Supporting Information

Macrocycle-Based Polymer Nanocapsules for Hypoxia-Responsive Payload Delivery

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Table of Contents

Experimental Procedures

Materials and Methods.

PerhydroxyCB[6] was synthesized by following a previously published method.[1] Spermine-folate (FA-SP) was also synthesized by following a previously published method.[2] 4,4'-Bis(bromomethyl)azobenzene (BBA) was purchased from Aikon International Limited, the ^{1}H NMR spectra and mass chromatogram of BBA were shown in Figure S1 and S2. Nile Red (99%), DOX·HCl (98%), sodium hydride (NaH, 60%), spermine (SP, 98%), FA (98%), sodium dithionite (SDT), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (97%), N-hydroxysuccinimide (NHS, 98%), nicotinamide adenine dinucleotide phosphate disodium salt (NADPH), and rat liver microsomes were purchased from Sigma-Aldrich (China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 98%) was supplied by Amresco. All reagents and solvents employed in this study were commercially available and used as supplied without further purification. Dialysis was performed using a Slide-A-Lyzer dialysis cassette (MWCO, 2 kD). Milli-Q water was purified using a Milli-Q Integral system from Merck Millipore.

^{1}H NMR spectrum were acquired on a Bruker Ultra Shield 600 PLUS NMR spectrometer. Mass spectrum were acquired on a MassHunter GC/MS, Agilent. UV-vis-NIR absorbance spectra were measured on IFS-66V/S and Shimadzu UV-1800 spectrophotometers. The size and zeta potential of NCs were determined by DLS at 25 °C with a Zeta Sizer (Malvern. Co., UK). TEM analysis was performed by a transmission electron microscopy (TEM, JEOL 2100F, Japan) at the operation voltage of 200 kV. SEM analysis was performed by a Zeiss Sigma FESEM. Cell uptake and cell apoptosis were analyzed immediately by a FACS flow cytometer (Beckman coulter). CLSM (Zeiss LSM710) was used to directly visualize the intracellular location of NCs. Cell viability was measured by a multimode microplate reader (FlexStation 3). Inverted fluorescent microscope (Olympus IX73) was employed to observe the fluorescence distribution and intensity in zebrafish embryos. The L02 cell line was obtained from the Committee of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The A12 cell line and 4T1 cell line were purchased from American Type Culture Collection (ATCC, Shanghai, China).

Cell culture.

The A12, L02 and 4T1 cell lines were incubated with DMEM and supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were sub-cultured regularly using trypsin/EDTA.

Biocompatibility study.

The A12, 4T1 and L02 cell lines were respectively seeded in a 96-well plate at a density of 8×10^3 cells per well in 100 μL of DMEM containing 10 % FBS and 1 % PS, respectively, and incubated in a humidified atmosphere with 5 % CO_2 at 37 °C for 24 h. The cell culture medium was replaced with 100 μL of a fresh one containing 6, 12, 25, 50, and 100 μg/mL of AZO-NCs and incubated for additional 36 h. After discarding the medium, the cells were incubated with 100 μL of a fresh medium containing 10 μL of MTT (5 mg/mL) for an additional 4 h at 37°C, and subsequently the medium was gently removed. The water-insoluble purple formazan crystals formed by live cells remaining at the bottom of the wells were dissolved in 100 μL DMSO and the solution was gently shaked for 10 min. UV absorption of the solution at 590 nm was measured by a multiwell plate reader. The same experiment without treatment of AZO-NCs was executed as control group. Each experiment was performed for 3 times to obtain a mean value and standard deviation.

Flow cytometry analysis.

In order to quantify the cellular uptake, 4T1 and A12 cells were treated with fresh culture media containing free DOX@AZO-NCs and DOX@FA-SP/AZO-NCs respectively, with different concentration of DOX·HCl (0.1, 0.2, 0.4, 0.8 μM) or different time lengths (4, 8, 12 and 24 h). After internalization, cells were harvested and analyzed by a flow cytometer. The PC5.5 channel was selected to detect the fluorescence signal of DOX·HCl.

Confocal imaging.

