Supplementary Information for:

Dual pH-triggered multistage drug delivery system based on host-guest interaction-associated polymeric nanogels

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Materials. 2-Hydroxyethyl methacrylate (HEMA, ≥ 99%, Fluka) was distilled before use. 2,2′-Azobisisobutyronitrile (AIBN, 98%, Fluka) was recrystallized from 95% ethanol. *β*-Cyclodextrin (*β*-CD) was recrystallized from distilled water. *N*-Hydroxysuccinimide (NHS, 97%, Aldrich) was recrystallized from toluene prior to use. Triethylamine (TEA), tetrahydrofuran (THF), 1,4-dioxane, *N*,*N*- dimethylformamide (DMF), dichloromethane (DCM), and dimethyl sulfoxide (DMSO) were dried and distilled prior to use. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 99%), *N*,*N*'-dicyclohexylcarbodiimide (DCC, 99%), 4-formylbenzoic acid (FBA, 98%), 1-adamantylamine hydrochloride (98%), 6-heptynoic acid (97%), and 4-dimethylaminopyridine (DMAP, 99%) were purchased from Aladdin Reagent Company and used as received. Poly(ethylene glycol) methyl ether (average M_n ~2,000, flakes), *t*-butyl carbazate (98%), copper(I) bromide (CuBr, 99%), and *N*,*N*,*N*',*N*'',*N*''-pentamethyldiethylenetriamine (PMDETA, 98%) were purchased from Sigma-Aldrich and used as received. Poly(ethylene glycol) monomethyl ether

methacylate (PEGMA),[1] *N*-(2-hydroxypropyl) methacrylamide (HPMA),[2] 4-cyanopentanoic acid dithiobenzoate (CTP),[3] 3-azidopropyl methacrylate (AzPMA),[4] and alkynyl group-substituted *β*-CD (*alkynyl*-CD)[5] were synthesized according to established procedures provided in the literatures. Fetal bovine serum (FBS), trypsin, phosphate buffered saline (PBS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO and used as received. Cell culture lysis buffer, 4',6-diamidino-2-phenylindole (DAPI), Lyso-Tracker Green, and 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All other commercially available solvents and reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. and used as received.

Characterization

All nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV300 NMR 300 MHz spectrometer using D_2O, DMSO-d_6 or $CDCl_3$ as the solvent. The molecular weights (MWs) and molecular weight distributions (M_w/M_n) of PPEGMA-*co*-PHEMA were determined by GPC using a series of three linear Styragel columns HT3, HT4 and HT5 with a column temperature of 35 °C. A Waters 1515 pump and Waters 2414 differential refractive index detector (set at 30 °C) were used. The eluent was THF at a flow rate of 1.0 mL/min. A series of six low polydispersity polystyrene standards were used for the GPC calibration. On the other hand, The molecular weights (MWs) and molecular weight distributions (M_w/M_n) of PHPMA-*co*-PAzPMA were determined by gel permeation chromatography (GPC) equipped with an Agilent1260 pump and an Agilent G1362A differential refractive index detector. The eluent was DMF with 1 g/L LiBr at a flow rate of 1.0 mL/min. A series of low polydispersity PEG standards were employed for calibration. Fourier transform infrared (FT-IR) spectra were recorded on a Bruker VECTOR-22 IR spectrometer. The spectra were collected over 64 scans with a spectral resolution of 4 cm^{-1}.

Sample preparation

Synthesis of alkynyl-DOX. The synthetic routes employed for the preparation *alkynyl*-DOX was shown in Scheme S1. Briefly, 6-heptynoic acid (1 g, 7.9 mmol) was dissolved in anhydrous

DCM (50 mL) and cooled in a water/ice bath. EDC•HCl (1.67 g, 8.7 mmol) and DMAP (0.1 g, 0.9 mmol) were added and the mixture was stirred at 0 ^{o}C for 30 min under the protection of nitrogen. NHS (1 g, 8.7 mmol) in 3 mL DCM was added dropwise into the reaction mixture. The reaction mixture was warmed to room temperature and stirred at room temperature for 4 h. Then *t*-butyl carbazate (1.15 g, 8.7 mmol) was added and stirred for 8 h. The mixture was washed with distilled water, brine, and dried over anhydrous sodium sulfate. The solvents were removed at reduced pressure and purified by flash column chromatography using DCM/ethyl ethanoate (10 : 1 v/v) as the eluent. After removal of the solvent, the final product **1** was obtained as a pale yellow solid (1.6 g, yield: 84.0%). ^{1}H NMR (δ, ppm, $CDCl_3$): 2.30-2.12 (4H, OC-C***H***$_2$CH$_2$CH$_2$C***H***$_2$C≡CH), 1.93 (1H, –C≡C***H***), 1.84–1.68 (2H, OC-CH$_2$C***H***$_2$CH$_2$CH$_2$C≡CH), 1.63-1.50 (2H, OC-CH$_2$CH$_2$C***H***$_2$CH$_2$C≡CH), 1.50-1.36 (9H, OC(C***H***$_3$)$_3$); ESI-MS calcd for ($C_{12}H_2O_3N_2$ + H)$^{+}$: 241.3; found: 240.7.

