Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information

Cucurbituril-based nanoparticles: a new efficient vehicle for targeted intracellular delivery of hydrophobic drugs

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General Methods.

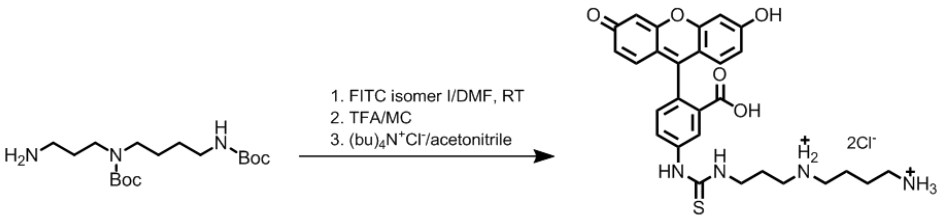
All the reagents and solvents were used as supplied without further purification. Photoreaction was performed using a RMR-600 (Rayonet) photochemical reactor equipped with four 254 nm lamps and four 300 nm lamps. NMR data were recorded on a DRX500 spectrometer (Bruker). Fluorescence measurements were performed with 10-mm quartz cells on a RF-5301PC spectrofluorometer (Shimadzu). UV-visible absorption was measured on a Hewlett-Packard 8453 diode array spectrophotometer. Dialysis was performed using a Spectra/Por® RC membrane (MWCO: 8,000). Dynamic light scattering experiments were performed on a ELS-8000 instrument (Otsuka Electronics). TEM images were recorded on a JEM-1011 (JEOL) electron microscope operating at 100 kV. Fluorescence images were observed on a LSM510 confocal scanning microscope (Carl Zeiss). Flow cytometry was performed with FACSCalibur (Becton Dickinson). UV absorption for MTT assay was measured on a Wallac Victor2 1420 multilable counter (Perkin Elmer). MALDI/TOF mass spectrometry was performed with a Reflex III mass spectrometer (Bruker). Human ovarian carcinoma HeLa cell line was obtained from American Type Culture Collection (ATCC). High-resolution mass (FAB) data were obtained with a JMS700 (JEOL) mass spectrometer at the Korea Basic Science Institute (Daegu).

Synthesis.

Synthesis of (3-(6-hydroxyhexanethio)propan-1-oxy)$_n$CB[6] (1). 6-Mercaptohexanol (150 mg, 1.1 mmol) was added to a solution of (allyloxy)$_{12}$CB[6] (30 mg, 1.8×10^{-2} mmol) in methanol (2 mL) in a quartz tube and degassed with N_2. After the mixture was irradiated with UV light (254 nm and 300 nm) for 3 d, the solvent was removed under a reduced pressure. The crude product was recrystallized from diethyl ether (10 mL). The solid was washed with diethyl ether and dried under a reduced pressure to give **1** (50 mg, 89%). The isolated product was a mixture of partially substituted **1** with a different degree of substitution. The MALDI-TOF mass spectrum of **1** revealed species with 9 - 12 substituents (3-(6-hydroxyhexanethio)propan-1-oxy) attached to a CB[6] core. The N/S ratio in elemental analysis suggested that the average degree of substitution is 11.4, which was consistent with the ^{1}H NMR integration. ^{1}H NMR (500 MHz, CD_3OD, 25 ^{o}C,): δ = 1.46 (br, 48H), 1.58 (br, 24H), 1.65 (br, 24H), 2.08 (br, 24H), 2.61 (br, 24H), 2.72 (br, 24H), 3.58 (br, 24H), 3.79 (br, 24H), 4.41 (br, 12H), 5.83 (br, 12H). ^{13}C NMR (125MHz, CD_3OD, 25 ^{o}C): δ = 25.7, 28.1, 28.9, 29.8, 32.1, 32.6, 61.9, 96.7, 153.1. MS (MALDI-TOF, m/z): 3330.4 $[M+Na]^+$, 3166.4 $[M-L+Na]^+$, 3032.8 $[M-2L+Na]^+$, 2898.3 $[M-3L+Na]^+$, ($M = C_{144}H_{252}N_{24}O_{36}S_{12}$, $L = C_6H_{13}OS$). Elemental Analysis data was calculated based on the average degree of

substitution (n = 11.4). Anal. Calcd. for $(C_{72}H_{84}N_{24}O_{24})(C_6H_{14}OS)_{11.4}(H_2O)_7$: C, 50.70; H, 7.81; N, 10.11; S, 10.99; found: C, 50.31; H, 7.41; N, 10.30; S, 11.32.