For cellular uptake study, 4T1 and A12 cells were first incubated in 35 mm confocal dishes for 24 h, respectively, and subsequently treated with DOX@AZO-NCs and DOX@FA-SP/AZO-NCs containing the same concentration of DOX·HCl. Confocal images were recorded by an inverted confocal laser scanning microscope after the cells were incubated for 4, 8, 12 and 24 h, respectively. Before hiring confocal imaging, all treated cells were washed with PBS in order to avoid accumulation of the non-internalized DOX·HCl. Cell nucleuses were stained with DAPI. For cellular hypoxic responsive release study, 4T1 and A12 cells were first incubated in 35 mm confocal dishes for 24 h, respectively, and subsequently treated with the same concentration of NR@AZO-NCs for 12 h, and finally, incubated with fresh medium and covered a cover glass on top for 12 or 24 h.

Zebrafish embryos raised.

Prepare embryo medium: 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 0.00001% methylene blue. Maintain and breed WT AB strain *Danio rerio* at 26.5 °C. Collect the embryos on the morning of fertilization, raise until 3 d post fertilization (dpf) at 28 °C in embryo medium prepared as described above, select and stage as previously described. After treating with NR@AZO-NCs for 24 h, carefully select the embryos for the experiment, use whole and alive embryos for the hypoxia incubation, zebrafish embryos 4 dpf in the experiments presented here. Place the embryo dishes into the incubator containing the gas incubation chamber and let them equilibrate to the temperature of the incubator. Maintain the gas incubation chamber under a constant infusion with the gas mixture of 5% O_2 and 95% N_2 for 24 h. For normoxic control group, the zebrafish embryos are raised in the normoxic medium corresponding to the embryo type for 24 h, after treating with NR@AZO-NCs for 24 h. Inverted fluorescent microscope was employed to observe the fluorescence distribution and intensity in zebrafish embryos.

Results and Discussion

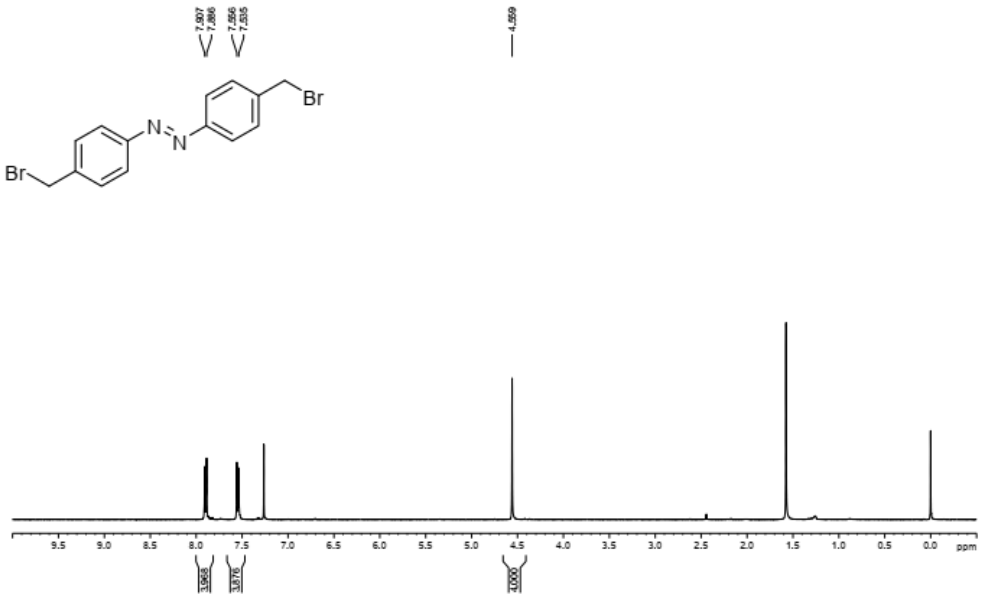
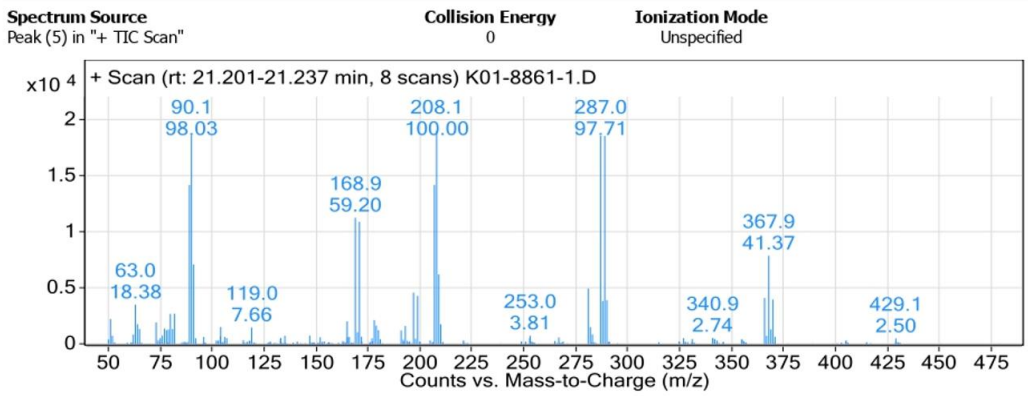