1 (0.3 g, 1.2 mmol) was stirred in a mixture solution of DCM and TFA (1/1) for 30 min at room temperature. Then the solvent was removed by rotary evaporator and the residue was co-evaporated three times with toluene to remove the excess TFA. The above product and Dox·HCl (0.24 g, 0.4 mmol) were dissolved in 10 mL of anhydrous methanol, and treated with a drop of TFA. After refluxed for 48 h under dark, the mixture was cooled down. The solvent was removed by rotary evaporator and the residue was precipitated in excess ethyl acetate. Precipitate was collected by centrifugation and washed with ether. The product **2** was obtained as a dark red solid (0.22 g, yield: 79.5%). ESI-MS calcd for ($C_{34}H_{39}O_{11}N_3$ + H)$^{+}$: 666.25; found: 666.26.

Synthesis of PPEGMA-co-PHEMA-AD. The synthetic routes employed for the preparation of PEGMA-*co*-PHEMA-AD were shown in Scheme S2. Briefly, PEGMA-*co*-PHEMA was prepared by RAFT polymerization of PEGMA and HEMA using CTP as the chain transfer agent (CTA). To a Schlenk tube equipped with a magnetic stirring bar, PEGMA (2.50 g, 1.2 mmol), HEMA (0.31 g, 2.4 mmol), CTP (17 mg, 0.06 mmol), and AIBN (1.25 mg, 7.6 × 10^{-6} mol) was dissolved in 1,4-dioxane (4 mL). The reaction tube was carefully degassed by three freeze–pump–thaw cycles, sealed under vacuum, and placed in an oil bath thermostated at 80 °C. After stirring for 15 h, the ampoule was quenched into liquid nitrogen to terminate the

polymerization. The mixture was precipitated into an excess of ether. The above dissolution-precipitation cycle was repeated for three times. The obtained solids were further purified by dialysis against distilled water for three days using a dialysis bag (MWCO: 10000 Da). The solution was lyophilized, affording PPEGMA-*co*-PHEMA as a pale pink powder (1.45 g, yield: 51.3%; $M_{n,GPC}$ = 31.6 kDa, M_w/M_n = 1.18, Fig. S4a). The actual DPs of PPEGMA and PHEMA segments were determined to be 13 and 21 by ^{1}H NMR analysis in $CDCl_3$ (Fig. S1a). Thus, the polymer was denoted as PPEGMA$_{13}$-*co*-PHEMA$_{21}$.

FBA was conjugated onto the polymer backbone of PPEGMA-*co*-PHEMA via DCC condensation reaction. Typically, to a solution of FBA (0.53 g, 3.5 mmol) in 10 mL DCM was added DCC (0.72 g, 3.5 mmol) and DMAP (0.43 g, 3.5 mmol). After being stirred for 30 min, PEGMA$_{13}$-*co*-PHEMA$_{21}$ (1.0 g, 3.35 × 10^{-5} mol) in 10 mL DCM was added dropwise into the reaction mixture at 0 ^{o}C. After warming to room temperature and stirring for 24 h, the solution was filtered. The filtrate was concentrated under reduced pressure. The residues were purified by basic alumina column chromatography using DCM as the eluent. Then, the solution was concentrated under reduced pressure and precipitated into excess ethyl ether. The dissolution-precipitation cycles were repeated twice affording the product, PPEGMA-*co*-PHEMA-*FBA*, as a white solid (0.91 g, Yield: 83.2%). The functionality of hydroxyl groups is 90.5% according to ^{1}H NMR analysis (Fig S1b). Thus, the polymer was denoted as PPEGMA$_{13}$-*co*-PHEMA$_{21}$-*FBA*$_{19}$.

2-Adamantylamine was conjugated onto the polymer, PPEGMA-*co*-PHEMA-*FBA*, via formation of benzoic imine between amino groups and aldehyde moieties. Briefly, to a solution of PPEGMA-co-PHEMA-*FBA* (0.8 g, 0.0558 mmol) in 20 mL DMSO was added 1-adamantanamine hydrochloride (0.44 g, 2.3 mmol), triethylamine (0.28 g, 2.8 mmol). After being stirred for 12 h at 40 °C, the DMSO was removed by distillation under reduced pressure. The crude product was purified by basic alumina column chromatography using DCM as the eluent. Then, the solution was concentrated under reduced pressure and precipitated into excess ethyl ether for three times. Then, the precipitate was dried under vacuum to afford final product, PPEGMA-*co*-PHEMA-*AD*, as a pale yellow solid (0.75 g, Yield: 87.0%). The quantitative transformation of aldehyde moieties into benzoic imine was confirmed by the disappearance of aldehyde signals in ^{1}H NMR spectrum (Fig S1c). Thus, the polymer was denoted as

PPEGMA$_{13}$-*co*-PHEMA$_{21}$-*AD*$_{19}$.