Synthesis of FITC conjugate spermidine (2).



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Scheme S1. Synthetic scheme of FITC conjugate spermidine **2**.

N^2,N^3-Di-*tert*-butoxycarbonylspermidine[1] (50 mg, 0.14 mmol) was added to a solution of fluorescein isothiocyanate (FITC) isomer I (60 mg, 0.15 mmol) in dimethylformamide (DMF) (5 mL). The mixture was stirred at room temperature for 8 h under a nitrogen atmosphere and evaporated under a reduced pressure to remove DMF. The dried mixture was filtered with chloroform to remove unreacted FITC isomer I and the filtrate was dried under a reduced pressure. To the dried product in methylene chloride (1 mL) was added to trifluoroacetic acid (1 mL) slowly. The reaction mixture was stirred at room temperature for 30 min and evaporated under a reduced pressure. After the residue was dissolved in acetonitrile (5 mL), tetrabutylammonium chloride (78 mg, 0.28 mmol) in acetonitrile (3 mL) was added to produce precipitate which was washed with acetonitrile and dried under a reduced pressure to give **2** (63.0 mg, 68%): ^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ = 1.80 (m, 4H), 2.09 (m, 2H), 3.07 (m, 2H), 3.14 (m, 4H), 3.78 (br, 2H), 6.81(m, 4H), 7.19 (m, 3H), 7.67 (d, J = 7.2 Hz, 1H), 8.06 (s, 1H). ^{13}C NMR (125MHz, D_2O, 25 ^{o}C): δ = 181.1, 170.7, 166.0, 156.3, 140.4, 131.7, 130.3, 128.8, 124.5, 117.4, 114.0, 103.1, 47.5, 45.4, 41.7, 39.3, 26.2, 24.5, 23.3. HRMS-FAB calcd. for $C_{28}H_{32}C_{l2}N_4O_5S$ $[M+H-2HCl]^+$: 535.2010; found: 535.2013.

Transmission electron microscopy (TEM) experiments. The samples for electron microscopy were prepared on a 400 mesh carbon-coated copper grid. A drop of nanoparticle solution (1.0×10^{-5} M) was located on the grid for 5 min, and then gently blotted with a filter paper. The specimen was stained with a drop of 5% (w/w) uranyl acetate solution, incubated for 5 min, and gently blotted with a filter paper.

Fluorescence emission spectroscopy. Fluorescence from the nanoparticle was measured by a fluorometer using 450 nm, 350 nm or 540 nm for FITC-spermidine conjugate **2**, folate-spermidine conjugate **3** or Nile Red, respectively.

Confocal laser scanning microscopy. A drop of nanoparticle solution on a slide glass was observed by a confocal laser scanning microscope using 488 nm or 543 nm for excitation of FITC-spermidine conjugate **2** or Nile Red, respectively.

Preparation of CB[6] nanoparticles (CB[6]NPs). A solution of **1** (3.3 mg, 1.0×10^{-3} mmol) was dissolved in ethanol (100 μL) in a 20 mL glass vial and the solvent was evaporated under a reduced pressure to give a thin film. Addition of distilled H_2O (10 mL) to the vial followed by sonication for 30 min resulted in the formation of nanoparticles (1.0×10^{-4} M). The successful formation of nanoparticles was confirmed by TEM and DLS experiments.