Figure S1. ^{1}H NMR spectrum of 4,4'-Bis(bromomethyl)azobenzene in $CDCl_3$.

^{1}H NMR (400 MHz, $CDCl_3$) δH (ppm) =7.907 (d, J = 8.4 Hz, 4H), 7.556 (d, J = 8.4 Hz, 4H), 4.559 (s, 4H).



Peak List

m/z	z	Abund
89.1		14170.75
90.1		18631.75
91.1	1	7080
168.9	1	11251.75
170.9	1	10894.88
207		14182.25
208.1		19005.5
287	1	18569.88
289	1	18553.38
367.9	1	7862

Figure S2. Mass spectrum of 4,4'-Bis(bromomethyl)azobenzene.

MS (ESI^{+}) Calcd for [M^{+}], 368.0, Found, 367.9.

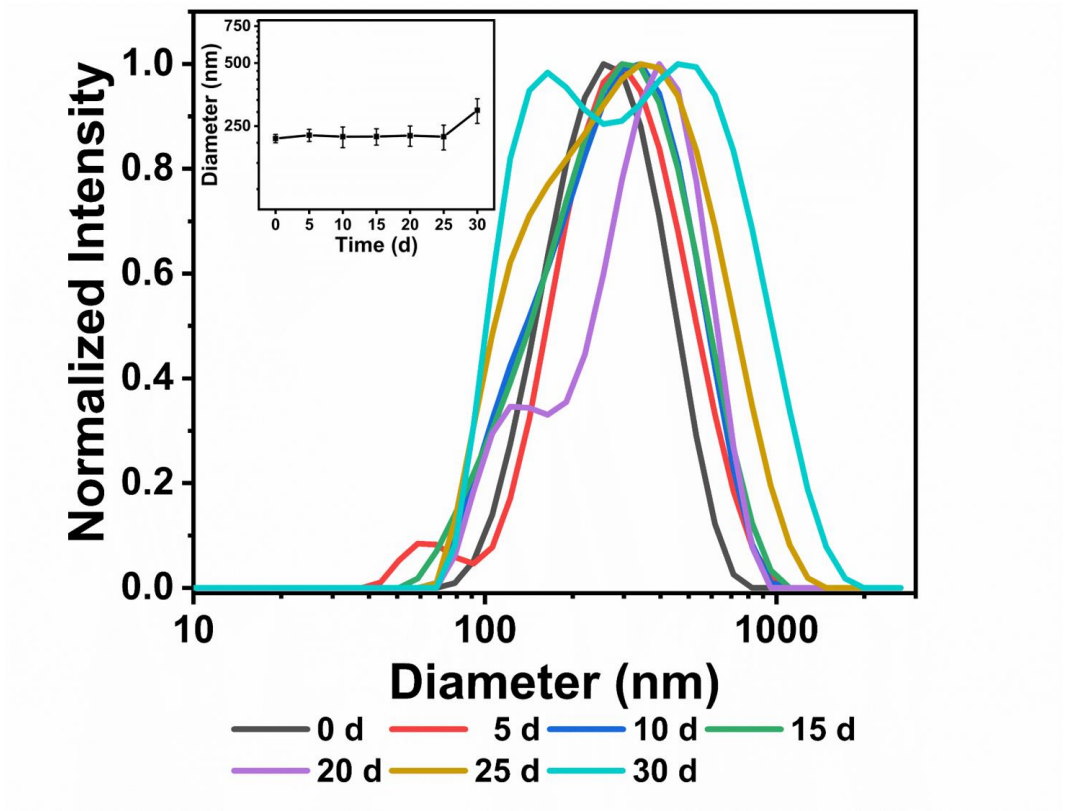


Figure S3. Time-evolved DLS analysis of AZO-NCs under ambient conditions in PBS solutions. Data were expressed as the mean values (n = 3) ± standard deviation.