Synthesis of PHPMA-co-PPMA-DOX-CD. The synthetic routes employed for the preparation of *β*-CD and DOX-conjugated polymer, PHPMA-*co*-PPMA-*DOX-CD*, were shown in Scheme S2. Firstly, copolymer, PHPMA-*co*-PAzPMA, was synthesized via RAFT polymerization of HPMA and AzPMA using CTP as the chain transfer agent. Briefly, HPMA (2.15 g, 15 mmol), AzPMA (0.51 g, 3 mmol), CTP (28 mg, 0.1 mmol), and AIBN (2 mg, 1.25×10^{-5} mol) were charged into a glass ampoule containing 2 mL 1,4-dioxane and 2 mL methanol solvents. The ampoule was then degassed via three freeze-pump-thaw cycles and flame-sealed under vacuum. It was then immersed into an oil bath thermostated at 80 °C to start the polymerization. After 15 h, the ampoule was quenched into liquid nitrogen to terminate the polymerization. The mixture was precipitated into an excess of ether. The above dissolution-precipitation cycle was repeated twice affording the final product as a pink solid (1.3 g, Yield: 48.4%; $M_{n,GPC}$ = 15 kDa, M_w/M_n = 1.14, Fig. S4b). The actual DPs of PHPMA and PAzPMA segments were determined to be 78 and 13 by ^{1}H NMR analysis in D_2O (Fig. S2a). Thus, the polymer was denoted as PHPMA$_{78}$-*co*-PAzPMA$_{13}$.

β-CD and DOX were conjugated onto the polymer, PHPMA-*co*-PAzPMA, via click reaction. Briefly, **2** (0.2 g, 0.3 mmol), and *alkynyl*-CD (0.34 g, 0.3 mmol), PHPMA-*co*-PAzPMA (0.5 g, 3.7×10^{-5} mol), PMDETA (51 mg, 0.3 mmol), and DMF (6 mL) were charged into a reaction flask. The mixture was degassed by three freeze-pump-thaw cycles and then CuBr (42 mg, 0.3 mmol) was introduced under N_2 protection. After thermostating at 40°C in an oil bath and stirring for 20 h, the ampoule was quenched into liquid nitrogen. The mixture was precipitated into an excess of ether to generate residues which were further dissolved in methanol and precipitated into ether. The crude product was passed through a basic alumina column chromatography to remove copper salt catalyst using methanol as the eluent. Then, the solution was concentrated under reduced pressure and precipitated into excess ethyl ether. The obtained solids were further purified by dialysis (MWCO: 6000 Da) against deionized water at pH 8 for 48 h under dark, and then lyophilized to afford the final product as a red solid (0.84 g, Yield: 90.5%). The precise amount of conjugated DOX on each polymer chain was determined to be 7 according to the UV-vis absorbance standard curve of DOX·HCl. The conjugation efficiency

was determined to be 86.4%. The β-CD amount was calculated to be 6 due to the disappearance of azido moiety signals on the FT-IR spectra (Fig. S3). Thus, the polymer was denoted as $PHPMA_{78}$-*co*-$PPMA_{13}$-DOX_7-CD_6.

Preparation of polymeric nanogels based on PPEGMA-*co*-PHEMA-*AD* and PHPMA-*co*-PPMA-*DOX-CD*. PPEGMA-*co*-PHEMA-*AD* and PHPMA-*co*-PPMA-*DOX*-*CD* were dissolved in DMSO at the AD/CD molar ratio of 1:1 at the concentration 8 mg/mL, followed by dialysis against PBS buffer (pH 7.4) for 12 h under dark. Fresh osmosis PBS buffer was replaced every 3 h. The final concentration of the nanogel solution was adjusted to 1 mg/mL.

Particle size measurements. The particles size and zeta potential change of the nanogels were evaluated at varying pH values (pH 6.5 and pH 7.4) using a Zetasizer Nano ZS instrument, equipped with a He-Ne ion laser (λ = 632 nm) at a scattering angle of 173^o.

Transmission Electron Microscopy (TEM) Observation. TEM observation was conducted using a Hitachi H-800 electron microscope at an acceleration voltage of 200 kV for insight on the morphology of the nanogels. Notably, the nanogels were investigated after incubation at pH 7.4 and pH 6.5 for 1 h, respectively. The copper TEM grids were dipped into desired samples. The sample grids were blotted by filter paper to remove excess complex solution, followed by drying for 30 min.

Collagen gel diffusion. Collagen hydrogels were prepared according to the manufacturer's protocol by mixing 50 μL of 8 mg/mL rat tail collagen I and 3 μL of 0.1 M sodium hydroxide on ice. The final concentration of collagen was 7.54 mg/mL. After vortexing, the collagen hydrogel was added to partially fill a microslide capillary tube followed by overnight incubation at 37 °C. After preincubation at pH 7.4 or pH 6.5 for 2 h, 3 μL nanogel solution was added into the capillary tube to ensure contact with the surface of the collagen gel. The sample was incubated for diffusion at 37 °C for 4 h and then imaged using CLSM.