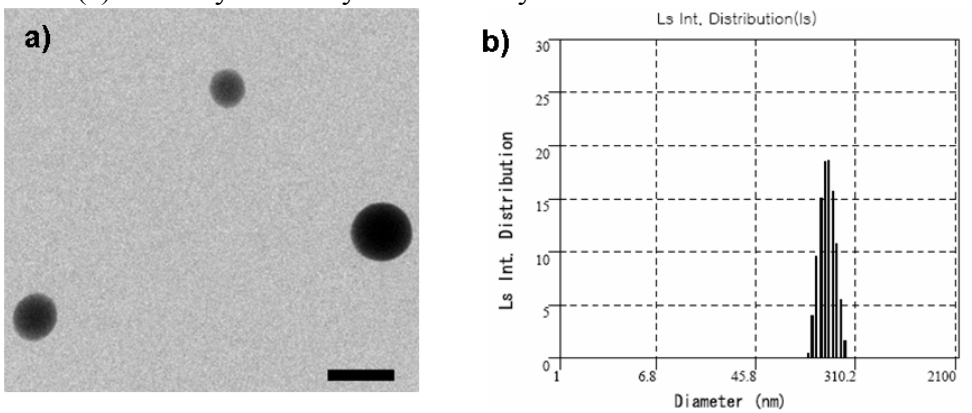

Fig. S1. a) TEM image of CB[6]NPs (scale bar = 300 nm) and b) size distribution of CB[6]NPs measured by DLS (polydispersity 0.157).

Preparation of Nile Red loaded CB[6]NPs (CB[6]NPs⊃NR). A solution of CB[6]NPs⊃NR was prepared with a solution of **1** (3.3 mg, 1.0×10^{-3} mmol) and Nile Red (10 μg, 0.03 μmol) in ethanol (100 μL) by using the same procedure as above. The successful entrapment of Nile Red in the CB[6]NPs was confirmed by fluorescence emission spectrometry and confocal laser scanning microscopy.

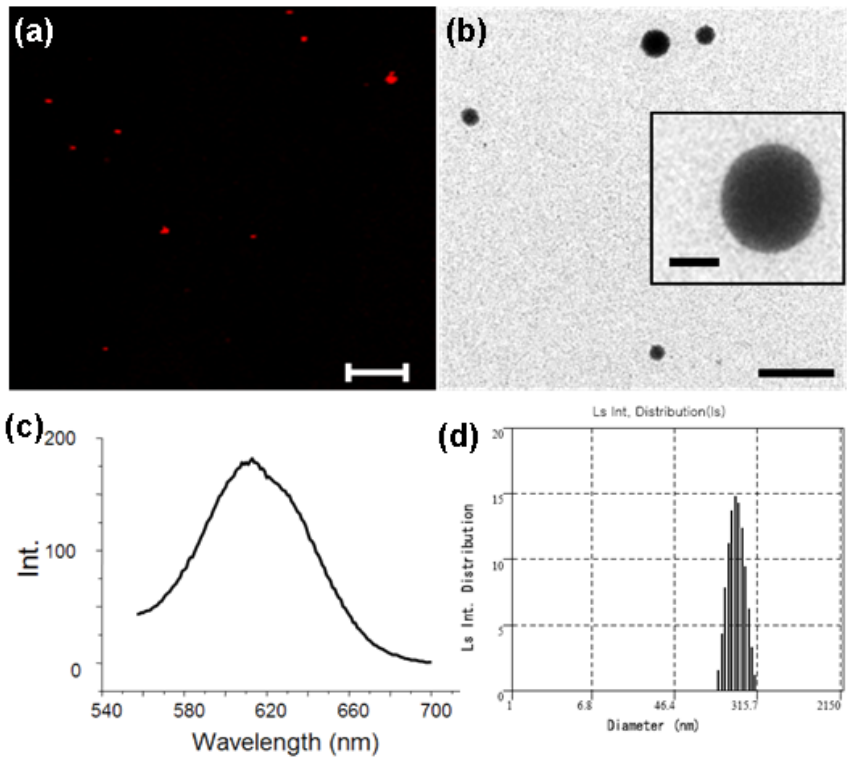

Fig. S2. (a) Confocal laser scanning microscopy (scale bar = 5 μm), (b) TEM image (scale bar = 500 nm, inset scale bar = 100 nm) (c) fluorescence emission measurement (ex. 540 nm) of CB[6]NPs ⊃ NR and (d) size distribution of CB[6]NPs ⊃ NR measured by DLS (polydispersity 0.148).

