Supplementary Information

Dual-Signal Chemical Exchange Saturation Transfer (Dusi-CEST): An Efficient Strategy for Visualizing Drug Delivery Monitoring in Living Cells

Chenlu Yuan[‡1], Qianni Guo[‡* 1,2,3], Qingbin Zeng [1,2], Yaping Yuan [1], Weiping Jiang[1,2], Yuqi Yang[1,2], Louis-S. Bouchard[4], Chaohui Ye [1,2,3], Xin Zhou [*1,2,3,5]

[1]*Key Laboratory of Magnetic Resonance in Biological Systems, State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, National Center for Magnetic Resonance in Wuhan, Wuhan Institute of Physics and Mathematics, Innovation Academy for Precision Measurement Science and Technology, Chinese Academy of Sciences–Wuhan National Laboratory for Optoelectronics, Huazhong University of Science and Technology, Wuhan, 430071, China. E-mail: qianniguo@wipm.ac.cn, xinzhou@wipm.ac.cn.*

[2]*University of Chinese Academy of Sciences, Beijing 100049, P.R. China.*

[3]*Optics Valley Laboratory, Hubei 430074, P.R. China.*

[4]*Departments of Chemistry and Biochemistry and of Bioengineering, California NanoSystems Institute, Jonsson Comprehensive Cancer Center, The Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA.*

[5]*Hainan University, Haikou, Hainan 570228, China.*

‡These authors contributed equally.

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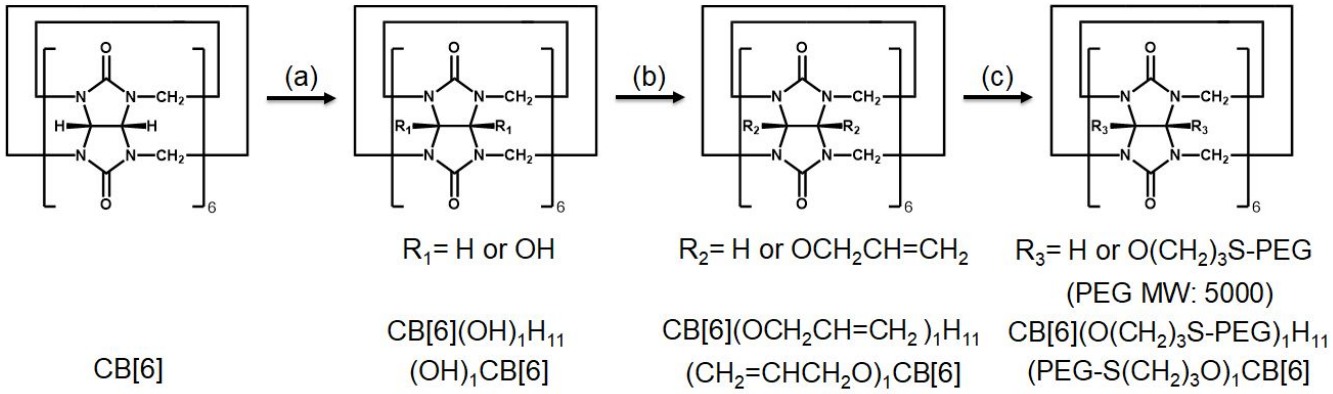
Experimental Procedures

1. Materials and Instruments

Cucurbit[6]uril, NaH (purity 60%, dispersed in mineral oil), acetic acid, formic acid, hydrogen peroxide solution, concentrated hydrochloric acid, methanol, paclitaxel, Nile Red, N-(tert-Butoxycarbonyl)-1,5-diaminopentane, fluorescein isothiocyanate (FITC), dimethylformamide, chloroform, ethyl acetate are purchased from Sinopharm, allyl bromide purchased from Sarn Chemical Technology (Shanghai) Co., Ltd., for analytical pure grade. HS-PEG (MW: 5000) purchased from Shanghai Qiansheng Biotechnology Co., Ltd., the name is methoxypolyethylene glycol sulfhydryl group, for chemical pure grade. Paclitaxel and Nile Red are formulated into 1 mmol/L ethanol solution. Other chemicals were used without further purification unless otherwise mentioned.

2. Synthesis of monofunctionalized CB[6].

Synthesis schematic



Scheme S1. Synthesis route of (PEG-S$(CH_2)_3O)_1$CB[6]. Reagents and conditions: (a) H_2O_2, UV light, 2 h; (b) NaH, DMSO, 4 h; allyl bromide, DMSO, 12 h; (c) PEG, MeOH, UV light, 3 d.

2.1. Synthesis of $(HO)_1$CB[6]. Reference to previous literature [1]. Cucurbit[6]uril (1 g, 1 mmol) was introduced in a 50 mL Round-bottomed flask in a 50 mL solution (1/1 vol. %) of HPLC grade water and 10 M HCl aqueous solution under nitrogen. Sonicate, and add hydrogen peroxide solution (50 μL, 0.5 mmol) after cucurbituril is completely dissolved. The solution was vigorously stirred and subjected to UV light at a wavelength of 254 nm for 2 h. The solvent was then evaporated under reduced pressure affording a white solid. The crude product was purified by column chromatography on silica gel eluting with the following mixture water/acetic acid/formic acid (10/10/1.0 respectively) and affording a white solid (CB[6]-$(OH)_1$, 0.316 g, 31% yield).
^{1}H NMR (500 MHz, D_2O) δ 5.72 (d, J=15.1 Hz, 2H), 5.66 (d, J=15.6 Hz, 8H), 5.53 (m, 12H), 5.28 (s, 1H), 4.53 (d, J=15.6 Hz, 2H), 4.27 (d, J=15.6 Hz, 10H). ^{13}C NMR (125 MHz, D_2O) δ 156.60, 154.99, 93.40, 77.14, 70.39, 51.44, 46.10. HRMS (ESI) m/z: [M+1,7-Diaminoheptane +2H]$^{2+}$, calcd. for $C_{43}H_{54}N_{26}O_{13}$, 572.22601; found, 572.22429.

