Electronic Supplementary Information

Amphiphilic Sugar Poly(orthoesters) as pH-Responsive Nanoscopic Assemblies for Acidity-Enhanced Drug Delivery and Cell Killing

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1. General Information

All chemicals and solvents were purchased from Sigma-Aldrich, unless otherwise noted. Anhydrous solvents (dichloromethane, CH_2Cl_2, DCM, 99.8%; tetrahydrofuran, THF 99.9%) were purchased in capped Sureseal™ bottles and were purified with a Mbraun solvent purification system. Other solvents and reagents were used without further purification. All glassware utilized was flame-dried before use. Glass-backed TLC plates (Silica Gel 60 with a 254 nm fluorescent indicator) were used without further manipulation and stored over desiccant. Silica gel column chromatography was performed using flash silica gel (32-63 μm) and employed a solvent or solvent mixture with a polarity correlated with TLC mobility.

Doxorubicin was purchased from Carbosynth Co. Ltd. HEK293 cells were purchased from American Type Culture Collection. Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals. Dulbecco's modified eagle's medium (DMEM) was purchased from Lonza Co. Ltd. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT reagent)

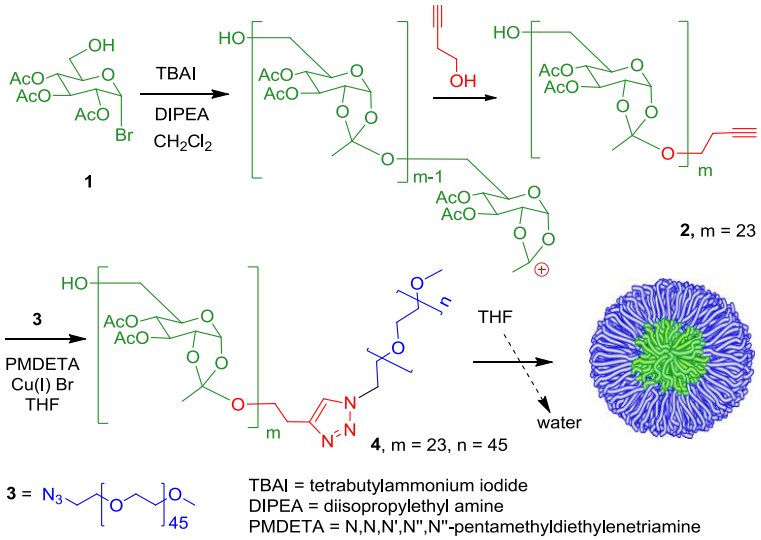
was purchased from Promega Co. Ltd.

^{1}H-NMR spectra were recorded at 300 MHz or 500 MHz on a Varian Mercury 300 or Varian Inova 500 spectrometer, respectively, with tetramethylsilane (TMS) proton signal as the reference. ^{13}C-NMR spectra were recorded at 126 MHz on a Varian Inova 500 spectrometer, with tetramethylsilane (TMS) carbon signal as the reference.

Gel permeation chromatography (GPC) analyses were conducted using a Viscotek GPC system equipped with a TDA270 dual-detector and a column system comprised of one PAS102 and one PAS103 column (Polyanalytik Inc.). The system was equilibrated at 35 °C in THF, which served as the polymer solvent and eluent with a flow rate of 1.0 mL/min. Polymer solutions were prepared at a known concentration (*ca.* 3 mg/mL) and an injection volume of 100 μL was used. Data collection and analyses were performed by OmniSEC software system from Malvern Inc.