Preparation of 2 decorated CB[6]NPs⊃NR (2@CB[6]NPs⊃NR). 2 (0.60 mg, 1.0 equiv with respect to **1** forming CB[6]NPs) was added to the solution of CB[6]NPs⊃NR prepared by using the same procedure as above and the mixture was gently shaken for 1 h at room temperature to allow host-guest interactions between **2** and the accessible cavity of

CB[6]NPs. The resulting solution was dialyzed against water to give **2**@CB[6]NPs⊃NR. The successful decoration of **2** on the surface of CB[6]NPs and Nile Red loading in the CB[6]NPs were confirmed by fluorescence emission spectroscopy and confocal laser scanning microscopy.

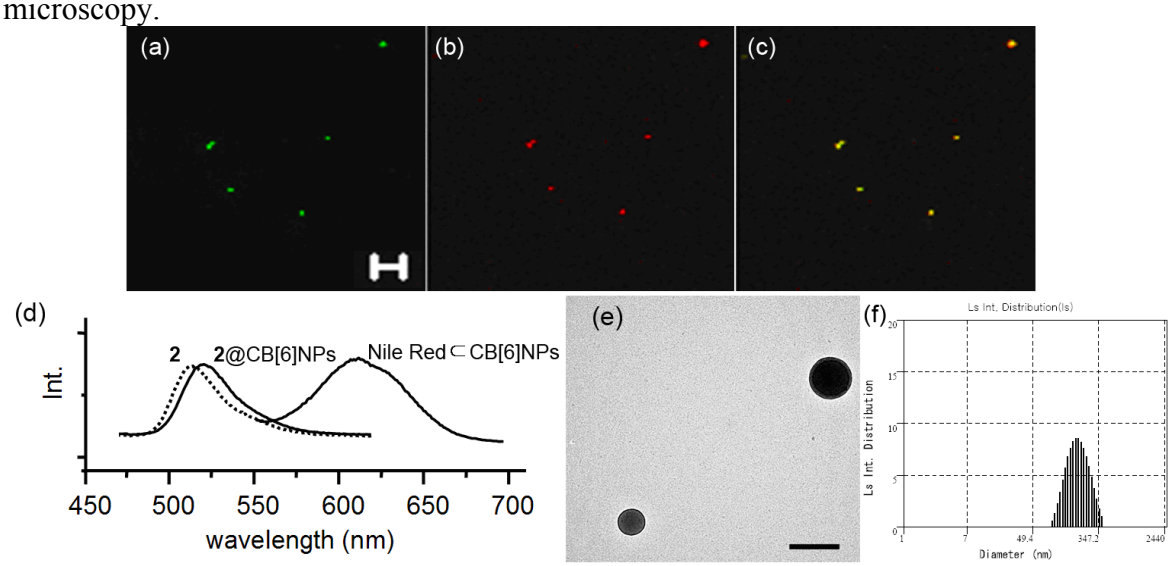

Fig. S3. Confocal laser scanning microscopy images of **2**@CB[6]NPs⊃NR (a) FITC channel (scale bar = 5 μm), (b) Nile Red channel, (c) overlay of (a) and (b), (d) fluorescence measurement of free **2** (dashed line) and **2**@CB[6]NPs⊃NR (solid line) (e) TEM image of **2**@CB[6]NPs⊃NR (scale bar = 200 nm) and (f) size distribution of **2**@CB[6]NPs⊃NR measured by DLS (polydispersity 0.214).

Preparation of 2 and 3 decorated CB[6]NPs⊃NR ((2+3)@CB[6]NPs⊃NR). **2** and **3** (0.30 mg, 0.5×10^{-3} mmol and 0.32 mg, 0.5×10^{-3} mmol, respectively, 1 equiv with respect to **1** (3.3 mg, 1×10^{-3} mmol) forming CB[6]NPs) were added to a solution of CB[6]NPs⊃NR prepared by using the same procedure as above and the solution was dialyzed against water to remove unbound **2** and **3**. The successful surface decoration of **2** and **3** on CB[6]NPs⊃NR was confirmed by fluorescence emission spectroscopy and confocal laser scanning microscopy.