2.2 Synthesis of $(allyloxy)_1$CB[6]. Reference to previous literature [2]. To a solution of $(HO)_1$CB [6] (0.30 g, 0.30 mmol) in anhydrous DMSO (200 mL) was added NaH (14.2 mg, 0.59 mmol) under nitrogen, and the mixture was stirred at room temperature for 4 h. Allyl bromide (71.7 mg, 0.59 mmol) was added to the reaction mixture at 0°C and stirred at room temperature for 12 h. Addition of ether (200 mL) to the reaction mixture produced a fluffy solid material, which was carefully washed with methanol (20 mL) and subsequently dried in vacuum. Further purification of the product with neutral alumina column yielded $(allyloxy)_1$CB [6] (56.8 mg, 18%).
^{1}H NMR (500 MHz, D_2O) δ 5.98-5.85 (m, 1H), 5.76-5.61 (m, 10H), 5.55-5.39 (m, 13H), 5.35-5.21 (dd, 2H), 4.41 (d, J=15.8 Hz, 2H,), 4.29-4.16 (m, 10H), 3.95 (d, J=5.7 Hz, 2H,); ^{13}C NMR (125 MHz, D_2O) δ 155.02, 131.91, 118.68, 72.38, 70.19, 64.54, 51.26, 46.37; HRMS (ESI) m/z: [M+Na]$^+$, calcd. for $C_{39}H_{40}N_{24}O_{13}Na^+$, 1075.3104; found, 1075.3099.

2.3 Synthesis of (PEG-S$(CH_2)_3O)_1$CB[6]. Reference to previous literature [3]. SH-PEG-OH (MW:5000, 285.1 mg, 57.0 μmol) was added to a solution of $(allyloxy)_1$CB[6] (30 mg, 28.5 μmol) 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 (PEG-S$(CH_2)_3O)_1$CB[6] (98.6 mg, 57.2%).

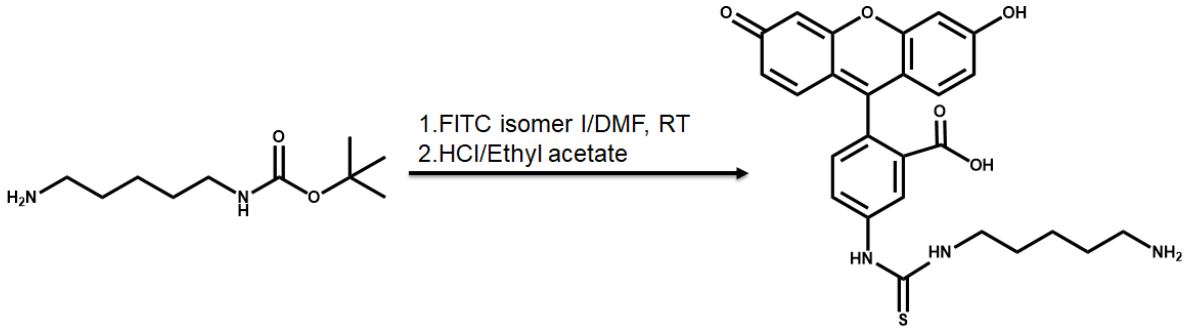
2.4 Preparation of CB[6] nanoparticles (CB[6]NPs). A solution of (PEG-S$(CH_2)_3O)_1$CB[6] (4 mg, 0.6 μmol) was dissolved in ethanol (100 μL) in a 5 mL glass vial and the solvent was evaporated under a reduced pressure to give a thin film. Addition of distilled H_2O (2 mL) to the vial followed by sonication for 30 min resulted in the formation of nanoparticles (2 mg/mL).

2.5 Preparation of paclitaxel loaded CB[6]NPs (CB[6]NPs⊃ PTX). A solution of (PEG-S$(CH_2)_3O)_1$CB[6] (4 mg, 0.6 μmol) and paclitaxel (80 μL, 1 mmol/L in ethanol) [3] was dissolved in ethanol (100 μL) in a 5 mL glass vial and the solvent was evaporated

under a reduced pressure to give a thin film. Addition of distilled H_2O (2 mL) to the vial followed by sonication for 30 min resulted in the formation of CB[6]NPs⊃ PTX (2 mg/mL).

2.6 Preparation of Nile Red loaded CB[6]NPs (CB[6]NPs⊃ NR). A solution of $(PEG\text{-}S(CH_2)_3O)_1CB[6]$ (4 mg, 0.6 μmol) and Nile Red (160 μL, 1 mmol/L in ethanol) was dissolved in ethanol (100 μL) in a 5 mL glass vial and the solvent was evaporated under a reduced pressure to give a thin film. Addition of distilled H_2O (2 mL) to the vial followed by sonication for 30 min resulted in the formation of CB[6]NPs⊃ NR (2 mg/mL).

3. Modification of FITC



Scheme S2. Synthesis route of FITC-(1, 5-Pentanediamine).

3.1 Synthesis of FITC-(1, 5-Pentanediamine). N-(tert-Butoxycarbonyl)-1, 5-diaminopentane (50 mg, 0.247 mmol) was added to a solution of fluorescein isothiocyanate (FITC) isomer I (104 mg, 0.260 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. A saturated solution of hydrogen chloride in ethyl acetate was added to the dried solid. The reaction mixture was stirred at room temperature for 30 min and evaporated under reduced pressure to give FITC-(1,5-Pentanediamine) (87.3 mg, 72%).
^{1}H NMR (500 MHz, MeOD) δ 8.58 (s, 1H), 8.09 (d, *J* = 6.8 Hz, 1H), 7.56 (d, *J* = 8.8 Hz, 2H), 7.40 (d, *J* = 7.8 Hz, 1H), 7.32 (s, 2H), 7.17 (d, *J* = 8.4 Hz, 2H), 3.70 (s, 2H), 3.37 (s, 3H), 3.01 (t, *J* = 7.3 Hz, 2H), 1.84–1.74 (m, 4H), 1.57 (d, *J* = 6.7 Hz, 2H), 1.30 (s, 1H). HRMS (ESI) m/z: $[M\text{-}H]^-$, calcd. for $C_{26}H_{24}N_3O_5S^-$, 490.14422; found, 490.14424.

3.1 Preparation of FITC decorated CB[6]NPs⊃ NR (FITC@CB[6]NPs⊃ NR). FITC-(1,5-Pentanediamine) (0.30 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 FITC-(1,5-Pentanediamine) and the accessible cavity of CB[6]NPs. The resulting solution was dialyzed against water to give FITC@CB[6]NPs⊃ NR.