Mass spectrometry was measured with a Waters LCT Premier™ XE unit. Fourier-transform infrared (FT-IR) spectra were obtained on a Thermo Scientific Nicolet™ iS™ 50 spectrometer used NaCl plates, with the sample being deposited from CH_2Cl_2 and allowing for evaporation of the solvent before measurement. The optical rotation values were measured on a DigiPol-DP1A11 polarimeter (Rudolph Instruments Inc.) Dynamic Light Scattering (DLS) measurements were performed by Zetasizer 3000HSA analyzer (Malvern Instruments Ltd.) Transmission Electron Microscopy (TEM) measurements were performed using a Philips CM-10 Unit with 60 kV accelerating voltage. Micrographs were collected at 21,000× magnification and calibrated using a 41 nm polyacrylamide bead from National Institute of Standards and Technology (NIST). Carbon-coated copper grids used for TEM were treated with oxygen plasma before deposition of the nanoparticle (NP) samples. The samples were deposited on the carbon grids for 1 min, and excess samples were wicked away. The samples were

allowed to dry under ambient conditions. The number-average particle diameters and standard deviations were generated from the analysis of a minimum of 80 particles. Dialysis was done with Spectra/Por® 3 (Spectrum Laboratories, Inc.) dialysis bags with mwco = 3.5 kDa. UV-vis spectroscopy was acquired on a Varian Cary 1 UV-vis system (Varian Inc., Palo Alto, CA) using quartz cuvettes.



Scheme 1S. The synthesis of a sugar poly(orthoester)-based amphiphilic block polymer, and the subsequent self-assembly to construct NPs.

2. The synthesis of alkyne-functionalized sugar poly(orthoester) (**2**)

The synthesis of the polymer was performed in a similar manner as that reported previously.[1] Briefly, to a 10-mL Schlenk flask was added monomer **1** (0.270 g, 0.73 mmol) in anhydrous CH_2Cl_2 (1 mL). *tetra*-Butylammonium iodide (TBAI, 0.271 g, 0.73 mmol) and N,N-diisopropylethylamine (DIPEA, 0.38 mL, 2.19 mmol) were added. The reaction mixture was stirred at reflux condition for 18 h. Subsequently, excess 3-butyn-1-ol (0.55 mL, 7.30 mmol) was added. The reaction mixture was stirred at reflux condition for another 18 h. The solvent was removed under reduced pressure. The polymer was precipitated three times using a mixture solvent of water/methanol (9/1, v/v) at rt to afford alkyne-functionalized sugar poly(orthoester) **2** as a slightly brown powder (0.126 g, 60%). M_n^{GPC} = 6.1 kDa; PDI = 1.3. $[\alpha]_D^{25}$ = +97.6 (*c* =1 in $CHCl_3$). ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 5.72 (d, *J* = 5.8, 1H, H-1),

5.21 – 5.07 (m, 1H, H-3), 4.92 – 4.88 (m, 1H, H-4), 4.32 – 4.28 (m, 1H, H-2), 3.85 – 3.74 (m, 1H, H-5), 3.65 – 3.52 (m, 2H, H-6'6), 2.43 – 2.47 (m, HC≡C-CH_2), 2.12 – 1.69 (m, 9H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ = 169.97 (C=O), 169.50 (C=O), 121.31 (orthoester C), 97.27 (C-1), 81.33 (HC≡*C*-CH_2), 72.78 (C-2), 70.54 (H*C*≡C-CH_2), 70.06 (C-3), 68.32 (C-5), 67.78 (C-4), 63.21 (C-6), 61.12 (HC≡C-CH_2-*C*H_2), 23.03 (HC≡C-*C*H_2), 21.15 (-OAc), 21.09 (-OAc), 20.64 (-CH_3). IR: 3330 (-OH), 2960 (C-H), 2873 (C-H), 1740 (C=O) cm^{-1}.