Preparation of 3 decorated CB[6]NPs⊃NR (3@CB[6]NPs⊃NR). **3**@CB[6]NPs⊃NR were prepared with **3** (0.64 mg, 1.0×10^{-3} mmol, 1 equiv with respect to **1** forming CB[6]NPs) by using the same procedure as above. The successful decoration of **3** on the surface of CB[6]NPs⊃NR was confirmed by fluorescence emission spectroscopy.

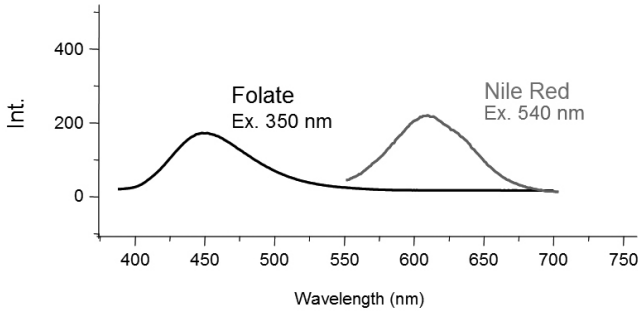

Fig. S4. Fluorescence emission spectrometry of **3**@CB[6]NPs⊃NR.

Measurement of the accessible CB[6] cavities on the surface of CB[6]NPs using 2 as a fluorescence probe. Following the procedure previously reported in the literature[2], the

amount of accessible CB[6] cavities on the surface of CB[6]NPs was measured using FITC-spermidine conjugate **2** as a fluorescence probe (0.37 mg, 0.61 μmol; 1.0 equiv with respect to **1** (2.00 mg) forming CB[6]NPs in water (6.25 mL)). The experiment was performed 3 times to obtain mean ± standard deviation (S.D). The amount of unbound **2** was measured to be 0.45 ± 0.02 μmol (approximately 0.74 equiv). It indicates that approximately 26 ± 4% of the host molecules **1** constituting CB[6]NP is accessible by the FITC-spermidine conjugate fluorescent probe **2**.

Targeted intracellular delivery of hydrophobic molecules measured by confocal laser scanning microscopy. HeLa cells were seeded on a poly-*L*-lysine coated cover glass in a 24-well plate at a density of 5×10^4 cells per well in 1 mL of folate deficient RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) and incubated in a humidified 5% CO_2 atmosphere at 37 ^oC for 24 h. The culture medium was replaced with 1 mL of a fresh one including 100 μL of water, CB[6]NPs⊃NR, **3**@CB[6]NPs⊃NR and **3**@CB[6]NPs⊃NR with a 1000-fold excess folic acid (1.3 mg, 0.2 μmol). The final concentration of CB[6]NPs in the medium was 1.0×10^{-5} M. After 1 h incubation at 37 ^oC, the cells were washed with phosphate buffer saline (PBS) and fixed with 1% (w/v) para-formaldehyde solution. The cells on the cover glass were examined by a confocal laser scanning microscope using a 543 nm excitation wavelength. To understand the mechanism of the intracellular delivery, the same experiments were performed with **3**@CB[6]NPs⊃NR at 4 ^oC, instead of 37 ^oC.

