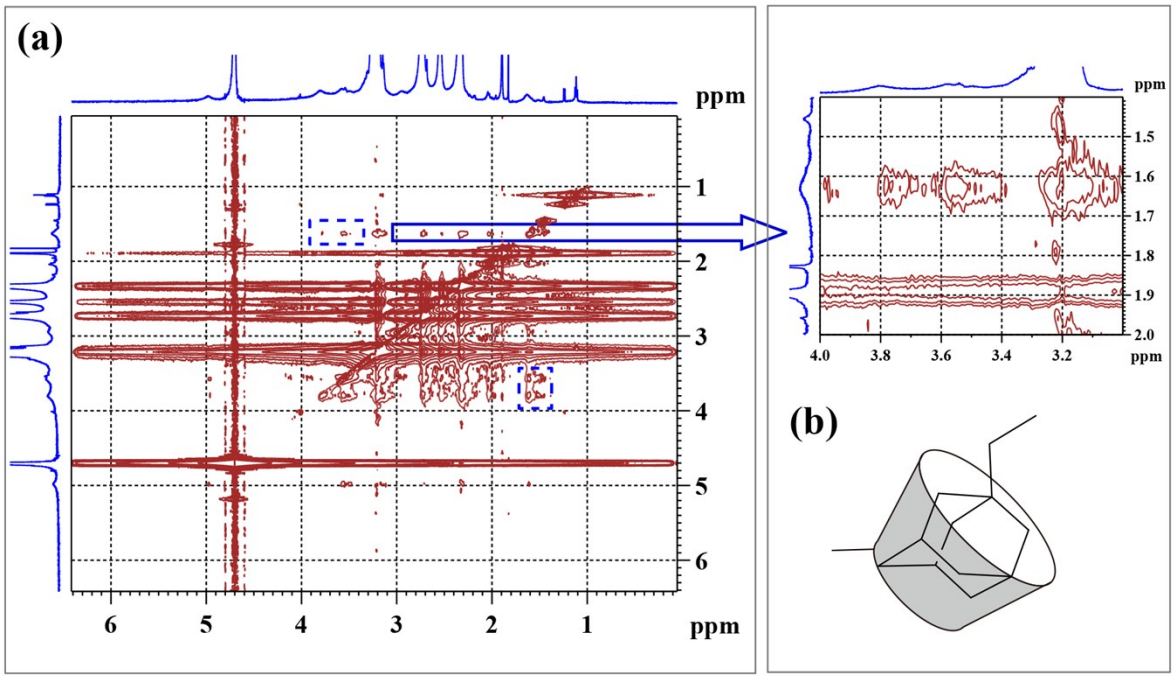

Figure S3. (a) 2D NOESY spectrum of G5.NHAc-CD/Ad-G3.NHAc dendrimers; (b) model of the interactions between Ad and β-CD and its corresponding enlarged 2D NOESY spectrum.