Drug release from the nanogel solution. In vitro release profiles of DOX from nanogels solution at varying pH values (pH 7.4, pH 6.5, and pH 5.0) was studied using a dialysis bag diffusion method. In brief, 2 ml of nanogels with the concentration of 1 mg/mL in 10 mM PBS (pH 7.4) was injected into a pre-swelled dialysis bag with a molecular weight cutoff of 6 kDa, followed by immersion into 18 ml of 10 mM PBS (pH 7.4). The dialysis was conducted at 37°C in a shaking culture incubator. Periodically, 1 mL aliquot of sample solution from the incubation medium was taken for measurement and compensated with 1 mL of fresh buffer to incubation medium. The concentration of DOX in the released solutions was quantified by UV-vis absorbance according to the standard curve of DOX·HCl. The DOX released from the nanogels was expressed as the percentage of cumulative DOX outside the dialysis bag to the total DOX in the nanogel solutions. The same drug release procedure was also applied for the quantification of release profiles of the nanogels at pH 6.5 and pH 5.0, respectively.

In vitro cytotoxicity evaluation. HeLa cells were used for in vitro cytotoxicity evaluation of the nanogels loading DOX based on a MTT assay. HeLa cells were seeded onto 96-well plates at a density of 1×10^4 cells/well in 100 μL DMEM with 10% FBS at 37 °C with 5% CO_2 humidified atmosphere. After 24 h incubation, the original medium was replaced with fresh culture medium. After preincubation at pH 7.4 or pH 6.5 for 2 h, the nanogel solutions or PHPMA-*co*-PPMA-*DOX*-*CD* polymer solution (1 mg/mL) were added into each well in a concentration-dependent manner. After 4 h incubation, the medium in each well was replaced with fresh cell culture medium for another 48 h incubation. MTT solution (20 μL, 5 mg/mL in PBS buffer) was added to each well and incubated 4 h for reaction. The medium in each well was then removed and 200 μL of DMSO was added to dissolve the internalized purple formazan crystals. The plate was subjected to gently agitation for 30 min until all the crystals were dissolved. The absorbance at wavelength of 480 nm was recorded by a microplate reader (Thermo Fisher).

Cellular internalization and intracellular distribution of the polyplex micelles using CLSM. Cellular internalization and intracellular distribution of the nanogels against HeLa cells

were observed using CLSM. HeLa cells were seeded at a density of 1×10^4 cells/well into a 35-mm glass-bottom culture dish (NEST, China) and cultured overnight in 2 mL DMEM medium with 10% FBS at 37 °C with 5% CO_2 humidified atmosphere. The medium was replaced by 2 mL fresh medium. After preincubation at pH 7.4 or pH 6.5 for 2 h, 150 μL of the nanogel solutions (1 mg/mL) were added into each dish. After 4 h incubation, part of the cells were washed three times with ice-cold PBS. Cell nuclei and lysosome were counterstained with DAPI (Blue) and Lyso-Tracker (Green), respectively. Then, they were washed three times with ice-cold PBS and observed under CLSM. On the other hand, for the other part of the cells after 4 h cellular uptake, the medium was exchanged with 2 mL fresh DMEM followed by another 24 h incubation. The cells were then washed three times with ice-cold PBS. Cell nuclei and lysosome were counterstained with DAPI (Blue) and Lyso-Tracker (Green), respectively. They were washed three times with ice-cold PBS and observed under CLSM.

Cellular uptake measured by flow cytometry. The cellular uptake efficiencies of the nanogels after preincubation at varying pH values (pH 7.4 and pH 6.5) were determined by flow cytometry. In brief, HeLa cells were seeded into 6-well plates at a density of 1×10^5 cells/well in 2 mL DMEM medium with 10% FBS at 37°C with 5% CO_2 humidified atmosphere and incubate overnight for cell attachment onto the substrate. After preincubation at pH 7.4 or pH 6.5 for 2 h, 150 μL of the nanogel solutions or PHPMA-*co*-PPMA-*DOX*-*CD* polymer solution (1 mg/mL) were added into each well. After 4 h incubation, the cells were washed three times with PBS to remove extracellular nanogel solutions. After detachment by trypsin treatment from the culture plate, the cells were harvested and resuspended in PBS for flow cytometry measurement. Data were analyzed with Flowjo software.