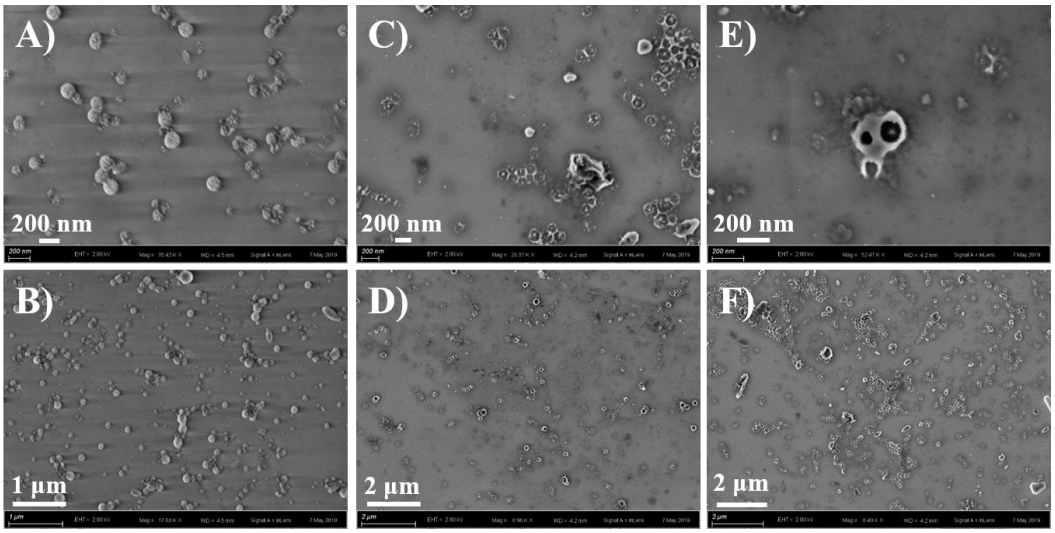


Figure S4. SEM images of AZO-NCs in the presence of rat liver microsomes (80 µg/ml) and NADPH (50 µM) under hypoxia conditions, (A) and (B) for 30 min, (C) and (D) for 60 min, and (E) and (F) for 120 min,

Under hypoxia, after adding rat liver microsomes (80 µg/ml) and NADPH (50 µM) as a cofactor for the reductases to AZO-NCs aqueous solutions, the size of AZO-NCs reduced firstly and then increased was observed by SEM.

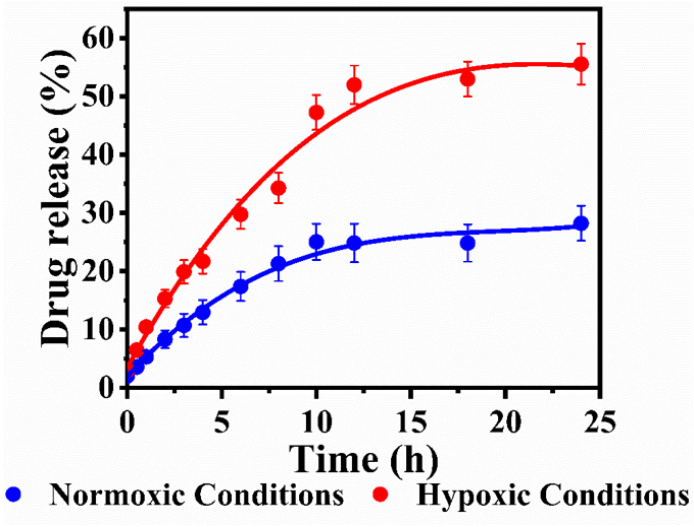


Figure S5. Drug (DOX·HCl) release profile of DOX@AZO-NCs under normoxic and hypoxic conditions at 37 ℃, respectively. The hypoxic conditions were prepared by bubbling nitrogen gas into the solution for 30 min.