4. Electron Microscopy Imaging
Transmission Electron Microscopy (TEM) imaging of CB[6] nanoparticles
CB[6] nanoparticles samples were dispersed in H_2O and sonicated for 30 s. The sample dispersion was then dropped on to the ultrathin carbon film. The TEM was performed on a Cs-corrected JEMARM200F with accelerating voltage of 200 kV. The results were shown in Fig. S2.

5. Fluorescence confocal microscopy
The Nile Red (540 nm excitation and 580-610 nm emission) and FITC (488 nm excitation and 500-550 nm emission) fluorescence signals were observed by confocal laser scanning microscope (A1R/A1, Nikon, Japan). CB[6]NPs⊃ NR in water was shown in Figs. S9, FITC@CB[6]NPs⊃ NR was shown in Figs. S11.

6. Hyperpolarized ^{129}Xe NMR
Hyperpolarized ^{129}Xe gas was generated using a home-built ^{129}Xe hyperpolarizer (hyperpolarized ^{129}Xe nuclear spin polarization was 100,000 times greater than its thermal equilibrium polarization). All ^{129}Xe NMR experiments used a 9.4 T Bruker AV400 wide bore NMR spectrometer (Bruker Biospin, Ettlingen, Germany). A gas mixture of 10% N_2, 88% He, and 2% Xe (26.4% ^{129}Xe natural abundance) (Spectra Gases) was used. Using the pressure from the polarizer (3.5 bar), the gas flow is 0.10 SLPM, and the gas mixture was directly bubbled into the sample solution by using a spectrometer triggered bubble dispenser. The results were shown in Figs. S3.

6.1 Hyperpolarized ^{129}Xe NMR of CB[6] Nanoparticles in Water. Two milliliters of prepared CB[6] NP aqueous solution was added to 0.1% of defoamer (L-81) and transferred to a 10 mm NMR tube. During the test, after the hyperpolarized Xe gas (gas composition: 2% natural abundance Xe, 10% N_2, 88% He) was directly bubbled into the sample for 20 s, a 3 s delay to allow the bubbles to collapse. And then, a continuous-wave (cw) saturation pulse (13 μT, 10 s) was swept across the chemical shift range of 30-250 ppm (according to experience and literature reports, the saturation point is not completely uniform), and then the spectrum was acquired in a single scan. All the spectra were processed by 5 Hz Lorentz broadening. The sample temperature was kept constant at 298 K. The results were shown in Fig. 2A, S4.

6.2 Hyperpolarized ^{129}Xe NMR of CB[6] Nanoparticles with PTX in Cell. A549 lung cancer cells were inoculated into a culture flask, and 4 mL of 4 mg/mL CB[6]NP⊃ PTX aqueous solution was added and incubated at 37°C for 3 h. The cells were moved to a 2

mL centrifuge tube and centrifuged at 3000 rpm for 3 min, followed by removing the medium and nanoparticle mixture. The supernatant was removed, and 2 mL distilled water was added to the centrifuge tube. Then, the cells were carefully pipetted to disperse evenly. Cells were transferred to a 10 mm NMR tube for the Hyper-CEST experiment. The saturation irradiation range was 30 ppm-250 ppm, and the saturation pulse was a continuous-wave (cw) pulse with 13 μT strength and 10 s length. The sample temperature was kept at 298 K. The results were shown in Fig. 2B, S7.

6.3 Hyperpolarized ^{129}Xe NMR of cell incubation solution. Two milliliters of the cell incubation solution were added to 0.1% of defoamer (L-81) and transferred to a 10 mm NMR tube for experiment. The other procedures were same to the hyperpolarized ^{129}Xe NMR of CB[6] nanoparticles in water. The results were shown in S8.

6.4 Hyperpolarized ^{129}Xe NMR of Three Prepared Types of Nanoparticles in Water. Two milliliters of the three prepared types of nanoparticle aqueous solution were added to 0.1% of defoamer (L-81) and transferred to a 10 mm NMR tube for experiment. The other procedures were same to the hyperpolarized ^{129}Xe NMR of CB[6] nanoparticles in water. The results were shown in Fig. 3A, S5, S10.

7. Cell experiment

7.1 Cell Culture

A549 lung cancer cells were cultured in complete F12K medium in the presence of 10% fetal bovine serum and 1% antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin) at 37°C in a humidified environment with 5% CO_2.

7.2 Cytotoxicity of CB[6]NPs and CB[6]NPs⊃ PTX to A549 cells.

A549 cells were seeded in a 96-well plate at a density of 5 × 10^3 cells per well in 200 μL 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 (0-250 μg/mL) of CB[6]NPs or (0-250 μg/mL) of CB[6]NPs⊃ PTX . The cells were incubated for 12 h at 37°C. Subsequently, the cells were incubated in a fresh medium containing methylthiazolyldiphenyl-tetrazolium bromide (MTT) (0.5 mg/mL) 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. The results were shown in Fig. S6.

7.3 Cell Experiments for Fluorescence.

Lung cancer cells A549 were incubated with FITC@CB[6] NPs⊃ NR for 1, 2, and 4 hours at 37°C. After washing 3 times with PBS, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min. The fixed cells were stained with DAPI for 5 min and washed 4 times with PBS. Finally, the cells were fixed on a glass slide and observed on a fluorescence confocal microscope. The results were shown in Fig. 3B.