References

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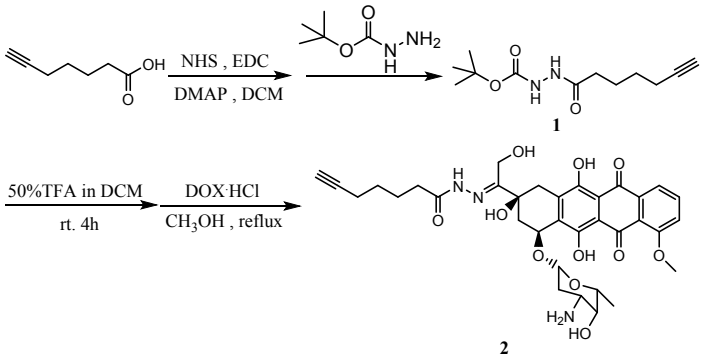
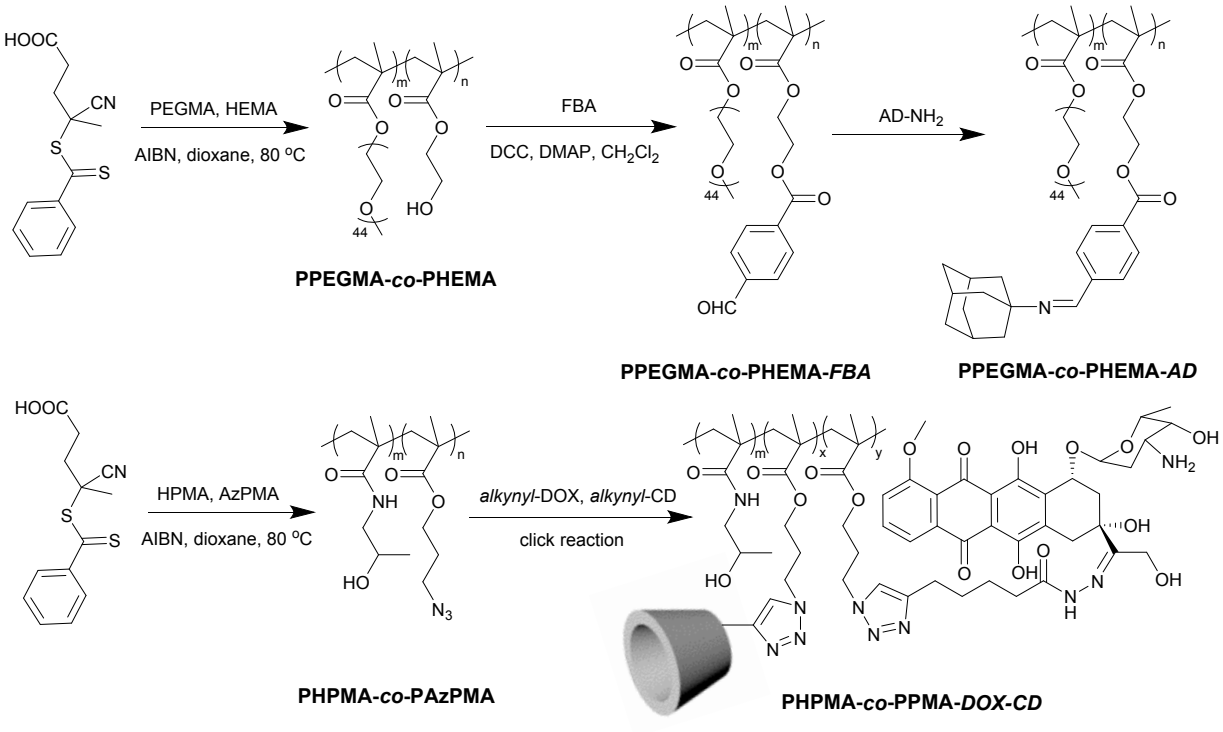


Scheme S1 Synthesis of *alkynyl*-DOX.



Scheme S2 Synthesis of AD-conjugated copolymer, PPEGMA-*co*-PHEMA-*AD* and *β*-CD and DOX-conjugated copolymer, PHPMA-*co*-PPMA-*DOX-CD*.

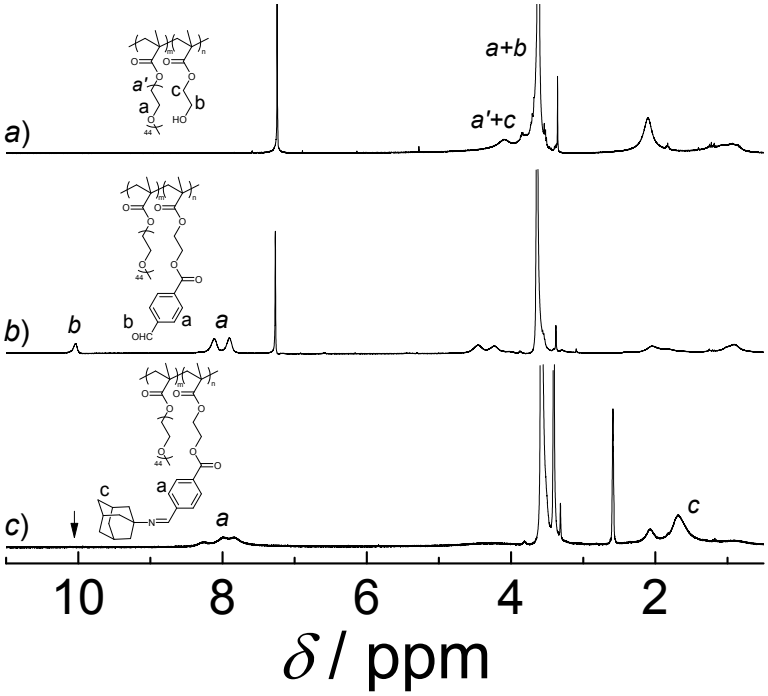


Fig. S1 ^{1}H NMR spectra of a) PPEGMA-*co*-PHEMA in $CDCl_3$, b) PPEGMA-*co*- PHEMA-*FBA* in $CDCl_3$, and c) PPEGMA-*co*-PHEMA-*AD* in DMSO-d_6.