The DOX release profile of DOX@AZO-NCs at normoxic or hypoxic conditions was monitored by HPLC, 2.5-fold DOX·HCl were released from AZO-NCs after incubation for 24 h at hypoxic conditions at 37 ℃.

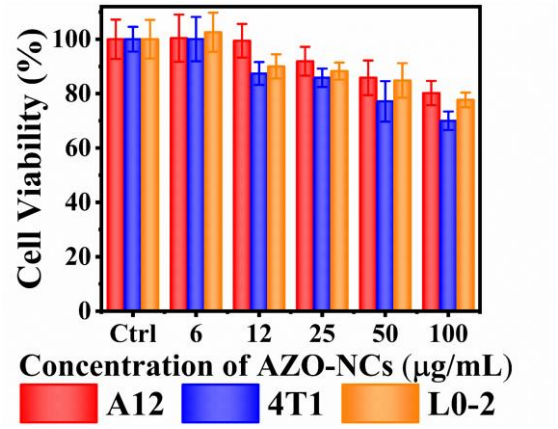


Figure S6. Cytotoxicity of AZO-NCs against A12, 4T1, and L02 cells after incubation for 36 h.

The cytotoxicity of AZO-NCs was evaluated first by MTT assays on A12 cell line (AML 12, derived from mouse liver), L02 cell line (derived from human liver), and 4T1 cell line (originated from mouse breast cancer) upon incubation for up to 36 h.

