Self-Immolative PEGylated Dendritic Drug Conjugates for Cancer Therapy with Enhanced Cellular Uptake

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Materials and methods

Materials

Chloroambucil (CB, 98%, J&K), Triethylamine (TEA, 99.5%, Aladdin), pyridine (99.5%, Lvshi), 1,3,5-benzenetrimethanol (96%, Bide), NH_2-PEG-OH (MW5000, Aladdin), (7-Azabenzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HATU, 99%, TRC), N, N-Diisopropylethylamine (DIPEA, 99%, Adamas) were used as received. Boc-D2, linker (L), and Azo-CB were synthesized following the procedure described previously, and the ^{1}H NMR spectrums were shown in Fig. S2-S4, respectively [1-3].

Synthesis of PEG-L

Briefly, TEA (21.25 μL) was added to a stirring solution of PEG-NH_2 (294 mg, 0.059 mmol) in 2 mL dry DMF at room temperature for further 30 min. Solution of linker (50 mg, 0.07 mmol) in 1 mL dry DMF was added dropwise, the reaction was stirred overnight at room temperature. After completion, the solution was dropped into ether for precipitation triplicate. The sediment was also collected by centrifugation to give white solid. The white solid was dried through a vacuum drying oven to get the PEG-L (276.05 mg, 84% yield).

Synthesis of BOC-D2-(Azo-CB)$_4$

Azo-CB (456.3 mg, 1.25 mmol) and HATU (760 mg, 2 mmol) were added to 10 mL dry DMF and stirred at 0 °C for 5 h to activated carboxyl group. DIPEA (348 mg, 2.7 mmol) was added and the mixture warmed to room temperature. Solution of Boc-D2

(105.6 mg, 0.125 mmol) in 5 mL dry DMF was added dropwise and further stirred for 48 h. The reaction was quenched with water, extracted with dichloromethane (DCM), The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel to give Orange-red solid Bco-D2-Aco-CB (128.3 mg, 46.2% yield).

Synthesis of D2-(Azo-CB)$_4$

TFA (1 mL) was added dropwise to solution of Bco-D2-Aco-CB (130 mg, 0.058 mmol) in 1 mL DCM and stirred for 2 h. The solvent and unreacted TFA was removed under reduced pressure and dried under vacuum gain orange-red solid D2-(Aoc-CB)$_4$ (112.2 mg, 90.4% yield).

Synthesis of PEG-L-(Azo-CB)$_8$

D2-Aoc-CB (120 mg, 0.056 mmol) and 50 μL pyridine was added to 2 mL DMF and stirred for 30 min at 0 °C. Solution of PEG-L (156 mg, 0.027 mmol) in 1 mL dry DMF was added dropwise and further stirred overnight. The solution was dropped into ether for precipitation triplicate. The sediment was dried overnight in a vacuum drying oven to obtain origine solid PEG-L-(Azo-CB)$_8$ (229 mg, 86.2% yield).

Fabrication of hypoxia-responsive nanomedicine NM-CB/Ce6

Firstly, PEG-L-(Azo-CB)$_8$ (2.0 mg) and Ce6 (1.0 mg) were dissolved in 1.0 mL DMF, and then stirred 6 h. Secondly, 10 mL distilled water was slowly added into the original solution, and kept stirring for overnight. Finally, the mixture solution was transferred to a dialysis tube (MWCO 3500 Da) to purify by dialysis against distilled water. The finally obtained nanomedicine was denoted as NM-CB/Ce6.

In vitro ROS generation

The 9, 10-anthracenediyl-bis(methylene) dimalonic acid (ABDA) (0.05 mg/mL) was added into the PBS of NM-CB/Ce6. For the NIR-triggered ROS release, the above solution was irradiated with 650 nm NIR (0.1 W/cm^2, 10 min). For that ABDA can effectively capture ROS through a rapid reaction with anthracene moiety, the original UV-Vis absorption of ABDA would gradually reduce in the environment of ROS.

Singlet oxygen generation was further evaluated using electron spin resonance (ESR) spectroscopy with 2,2,6,6-tetramethylpiperidine (TEMP) as a spin trapper. NM-CB/Ce6 (0.1 mg/mL) was dispersed into the TEMP solution (0.06 mM), then the solution was illuminated under 650 nm NIR (0.1 W/cm^2) for the different predetermined time, and measured by ESR immediately.