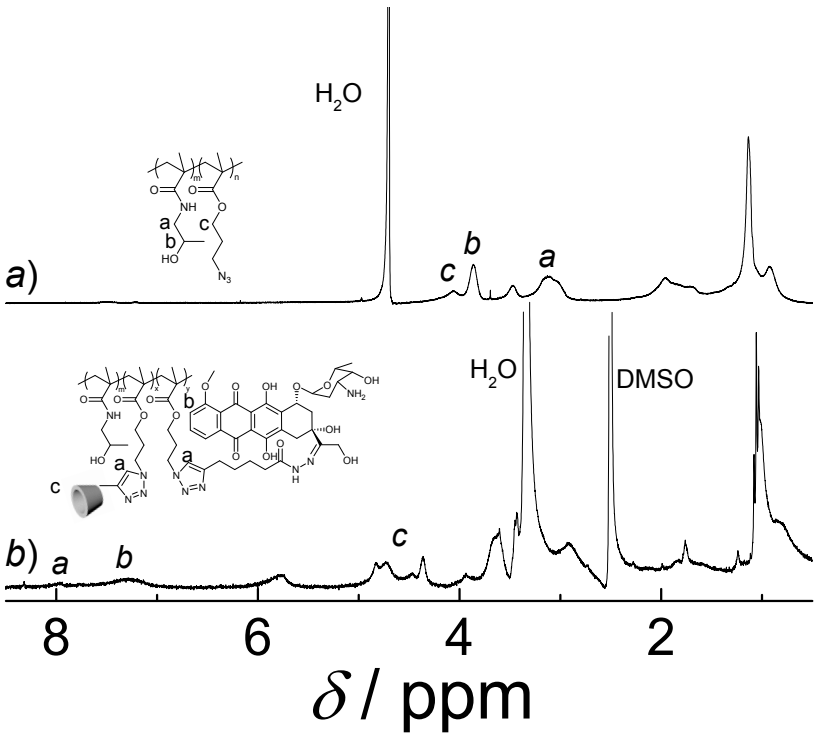


Fig. S2 ^{1}H NMR spectra of (a) PHPMA-*co*-PAzPMA in D_2O and (b) PHPMA-*co*-PPMA-*DOX*-*CD* in DMSO-d_6.

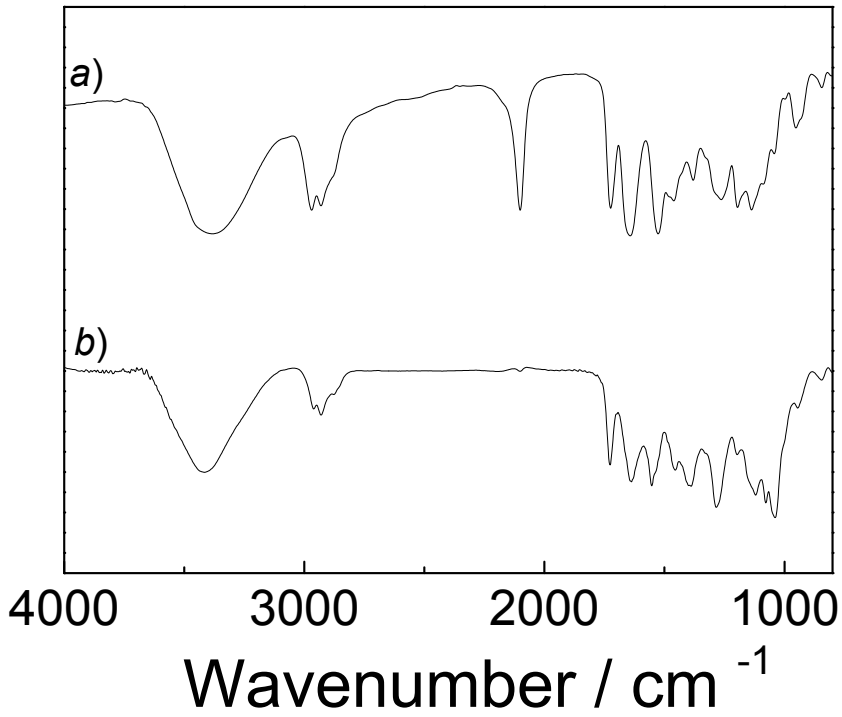


Fig. S3 FT-IR spectra of (a) PHPMA-*co*-PAzPMA and (b) PHPMA-*co*-PPMA-*DOX*-*CD*.