8. Supplementary Figures.

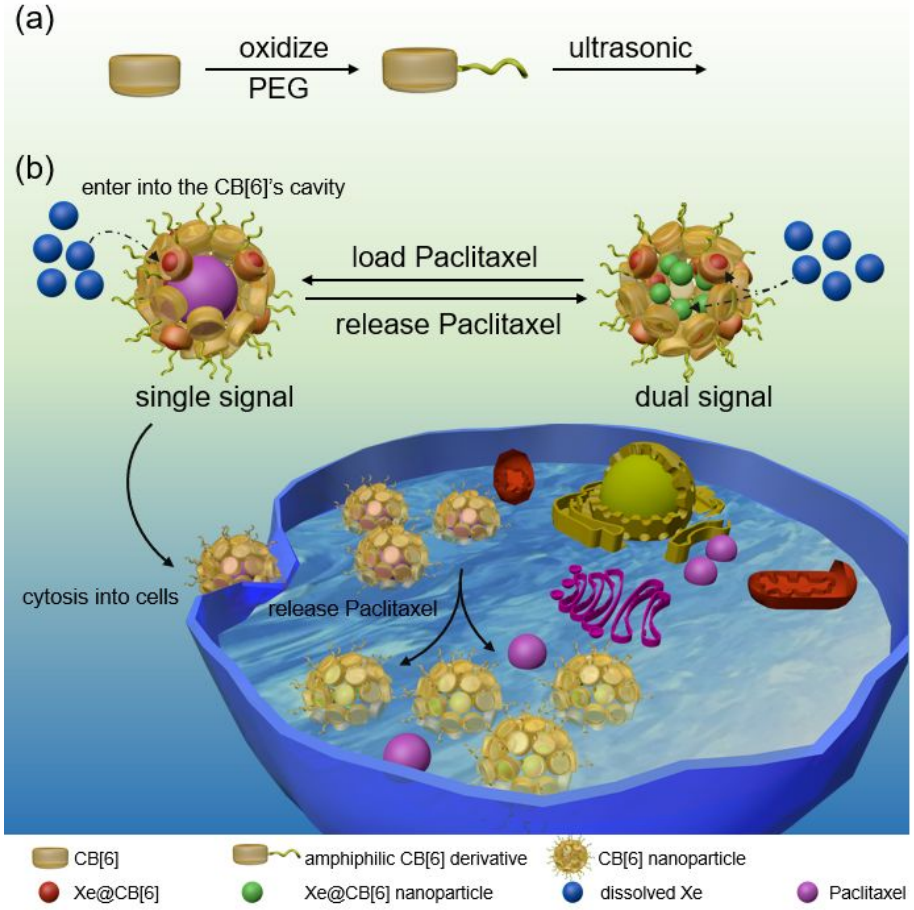


Fig. S1. Schematic diagram of CB[6] nanoparticles synthesis, drug loading, and drug release in cells. (a) Synthesis steps of CB[6] nanoparticles. (b) The source of the nanoparticle dual signals, the change of the dual signals after drug loading, and the change of the dual signals after the release of the drug in cells.

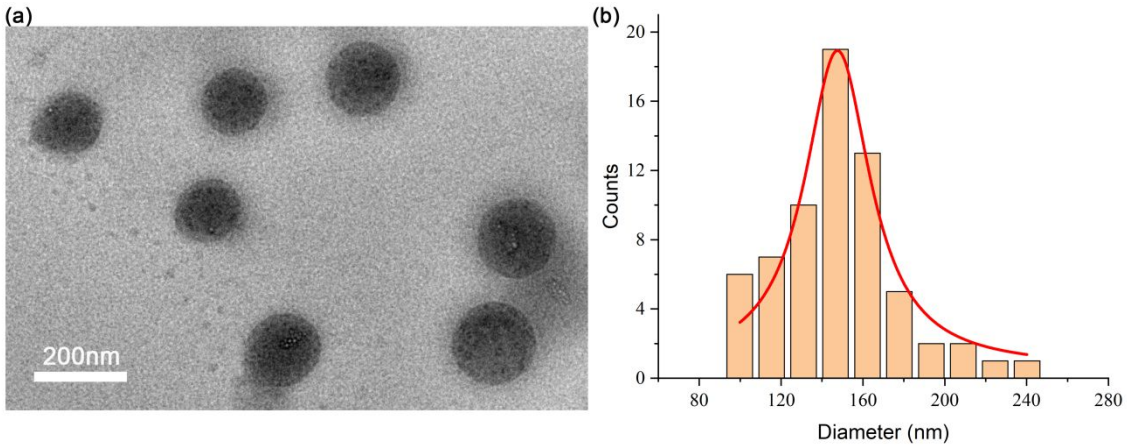


Fig. S2. Characterization of CB[6]NPs. (a) TEM images of CB[6]NPs (scale bar = 200 nm). (b) Size distribution counted by TEM.

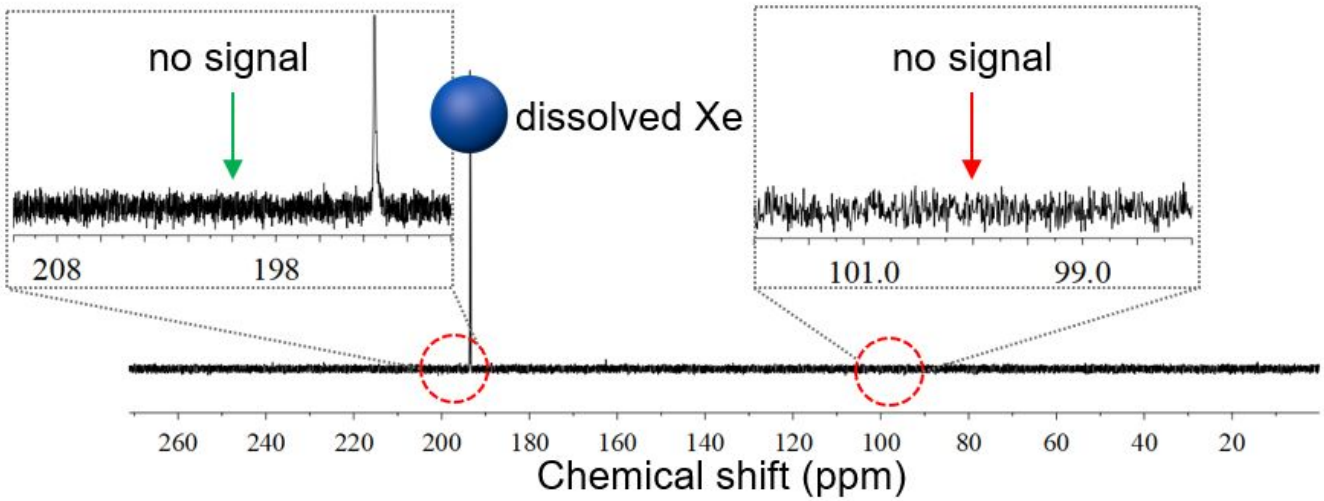


Fig. S3. Hyperpolarized ^{129}Xe NMR spectrum with 2 mg/mL CB[6]NPs in water at 298 K. Only the signal of dissolved Xe can be observed.