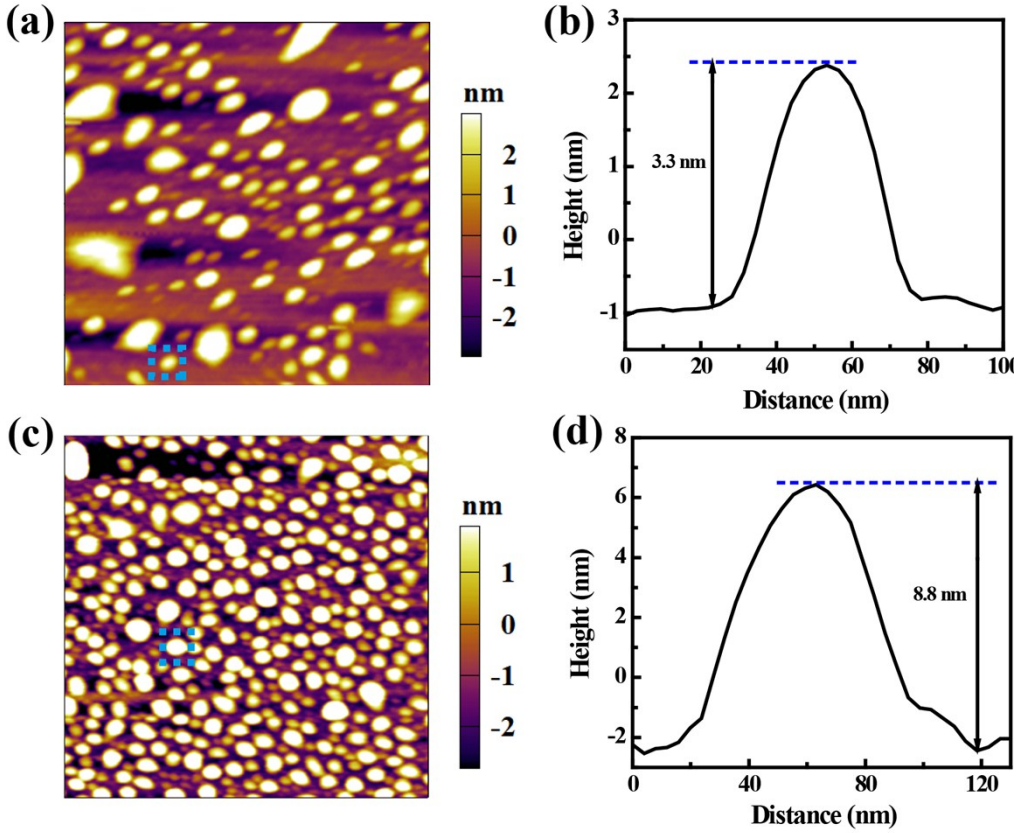

Figure S4. AFM images of G3.NHAc-Ad (a) and G5.NHAc-CD/Ad-G3.NHAc (c) dendrimers. Corresponding height profiles of G3.NHAc-Ad (b) and G5.NHAc-CD/Ad-G3.NHAc (d) dendrimers.

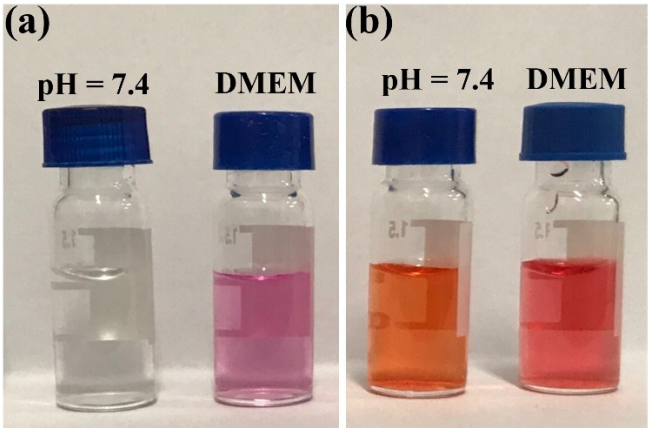

Figure S5. Photographs of the G5.NHAc-CD/BM-G3.NHAc CSTDs (a) and G5.NHAc-CD/BM-G3.NHAc/DOX complexes (b) dispersed in PBS (pH = 7.4) and DMEM for three weeks.

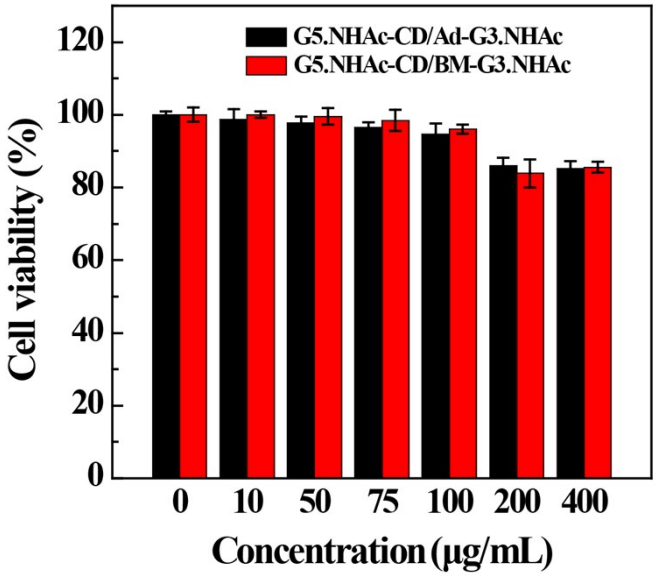

Figure S6. CCK-8 assay of the viability of HeLa cells after treated with CSTDs at various concentrations for 24 h.