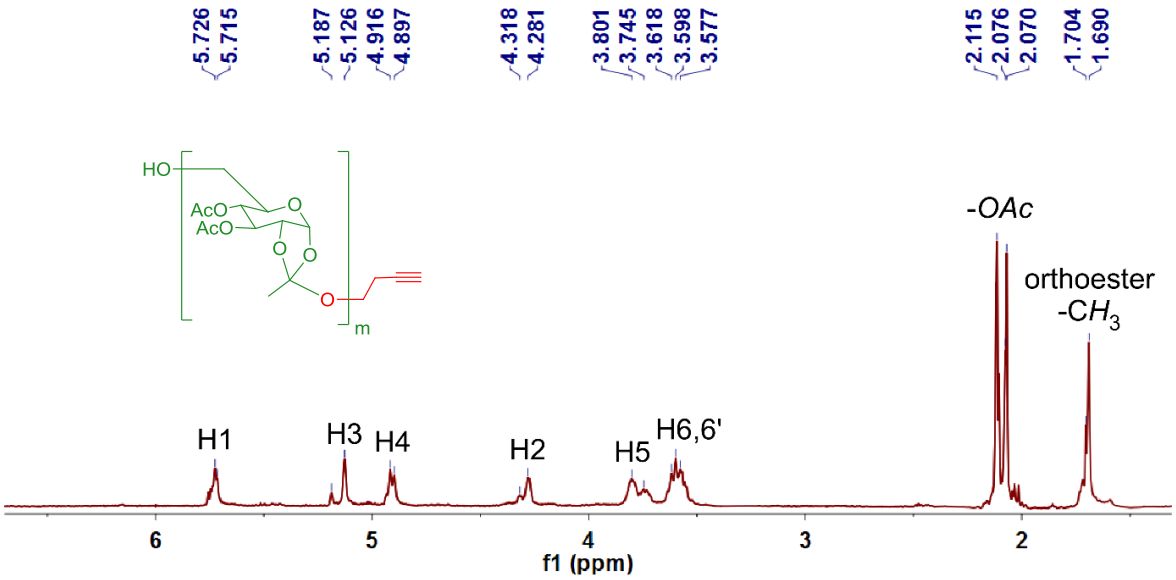


Figure 1S. ^{1}H-NMR spectrum of polymer **2**

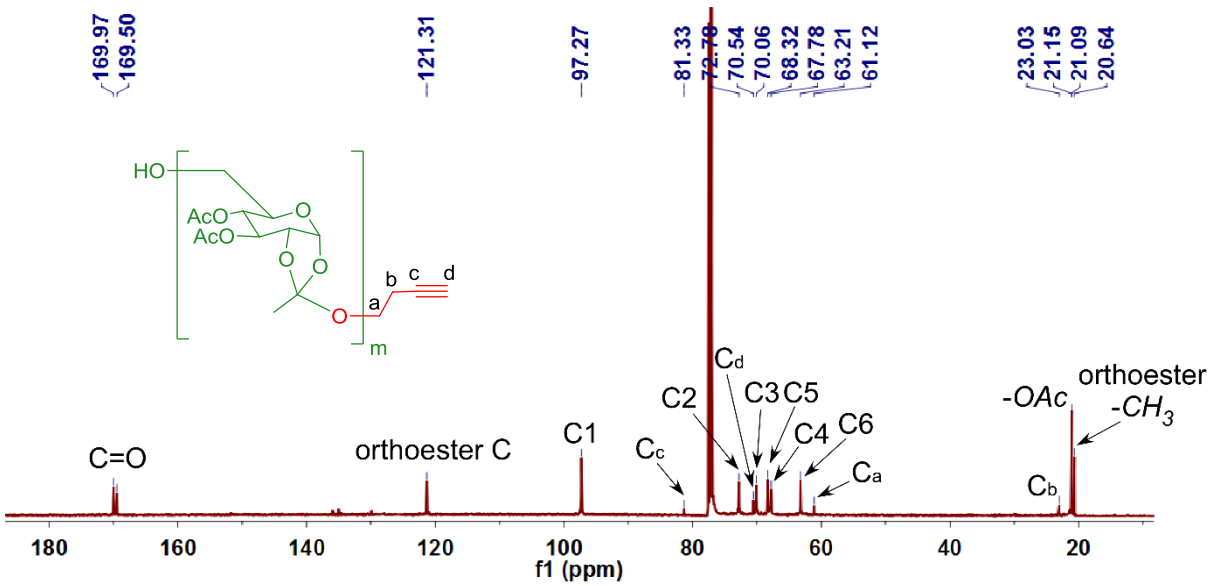


Figure 2S. ^{13}C-NMR spectrum of polymer **2**

3. The synthesis of sugar poly(orthoester)-*b*-poly(ethylene glycol) (**4**)

The synthesis of the polymer was performed in a similar manner as that reported previously.[2] Briefly, to a 10-mL Schlenk flask was added polymer **2** (0.050 g, M_n^{GPC} = 6.3 kDa, 0.008 mmol) and azido-functionalized poly(ethylene glycol) **3** (0.017 g, 0.009 mmol) in anhydrous THF (1.0 mL). After two cycles of freeze-pump-thaw, Cu(I)Br (0.002 g, 0.016 mmol) and N,N,N',N'',N''-pentamethyldiethylenetriamine (PMDETA, 0.003 g, 0.018 mmol) were added. The reaction mixture was stirred at 40 °C for 18 h. The reaction mixture was loaded into a short column of neutral alumina to remove the copper catalyst. The collected solution was then subjected to dialysis (mwco = 3.5 kDa) against nanopure water to remove excess **3** and other small molecule impurities. The solution was collected and the solvent was removed under reduced pressure to give the conjugated product **4** as a slightly yellow powder (0.046 g, 90%). M_n^{GPC} = 8.7 kDa; PDI = 1.3. ^{1}H-NMR (500 MHz, $CDCl_3$) δ = 5.71 (m, 1H, H-1), 5.12 (m, 1H, H-3), 4.90 (m, 1H, H-4), 4.27 (m, 1H, H-2), 3.79 (m, 1H, H-5), 3.71 – 3.46 (m, 14H), 3.38 (s), 2.08 (d, 6H), 1.68 (s, 3H). ^{13}C-NMR (126 MHz, $CDCl_3$) δ = 169.96 (C=O), 169.48 (C=O), 121.30 (orthoester C), 97.26 (C-1), 72.77 (C-2), 70.78 (PEO-*C*s), 70.05 (C-3), 68.32 (C-5), 67.77 (C-4), 63.21 (C-6), 59.18 (-*C*H_3), 50.84 (-*C*H_2N_3), 21.16 (-OAc), 21.08 (-OAc), 20.64 (-*C*H_3).