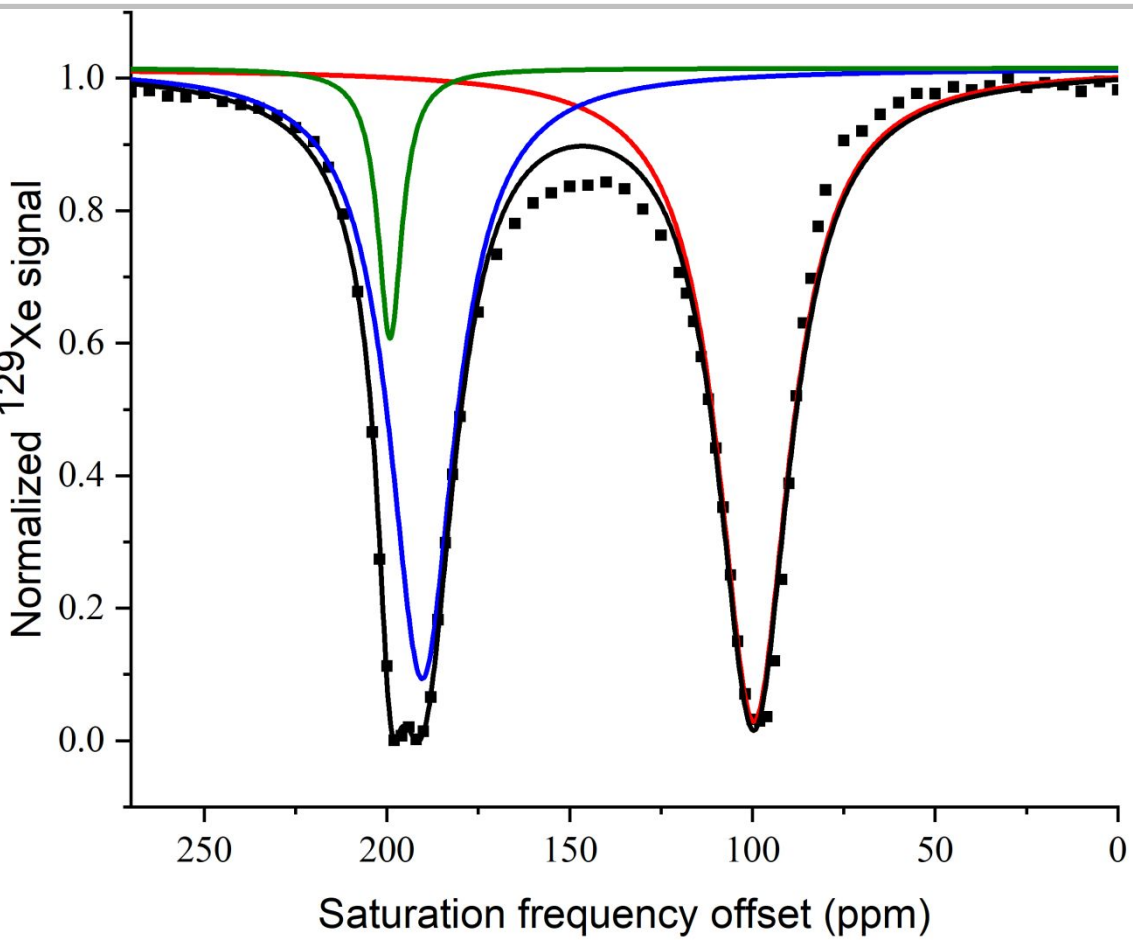


Fig. S4. Hyper-CEST frequency-scan profile of 2 mg/mL CB[6]NPs in water at 298 K. The black dots represent the original data points, and the smooth curve is the Lorentz fitting curve, where black is the sum of the fitted lines, red is the fitting line of the CB[6] cavity, blue is the fitting line of the dissolved Xe signal, and green is the fitting line of the CB[6] nanoparticle cavity.