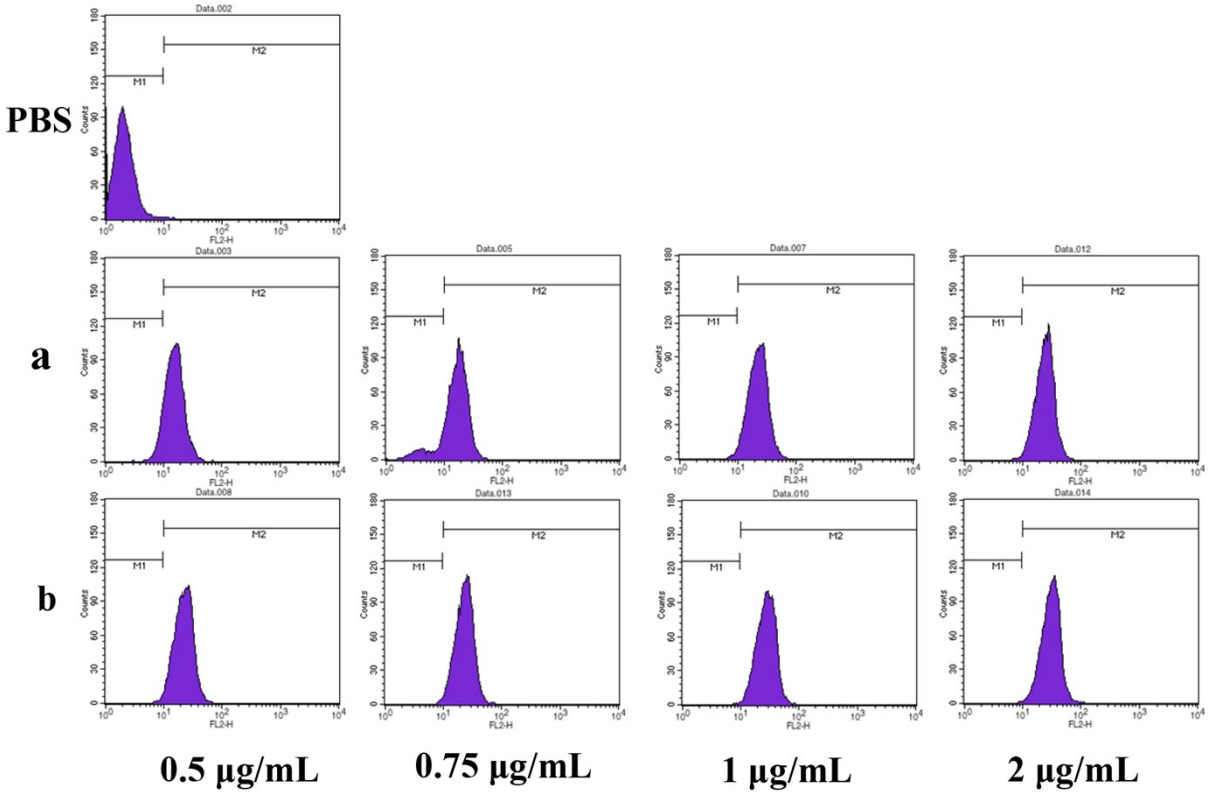

Figure S7. Flow cytometric analysis of HeLa cells after treatment with (a) G5.NHAc-CD/Ad-G3.NHAc/DOX and (b) G5.NHAc-CD/BM-G3.NHAc/DOX complexes at different DOX concentrations for 4 h. PBS was used as a control.

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