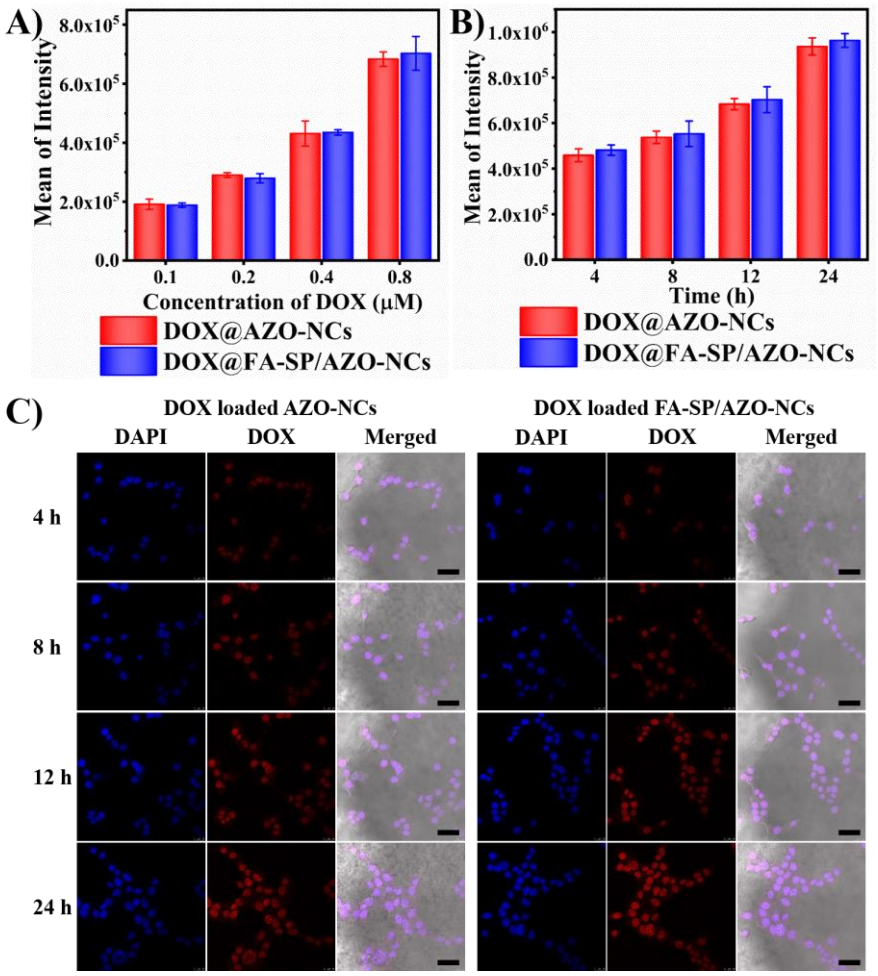


Figure S7. Cellular uptake of DOX@AZO-NCs and DOX@FA-SP/AZO-NCs by A12 cells determined by flow cytometry. A12 cells were treated with (A) different concentrations of DOX for 12 h, (B) 0.4 μM DOX for different time lengths. (C) CLSM images of A12 cells that were incubated with DOX@AZO-NCs and DOX@FA-SP/AZO-NCs at 37 ˚C for different time lengths with 0.4 μM DOX. Cell nucleuses were stained with DAPI. The scale bar is 50 μm.

The intracellular uptake of DOX@AZO-NCs and DOX@FA-SP/AZO-NCs in A12 cells was studied by flow cytometry and CLSM.

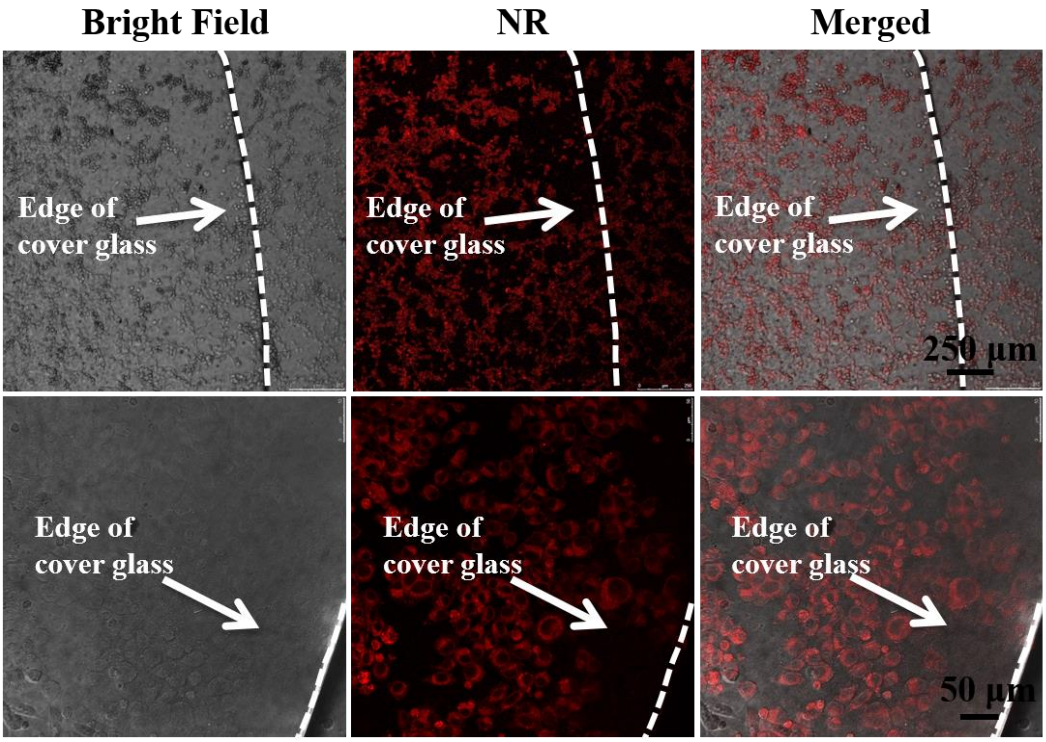


Figure S8. *In vitro* hypoxia-responsive release of NR from NR@AZO-NCs at a gradient cellular hypoxia conditions. CLSM images of 4T1 cells that were incubated with fresh medium under cover glass with normal culture conditions for 24 h, after treated with NR@AZO-NCs for 12 h.

The hypoxia-responsive release of NR@AZO-NCs in 4T1 cells for 24 h.

References

[1] Jon, S. Y.; Selvapalam, N.; Oh, D. H.; Kang, J.-K.; Kim, S.-Y.; Jeon, Y. J.; Lee, J. W.; Kim, K., Facile synthesis of cucurbit [n] uril derivatives via direct functionalization: expanding utilization of cucurbit [n] uril. *J. Am. Chem. Soc.* **2003,** *125*, 10186-10187.

[2] 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.