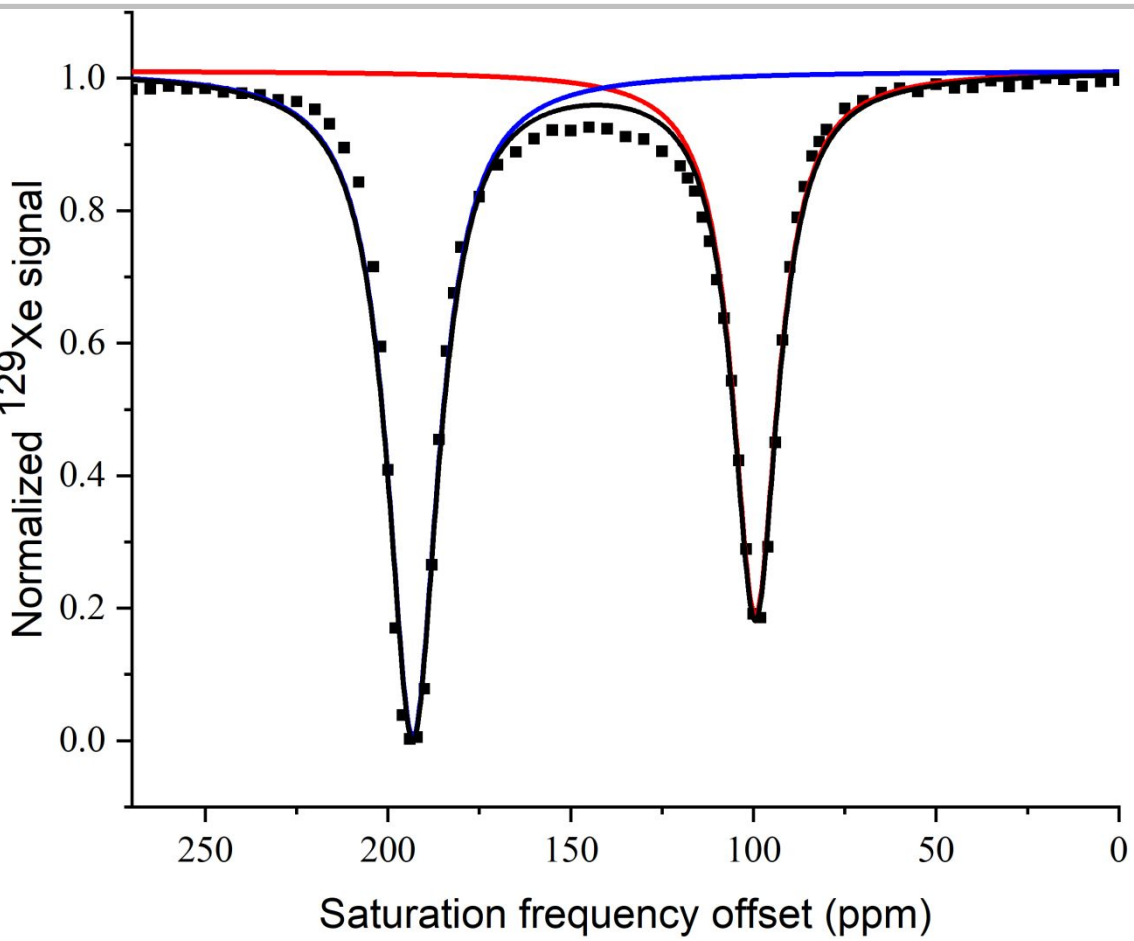


Fig. S5. Hyper-CEST frequency-scan profile of 2 mg/mL CB[6]NPs⊃ PTX in water at 298 K. The black dots represent the original data points, and the smooth curve is the Lorentz fitting curve, where black is the sum of the fitted lines, red is the fitting line of the CB[6] cavity, and blue is the fitting line of the dissolved Xe signal.

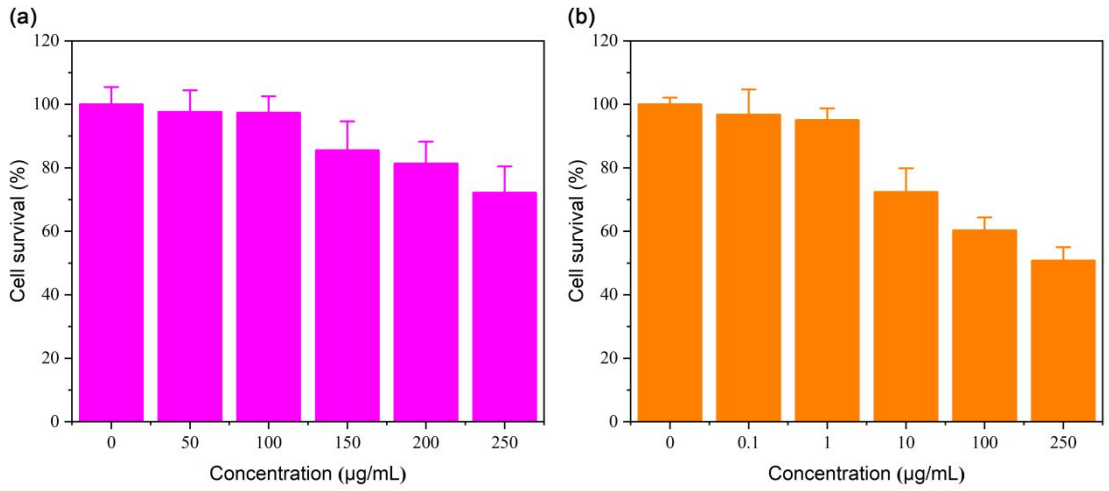


Fig. S6. (a) Cytotoxicity of CB[6]NPs measured by MTT assay (n = 3), (b) Cytotoxicity of CB[6]NPs⊃ PTX measured by MTT assay (n = 3).