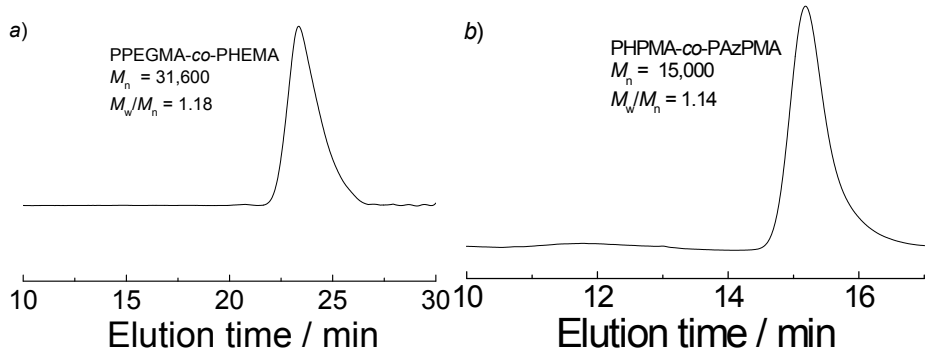


Fig. S4 a) THF GPC trace of PPEGMA-*co*-PHEMA (M_n = 31,600, M_w/M_n = 1.18), and b) DMF GPC trace of PHPMA-*co*-PAzPMA (M_n = 15,000, M_w/M_n = 1.14).

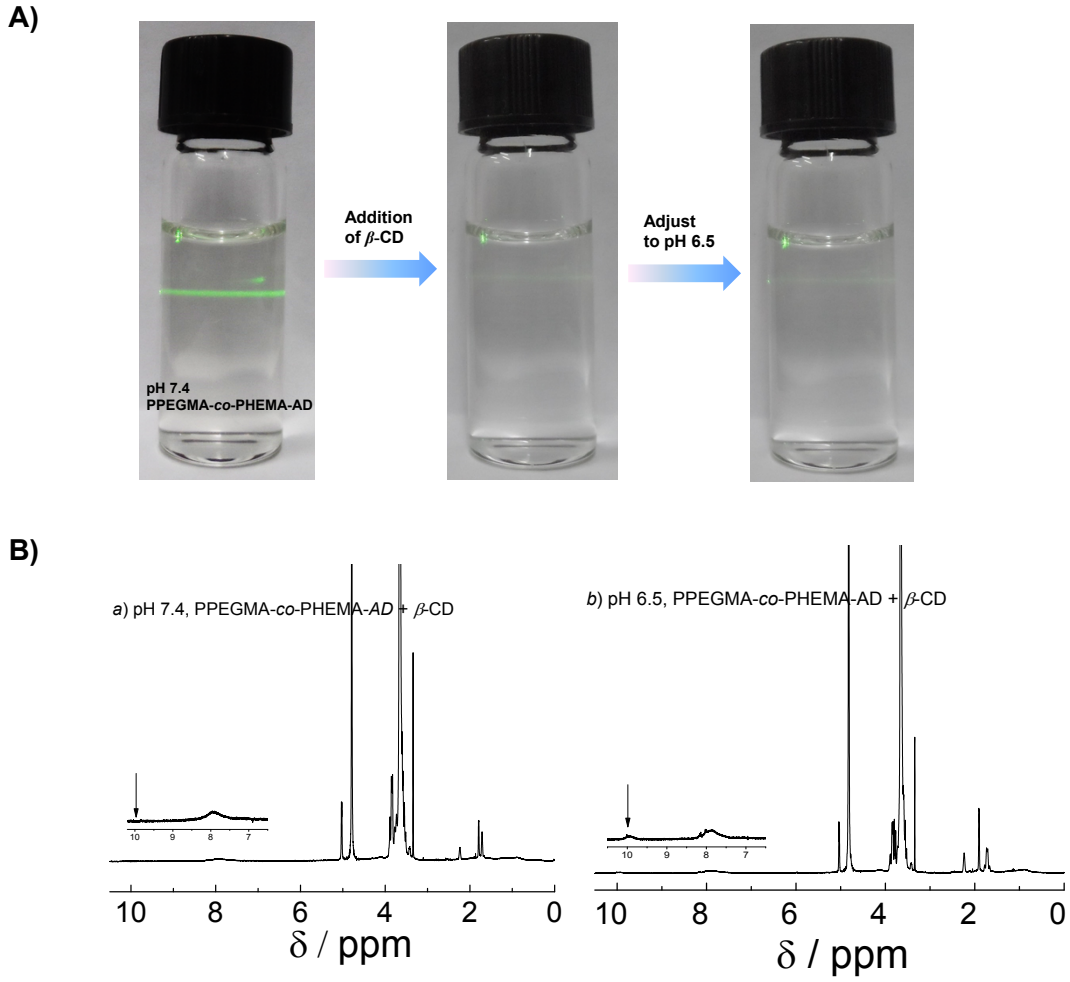


Fig. S5 A) Photographic images of laser light scattering of PEGMA-*co*-PHEMA-*AD* dispersed in aqueous solution at pH 7.4 followed by addition of *β*-CD and adjustment to pH 6.5. The concentration of the solution was fixed at 1 mg/mL. B) ^{1}H NMR spectra of PEGMA-*co*-PHEMA-*AD* in aqueous solution at a) pH 7.4 and b) pH 6.5 in the presence of *β*-CD.