***In vitro* drug release**

A solution of CSN-IR806/CB (1 mg/mL) was mixed with varying concentrations of $Na_2S_2O_4$ at 1.6, 3.2, 6.4, 12.8, or 25.6 mM. After stirring the mixture for 2 h, it was placed in a dialysis bag (MWCO: 500 Da) and immersed in methanol (15 mL) while stirring for 24 h. The methanol solution outside the dialysis bag was then evaporated and analyzed using HPLC.

***In vitro* cytotoxicity**

HepG2 cells were seeded in a 96-well plate at a density of 1 × 10^4 cells per well (200 μL) and incubated in DMEM for 12 h. After this period, the medium was replaced with fresh DMEM containing varying concentrations of NM-CB/Ce6 and incubated for an additional 6 h under either hypoxic or normoxic conditions. For the irradiation groups,

the cells received NIR exposure (650 nm, 0.1 W/cm^2, for 10 minutes), followed by another incubation period of 18 h. The cytotoxicity was then assessed using MTT assays. In addition, live/dead staining was performed on HepG2 cells using fluorescein diacetate and propidium iodide before imaging them with a fluorescent microscope.

Cell internalization

HepG2 cells were plated in a 12-well plate at a density of 1.0×10^5 cells per well and incubated in DMEM for 12 hours. Following this, the medium was replaced with fresh DMEM containing either NM-CB/Ce6 at a concentration of 4 μg/mL. After incubation for either 1 hour or 4 hours under hypoxic or normoxic conditions, the cells were stained with DAPI for 20 minutes and then observed using a confocal laser scanning microscope (CLSM).

Intracellular ROS generation

HepG2 cells were plated in a 12-well plate at a density of 1.0×10^5 cells per well and incubated in DMEM for 12 h. Subsequently, the medium was replaced with fresh DMEM containing NM-CB/Ce6 at an Ce6 concentration of 4 μg/mL. After a 4 h incubation period, the irradiation groups received NIR exposure (650 nm, 0.1 W/cm^2, 10 min)). Following this, the cells were stained with 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 minutes and examined using a fluorescence microscope.

Statistical analysis

The data were presented as mean ± SD, and the statistical significance of the differences between groups was assessed using Student's t-test.