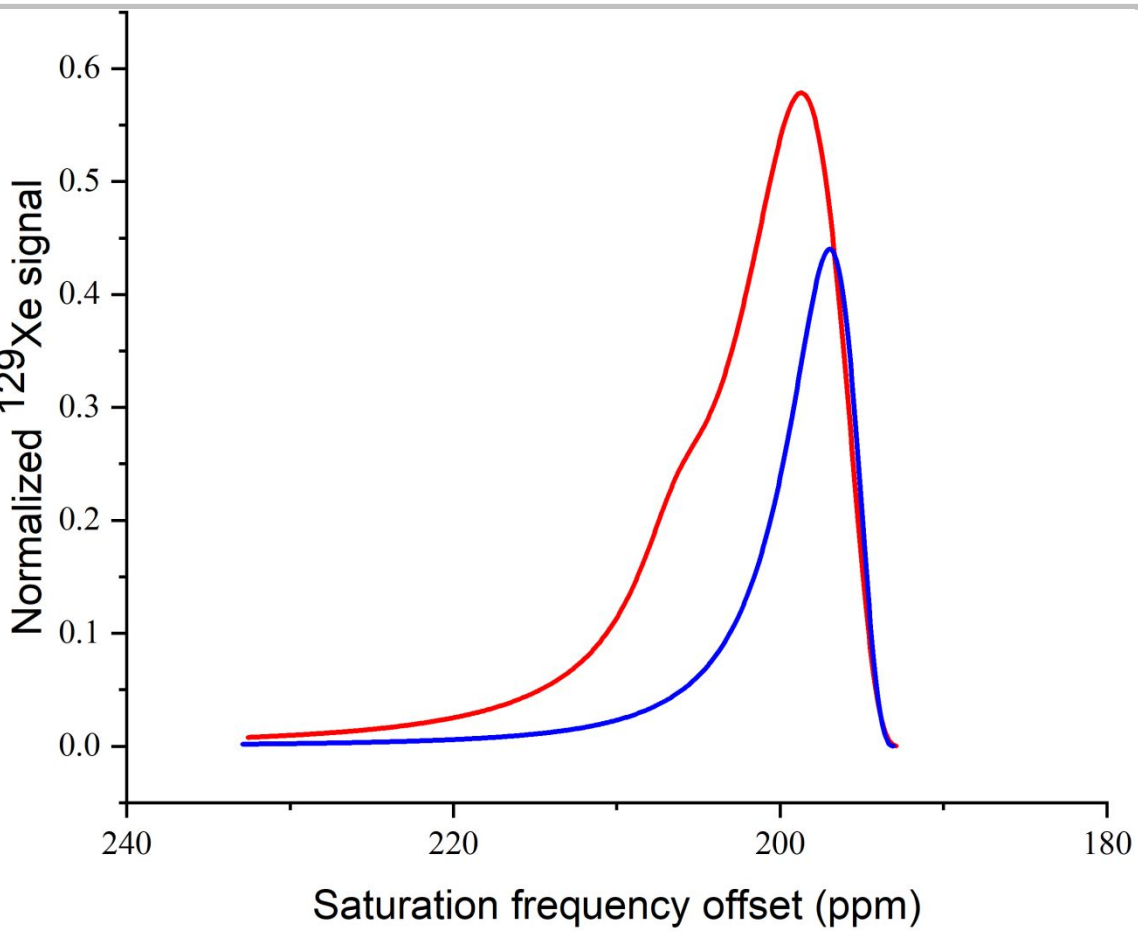


Fig. S7. MTR of A549 cell co-incubation with CB[6]NPs and CB[6]NPs⊃ PTX in PBS (pH 7.2) at 298 K. The blue line represents MTR of Xe in monomers in cells and the red line represents MTR of Xe in CB[6]NPs in cells.

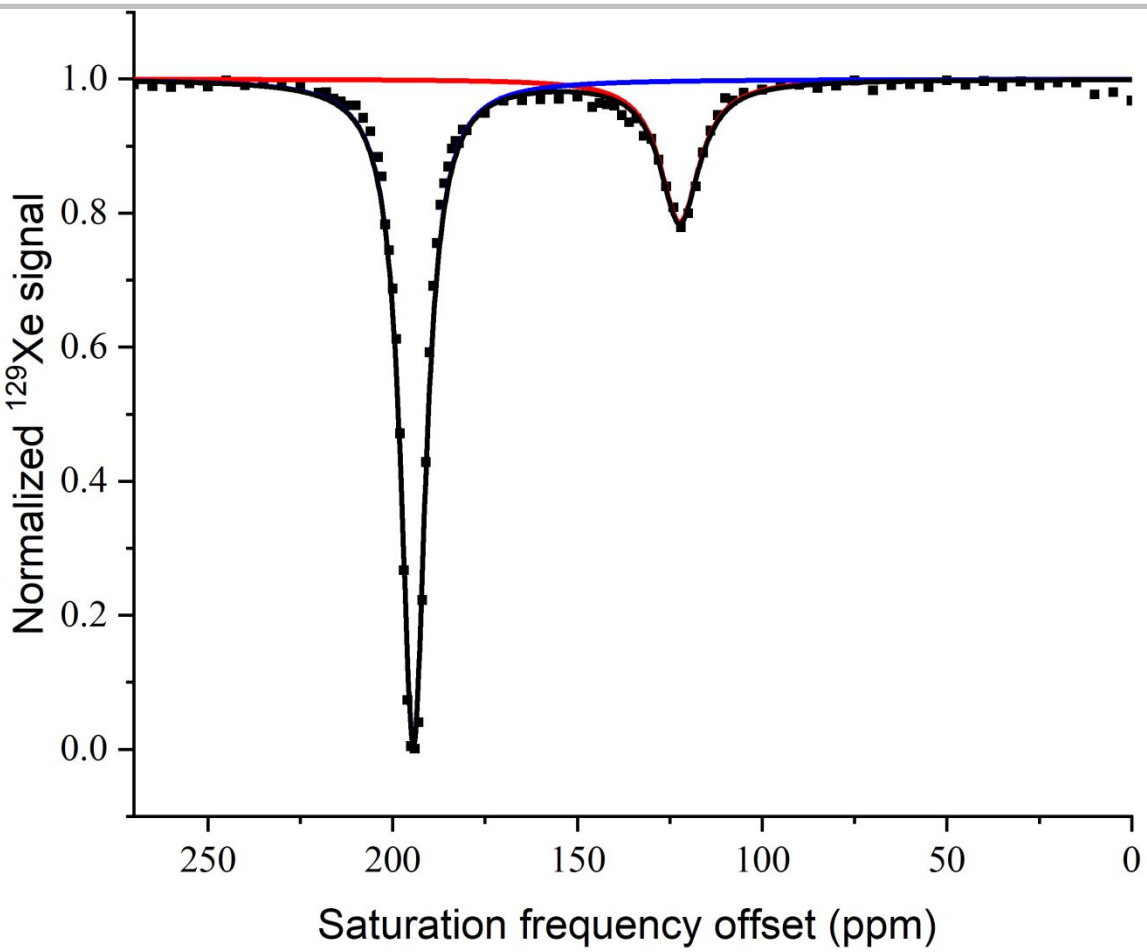


Fig. S8. Hyper-CEST frequency-scan profile of cell incubation solution at 298 K. The black dots represent the original data points, and the smooth curve is the Lorentz fitting curve, where black is the sum of the fitted lines, red is the fitting line of the CB[6] cavity, and blue is the fitting line of the dissolved Xe signal.

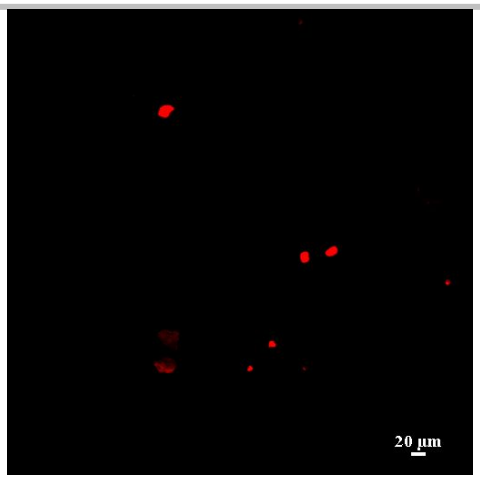


Fig. S9. Confocal laser scanning microscopy of CB[6]NPs⊃ NR in water (scale bar = 20 μm).