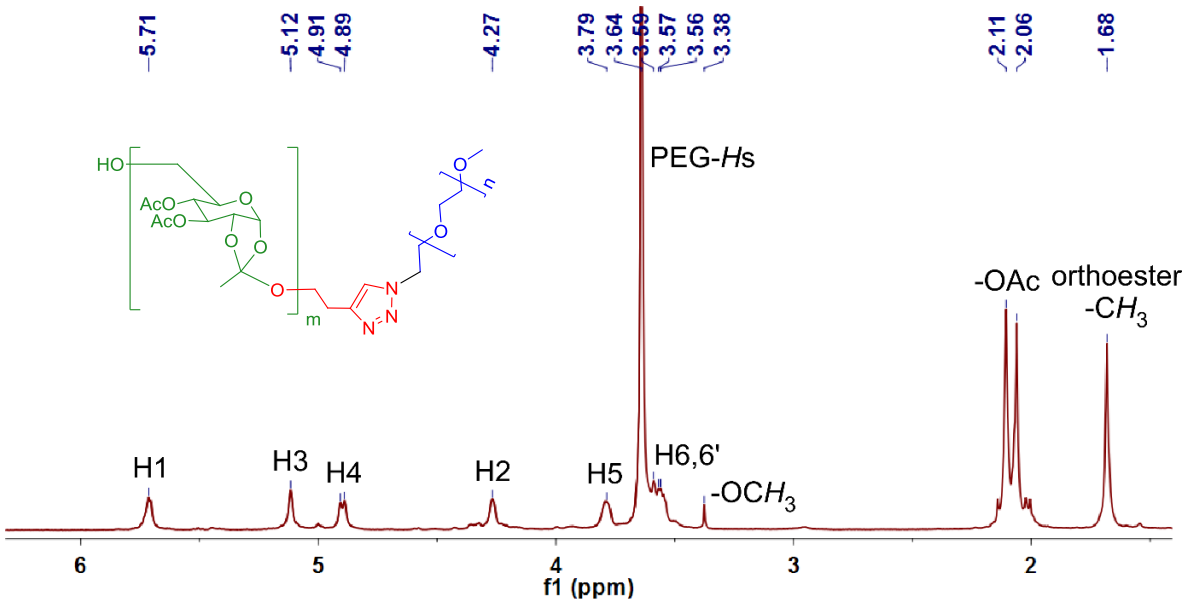


Figure 3S. ^{1}H-NMR spectrum of polymer **4**

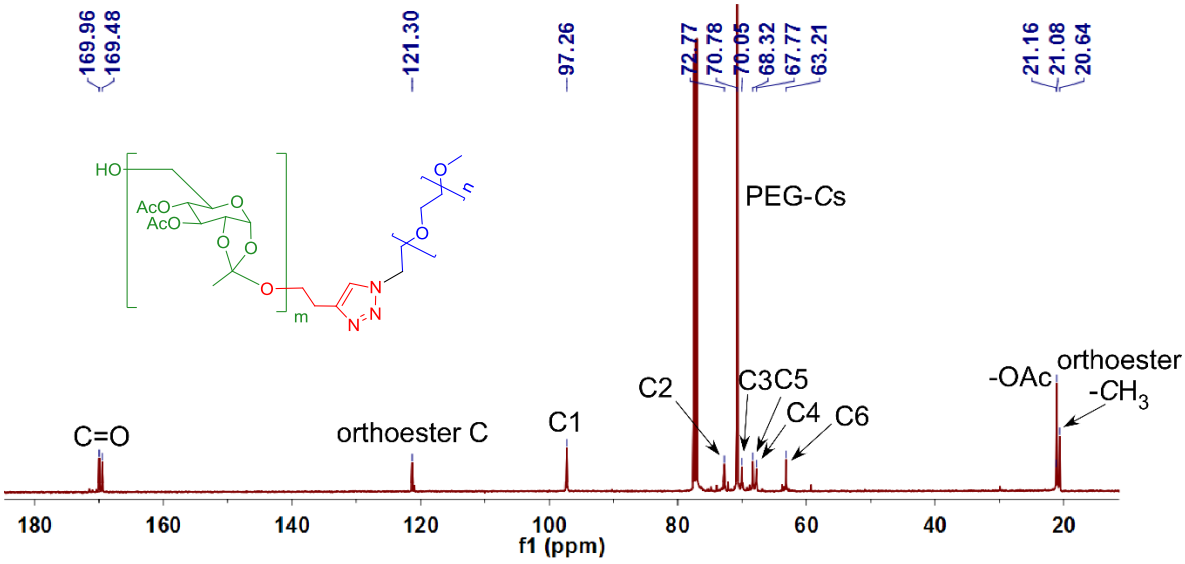


Figure 4S. ^{13}C-NMR spectrum of polymer **4**

4. Synthesis of the NPs

Amphiphilic block polymer **4** (6.0 mg) was dissolved in tetrahydrofuran (THF, 2.0 mL) and stirred at rt for 2 h. Nanopure water (2.0 mL) was then added dropwise over a time period of 2 h. The solution was stirred for another 4 h and then was subjected to dialysis against nanopure water for 48 h to afford a NP solution. The NP solution was re-constituted, by the addition of PBS buffer, to give a concentration of 1.0 mg/mL. TEM sizes: 56 ± 8 nm; Dynamic light scattering (DLS) sizes: D_h (intensity) = 96 ± 15 nm; D_h (volume) = 90 ± 25 nm.

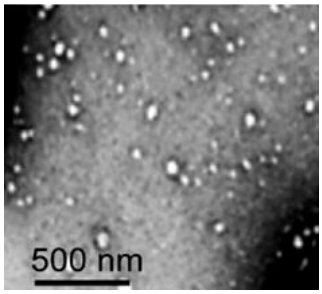


Figure 5S. The TEM image of the NPs derived from amphiphilic block polymer **4**.

5. The stability study of the NPs

The storage stability of the NPs was monitored using DLS after 1 day, 3 days and 7 days. There were no significant changes in the NP sizes during these time periods at rt.