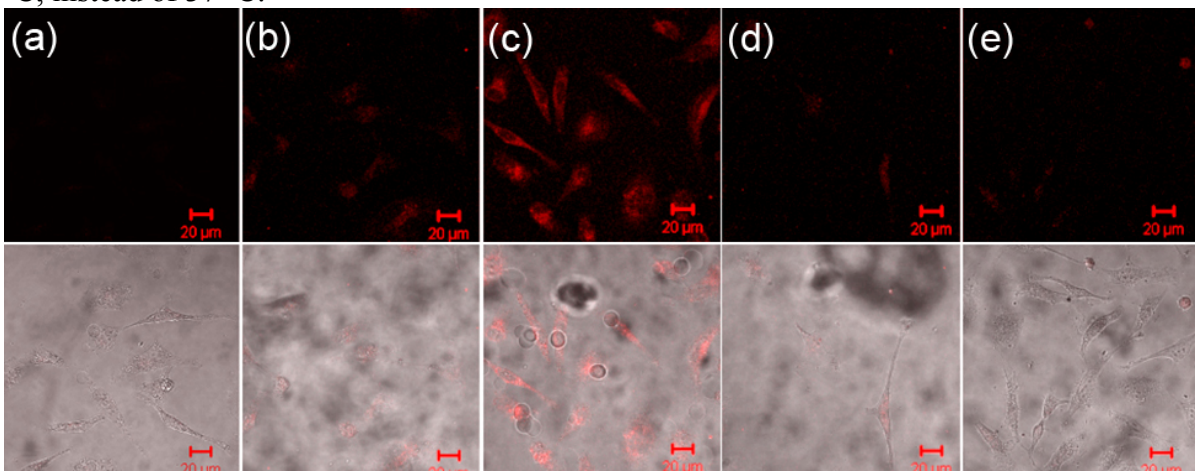

Fig. S5. Confocal laser scanning microscopy images of (a) HeLa cells after incubation for 1 h at 37 ^oC with (b) CB[6]NPs⊃NR, (c) **3**@CB[6]NPs⊃NR, (d) **3**@CB[6]NPs⊃NR with a 1000-fold excess folic acid and (e) **3**@CB[6]NPs⊃NR after incubation for 1 h at 4 ^oC.

Targeted intracellular delivery of hydrophobic molecules measured by flow cytometry. HeLa cells were seeded on a 6-well plate at a density of 2.5×10^5 cells per well in 2 mL of folate deficient RPMI 1640 medium containing 10% FBS and 1% PS and incubated in a humidified 5% CO_2 atmosphere at 37 ^oC for 24 h. The culture medium was replaced with 2 mL of folate deficient RPMI 1640 medium including 200 μL of water, CB[6]NPs⊃NR, **3**@CB[6]NPs⊃NR or **3**@CB[6]NPs⊃NR with 1000-fold excess amount of folic acid (2.5 mg, 0.4 μmol), respectively. The final concentrations of CB[6]NPs in the media were 1.0×10^{-5} M. After 1 h incubation at 37 ^oC. The cells were washed with PBS and fixed with 1% (w/v) para-formaldehyde solution. The extents of intracellular uptake of CB[6]NPs⊃NR by the cells were measured by flow cytometry. To understand the mechanism of the intracellular delivery, the same experiment was performed with **3**@CB[6]NPs⊃NR at 4 ^oC, instead of 37 ^oC.

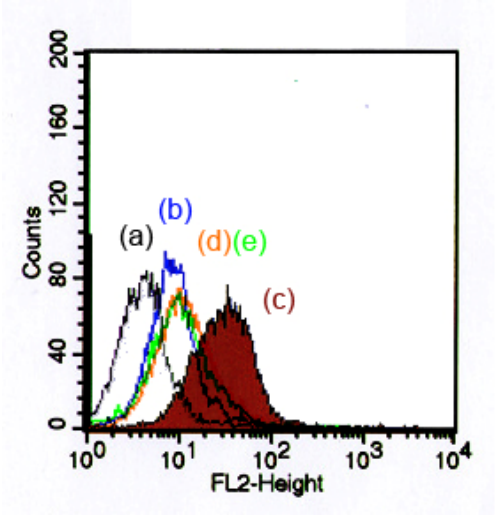

Fig. S6. Flow cytometry histograms of Nile Red fluorescence from (a) HeLa cells after incubation at 37 °C with (b) CB[6]NPs⊃NR, (c) **3**@CB[6]NPs⊃NR, (d) **3**@CB[6]NPs⊃NR with 1000-fold excess amount of folic acid and (e) **3**@CB[6]NPs⊃NR after incubation at 4 °C.