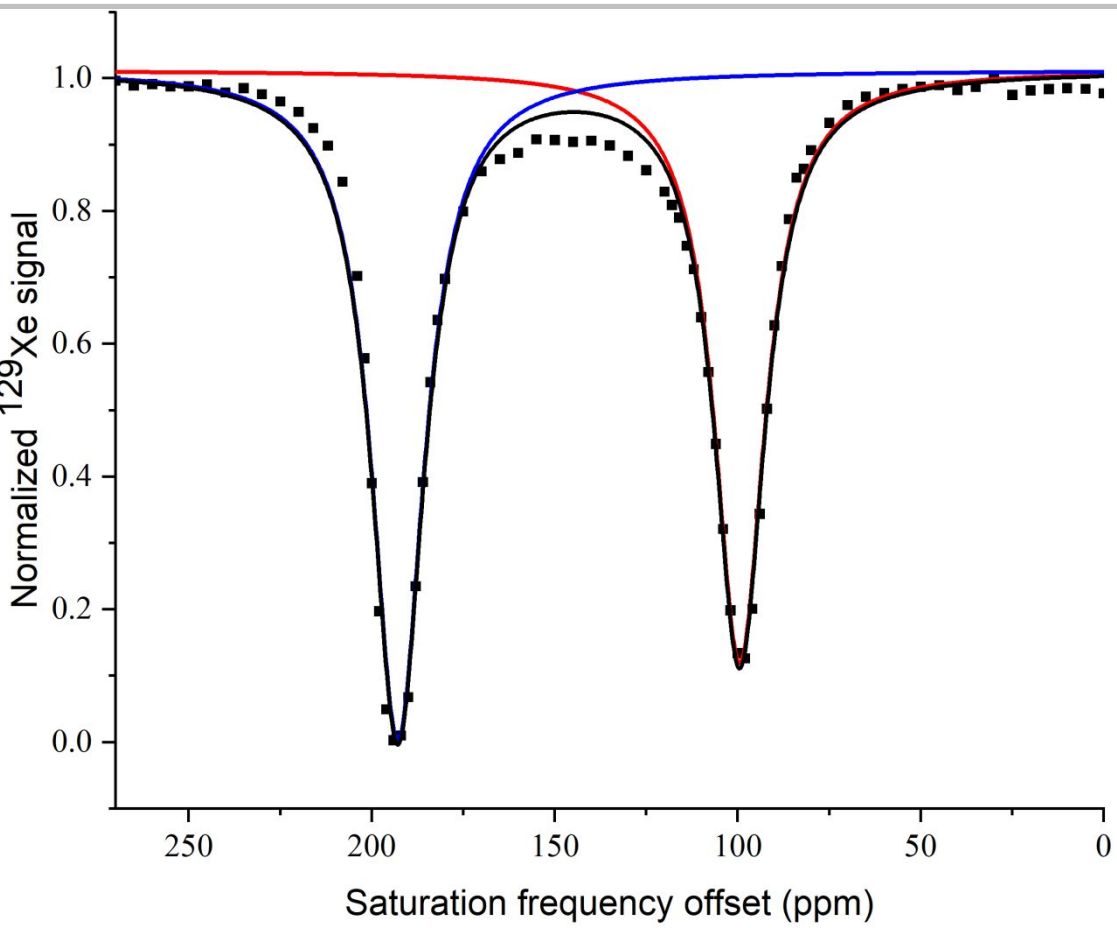


Fig. S10. Hyper-CEST frequency-scan profile of 2 mg/mL CB[6]NPs⊃ NR in water at 298 K. The black dots represent the original data points, and the smooth curve is the Lorentz fitting curve, where black is the sum of the fitted lines, red is the fitting line of the CB[6] cavity, and blue is the fitting line of the dissolved Xe signal.

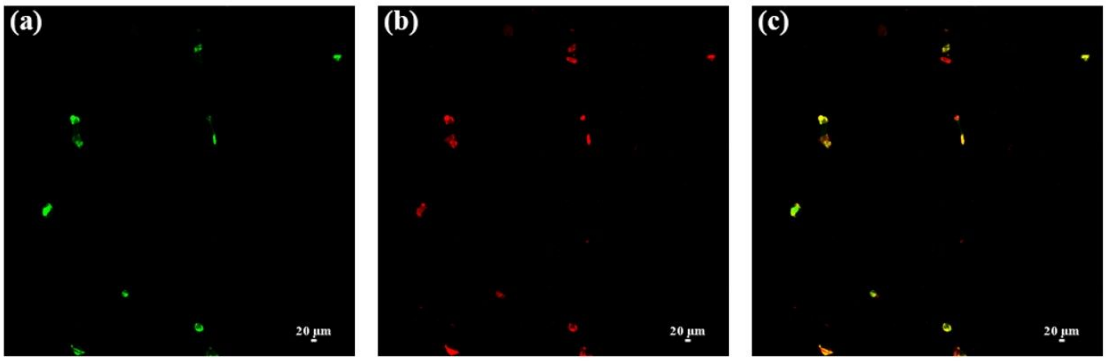


Fig. S11. Confocal laser scanning microscopy images of FITC@CB[6]NPs⊃ NR (a) FITC channel (scale bar = 20 μm), (b) Nile Red channel, (c) overlay of (a) and (b).

References

[1] a) Ayhan, M. M.; Karoui, H.; Hardy, M.; Rockenbauer, A.; Charles, L.; Rosas, R.; Udachin, K.; Tordo, P.; Bardelang, D.; Ouari, O. Comprehensive Synthesis of Monohydroxy-Cucurbit n urils (n=5, 6, 7, 8): High Purity and High Conversions. J. Am. Chem. Soc. **2015**, 137 (32), 10238-10245.; b) Zhao, N.; Lloyd, G. O.; Scherman, O. A. Monofunctionalised cucurbit 6 uril synthesis using imidazolium host-guest complexation. *Chem. Commun.* **2012**, 48 (25), 3070-3072.

[2] Ahn, Y.; Jang, Y.; Selvapalam, N.; Yun, G.; Kim, K. Supramolecular velcro for reversible underwater adhesion. *Angew. Chem. Int. Ed.* **2013,** *52* (11), 3140-3144.

[3] Park, K. M.; Suh, K.; Jung, H.; Lee, D. W.; Ahn, Y.; Kim, J.; Baek, K.; Kim, K. Cucurbituril-based nanoparticles: a new efficient vehicle for targeted intracellular delivery of hydrophobic drugs. *Chem. Commun.* **2009,** (1), 71-73.