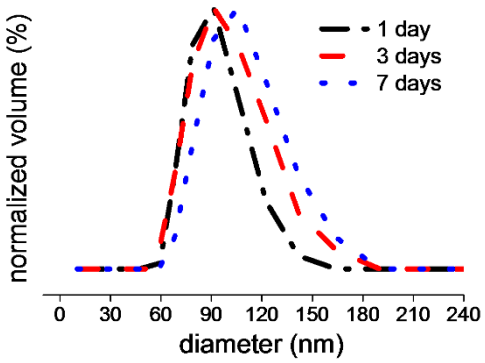


Figure 6S. The DLS analyses of the NP's sizes after 1 day, 3 days and 7 days at rt.

6. Acidolysis of the polymer

The measurement of the degraded products of the poly(orthoesters) was performed in a similar manner as that reported previously.[1] Briefly, to the polymer **2** solution in $CDCl_3$ (1 mL) was added trifluoroacetic acid to adjust the pH values to pH = 5. The reaction mixture was stirred at rt overnight. ^{1}H-NMR analyses was performed to determine the structure of the degraded products, which are a mixture of acetylated glucose.[3]

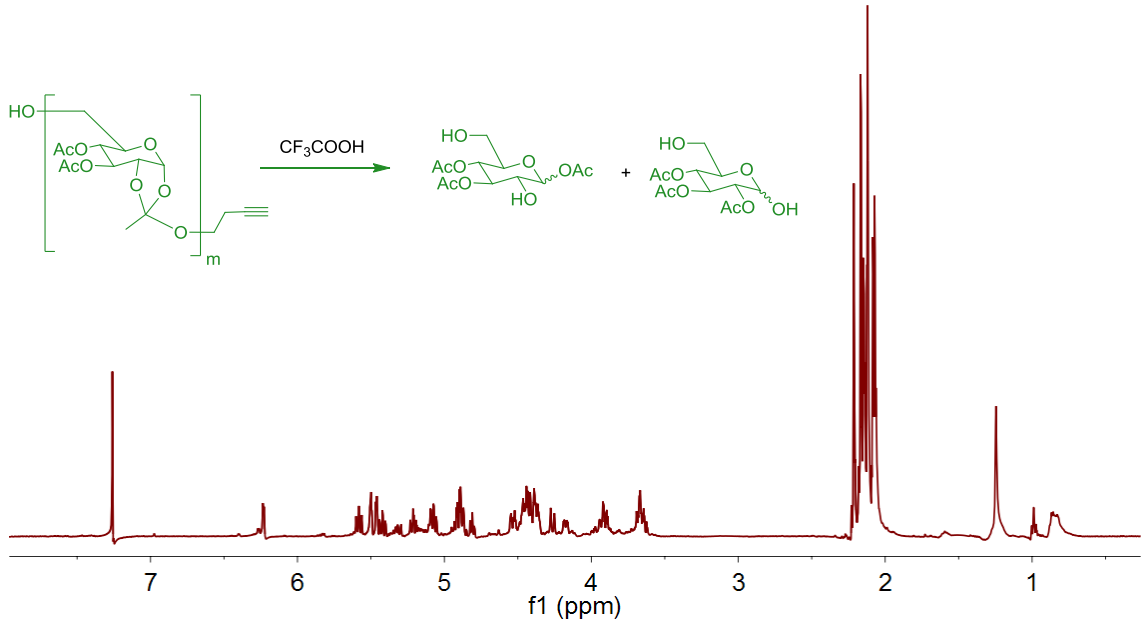


Figure 7S. The ^{1}H-NMR spectrum of the degraded product after acidolysis of the polymer 2.

7. Determination of the critical micelle concentration (CMC)

The measurement of CMC was performed according to the literature reported previously.[4] Briefly, a stock solution of Nile Red (10^{-4} mg/mL) was prepared by dissolving Nile Red in DCM. The Nile Red (10 μL) was added to a series of the NP **4** solutions (1.0 mL, with concentrations ranging from 1×10^{-3} to 2×10^{-2} mg/mL). This will give the Nile Red concentration in each vial of 1×10^{-6} M. The solutions were stirred at rt overnight to equilibrate the Nile Red with the NPs. The fluorescence emissions were measured with λ_{exc} = 544 nm and by λ_{emi} = 612 nm using SpectraMax M2 fluorimeter (Molecular Devices, LLC. Sunnyvale, CA). A graph of fluorescence intensity vs. log [concentration] was plotted. From the graph, the CMC was determined at the intersection of the two lines.