Location of CB[6]NPs in HeLa cells after endocytosis. HeLa cells were seeded on a poly-L-lysine coated cover glass in a 24-well plate at a density of 5×10^4 cells per well in 1 mL of folate deficient RPMI 1640 medium containing 10% FBS and 1% PS and incubated in a humidified 5% CO_2 atmosphere at 37 °C for 24 h. The culture medium was replaced with 1 mL of a fresh one including a 100 μL of (**2**+**3**)@CB[6]NPs (1.0×10^{-4} M) for 1 h at 37 °C, the cell culture medium was replaced with a fresh one containing 50 nM of LysoTracker Red and then further incubated for 30 min at 37 °C. The HeLa cells on the cover glass were examined by confocal laser scanning microscope using 488 nm and 543 nm for FITC-spermidine and LysoTracker Red, respectively.

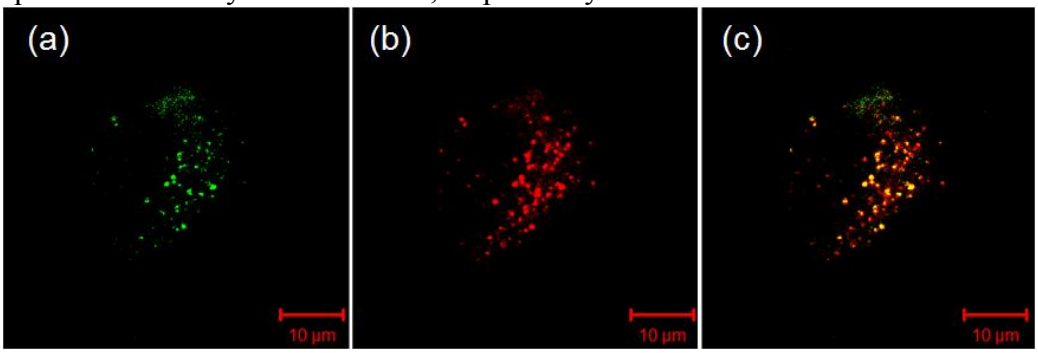

Fig. S7. Confocal laser scanning microscopy images of HeLa cells incubated with (**2**+**3**)@CB[6]NPs for 1 h and then with LysoTracker Red for 30 min at 37 °C: (a) FITC channel, (b) LysoTracker Red channel and (c) overlay of (a) and (b).

Release of hydrophobic molecules into cytoplasm after endocytosis. To understand the behavior of Nile Red loaded in CB[6]NPs after endocytosis, a 100 μL of (**2**+**3**)@CB[6]NPs ⊃NR (1.0×10^{-4} M) was treated to the HeLa cells cultured under the same condition as above and the cells were incubated for 1 h at 37 °C. The cells on the cover glass were examined by confocal laser scanning microscope using 488 nm and 543 nm for FITC-spermidine **2** and Nile Red, respectively.

Measurement of PTX loading capacity and efficiency of CB[6]NPs. CB[6]NPs⊃PTX were prepared with 1 (2.00 mg) and PTX (0.10 mg, 0.20 mg or 0.40 mg) in 5.00 mL of water by using the same procedure as CB[6]NPs⊃NR. The samples were syringe-filtered through a PTFE syringe filter having 1 μm pore diameter to remove insoluble PTX and dialyzed against water. The filtrate was lyophilized and PTX in CB[6]NPs was extracted with chloroform. After removal of chloroform under the reduced pressure, the sample was dissolved in methanol to measure the amount of PTX by UV-visible spectroscopy. The experiment was performed 3 times to obtain mean ± standard deviation (S.D.).

Loading capacity (%) = (weight of PTX loaded / weight of CB[6]NPs⊃PTX) × 100

Loading efficiency (%) = (weight of PTX loaded / weight of initially added PTX) × 100

Table S1. PTX loading efficiency and capacity of CB[6]NPs (*n* = 3).