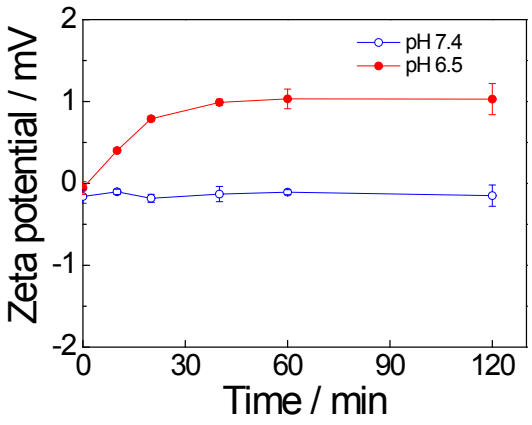


Fig. S6 Time-dependent zeta potential examination of PPEGMA-*co*-PHEMA-*AD*/PHPMA-*co*-PPMA-*DOX*-*CD* nanogels incubated at pH 7.4 and pH 6.5, respectively.

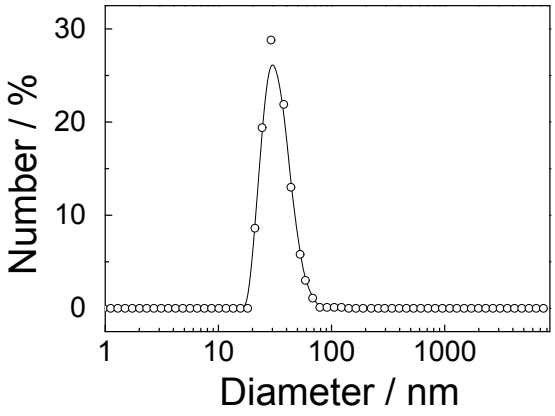


Fig. S7 Size distribution of the nanoparticles formed from PPEGMA-*co*-PHEMA-*AD*/PHPMA-*co*- PPMA-*DOX*-*CD* nanogels after incubation at pH 6.5 for 2 h in aqueous solution of 10% serum.

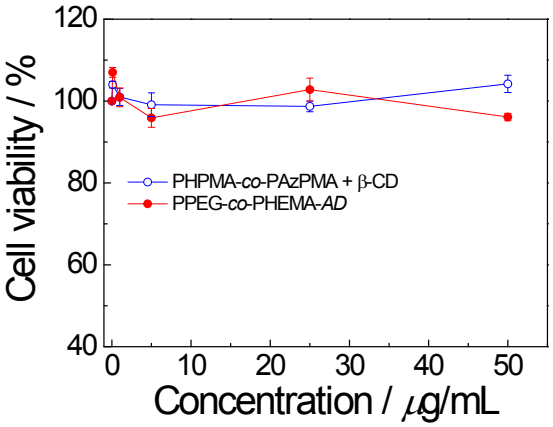


Fig. S8 Cytotoxicity of the polymers against HeLa cells (mean ± SEM, n = 4).

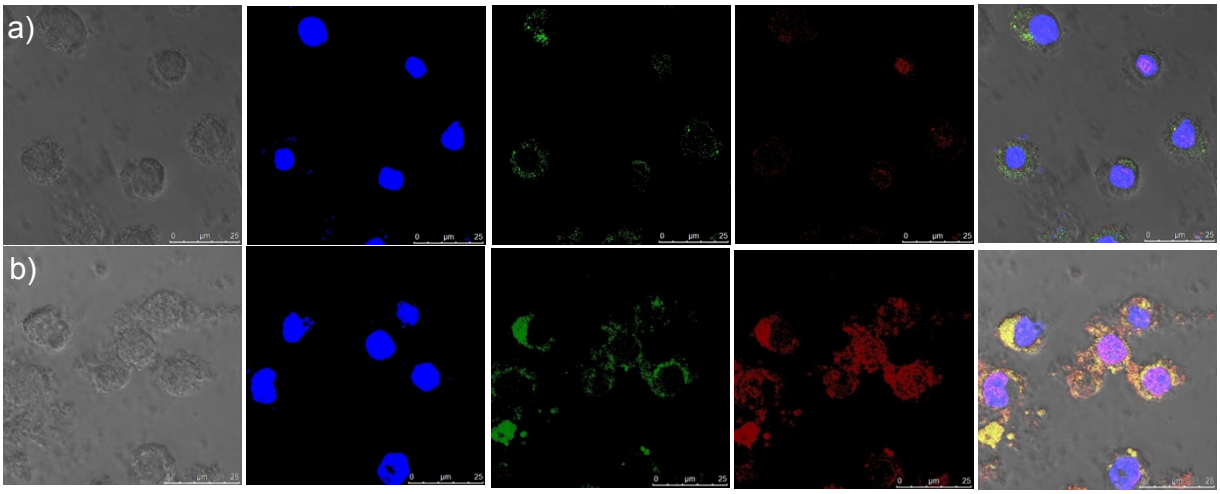


Fig. S9 CLSM observation of the intracellular distribution of DOX (red) from the nanogels with endo-/lysosomes (green) and nuclei (blue) in HeLa cells after preincubation in PBS at pH 7.4 (a) and pH 6.5 (b) for 2 h and incubation with cells for 4 h followed by replacing fresh medium and 24 h incubation.