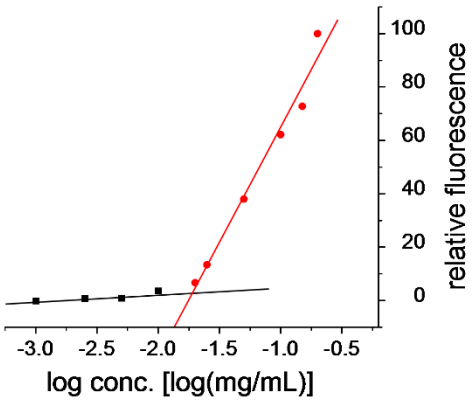


Figure 8S. Determination of the critical micelle concentration (CMC) from Nile Red fluorescence in PBS buffer (pH = 7.4).

8. Cell viability assay

All experiments were performed at 37 °C. The media formulation used for HEK293 cells was Dulbecco's modified eagle's medium (DMEM) + 10% FBS. HEK293 cells were seeded on 96-well plates at a density of 5000 cells per well for *ca.* 24 h. A full media replacement was then conducted, which contained various concentrations of NPs, DOX-loaded NPs, or DOX at either pH = 7.4 or pH = 5.0, depending on the experimental design (see manuscript). The treatments were performed for 4 h. Thereafter, full media replacements were conducted and the cells were incubated for 72 h. To perform the MTT assay, the existing media was replaced with 120 μL MTT reagents, which contained 100 μL cell media + 20 μL CellTiter 96 Aqueous One Solution® (Promega Co. Ltd.). The cells were incubated for another 2.5 h. To evaluate the cell viability, the absorbance at 490 nm was measured using a

PerkinElmer Wallac Victor3 1420 Multilabel Counter. The cell viability was normalized compared to those cells treated with media only.

9. DOX loading and release studies

DOX loading: To the NP solution (1.0 mg/mL) in a 15 mL vial was added DOX (3.0 mg/mL in CH_2Cl_2 with 30% trimethylamine, v/v %). The solution was stirred in dark conditions for 18 h with the vial being open. Insoluble DOX was removed by centrifugation (6000 rpm ×10 min) in a centrifugal filter device (Amicon® Ultr-4, mwco = 3.0 kDa, Merck KGaA, Germany). The DOX-loaded NPs were then reconstituted to a final volume of 4 mL. After adding 3 folds of methanol (v/v), the UV-vis absorbance at 480 nm were measured and the amount of incorporated DOX was determined using a calibration curve of DOX of varying concentration in MeOH/PBS 3:1. The Drug Loading Efficiency (DLE) and Drug Loading Content (DLC) were calculated according to the following equation:

DLE = the amount of drugs loaded in the NPs / the amount of drugs used

DLC = the amount of DOX loaded in the NPs / the amount of the polymer used

DOX release: The DOX-loaded NP solution (2 mL) was transferred to pre-soaked dialysis cassettes. The cassettes were then stirred in a 4-L beaker containing 4 L of 5 mM PBS at pH = 7.4 and pH = 5.0, respectively. Samples (~100 μL) were removed from the cassette at 0, 1, 2, 4, and 6 h and were analysed by UV-vis measurements at 480 nm. All release experiments were conducted in triplicate.

DOX release in FBS: The DOX-loaded NP solution (1 mL) was transferred to pre-soaked dialysis cassettes. The cassettes were then stirred in a 1-L beaker containing 600 mL FBS (33%, v/v, in PBS, pH = 7.4). Samples (~100 μL) were removed from the cassette at 0, 1, 2, 4, and 6 h and were analysed by UV-vis measurements at 480 nm. All the release experiments were conducted in triplicate.

10. References:

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