CB[6] derivative **1** (mg)	Amount of PTX initially added (mg)	Amount of PTX loaded (mg)	PTX loading capacity (%, w/w)	PTX loading efficiency (%, w/w)
2.00	0.10	0.10 ± 0.01	4.5 ± 0.2	95 ± 5
2.00	0.20	0.19 ± 0.01	8.6 ± 0.6	94 ± 6
2.00	0.40	0.17 ± 0.02	7.8 ± 0.9	43 ± 5

Cytotoxicity of CB[6]NPs to HeLa cells. HeLa cells were seeded in a 96-well plate at a density of 5×10^3 cells per well in 200 μL of folate deficient RPMI 1640 medium containing 10% FBS and 1% PS and incubated in a humidified 5% CO_2 atmosphere at 37 °C for 24 h. The cell culture medium was replace with a fresh one containing various concentration (0.04 - 40 μg mL^{-1}) of CB[6]NPs. The cells were incubated for 3 d at 37 °C. Subsequently, the cells were incubated in a fresh medium containing methylthiazolyldiphenyl-tetrazolium bromide (MTT) (0.5 mg mL^{-1}) for an additional 4 h at 37 °C. After the incubation with the MTT reagent, the medium was gently removed. The purple, water insoluble crystals formed by live cells remaining in the bottom of the wells were dissolved with 200 μL of dimethyl sulfoxide and the solution was gently shaken for 10 min. UV absorption of the solution at 590 nm was measured by a multi-well plate reader. The experiment was performed 3 times to obtain mean ± S.D.

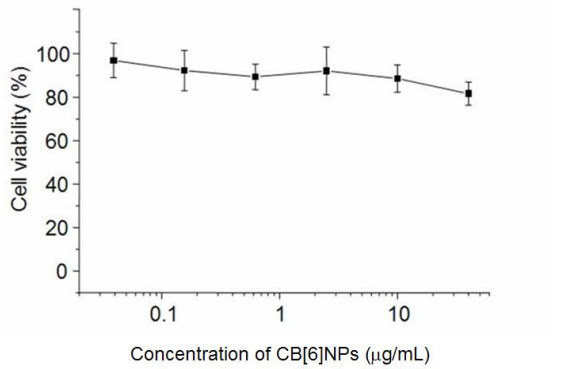

Fig. S8. Cytotoxicity of CB[6]NPs measured by MTT assay (*n* = 3).

In vitro cytotoxicity against HeLa cells to obtain IC_{50} values. HeLa cells were seeded in a 96-well plate at a density of 5×10^3 cells per well in 200 μL of folate deficient RPMI 1640 medium containing 10% FBS and 1% PS and incubated in a humidified 5% CO_2 atmosphere at 37 °C for 24 h. The cell culture medium was replaced with a fresh one containing various concentrations of PTX (0.01 - 4 μg mL^{-1}) in CB[6]NPs⊃PTX and **3**@CB[6]NPs⊃PTX. The same experiment was performed with free PTX (0.01 - 4 μg mL^{-1}) as a control. The cells were incubated for 1 h at 37 °C and the medium was again replaced with a fresh one and further incubated for 3 d at 37 °C. Subsequently, the cells were incubated

in a fresh medium containing methylthiazolyldiphenyl-tetrazolium bromide (MTT) (0.5 mg mL^{-1}) for an additional 4 h at 37 ^{o}C, and then the medium was gently removed. The purple, water insoluble crystals formed by live cells remaining in the bottom of the wells were dissolved with 200 μL of dimethyl sulfoxide and the solution was gently shaken for 10 min. UV absorption of the solution at 590 nm was measured by a multi-well plate reader. The experiment was performed 3 times to obtain S.D.

Table S2. IC_{50} values of PTX only, CB[6]NPs⊃PTX and **3**@CB[6]NPs⊃PTX toward HeLa cells measured by MTT assay (n = 3).

	PTX	CB[6]NPs⊃PTX	**3**@CB[6]NPs⊃PTX
IC_{50} (μg mL^{-1})	1.24 ± 0.20	0.33 ± 0.10	0.08 ± 0.02

References

1. M. Humora and J. Quick, *J. Org. Chem.*1979, **44**, 143.
2. D. Kim, E. Kim, J. Kim, K. M. Park, K. Baek, M. Jung, Y. H. Ko, W. Sung, H. S. Kim, J. H. Suh, C. G. Park, O. S. Na, D.-k. Lee, K. E. Lee, S. S. Han and K. Kim, *Angew. Chem., Int. Ed.* 2007, **46**, 3471.