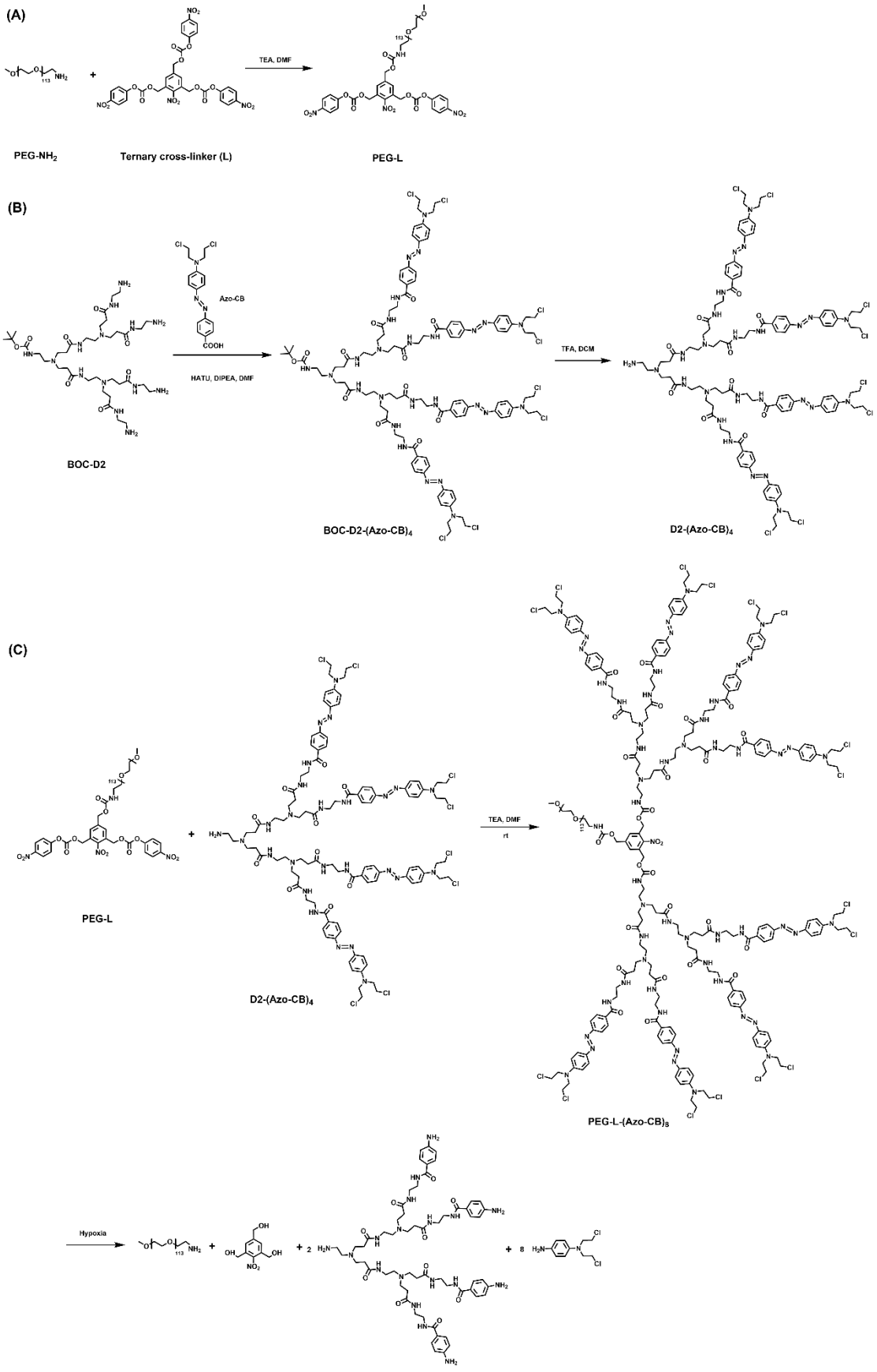

Fig. S1. Synthesis of (A) PEG-L, (B) D2-(Azo-CB)$_4$, and (C) PEG-L-(Azo-CB)$_8$.

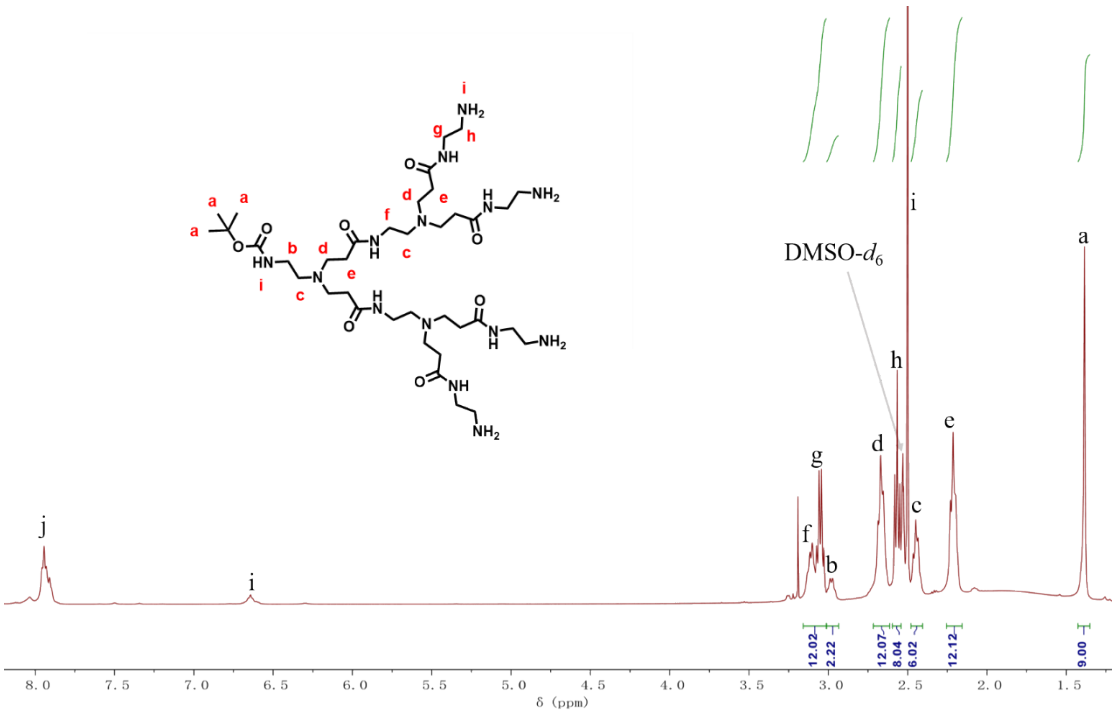

Fig. S2. ^{1}H NMR spectra of Boc- D2 (DMSO-d_6).

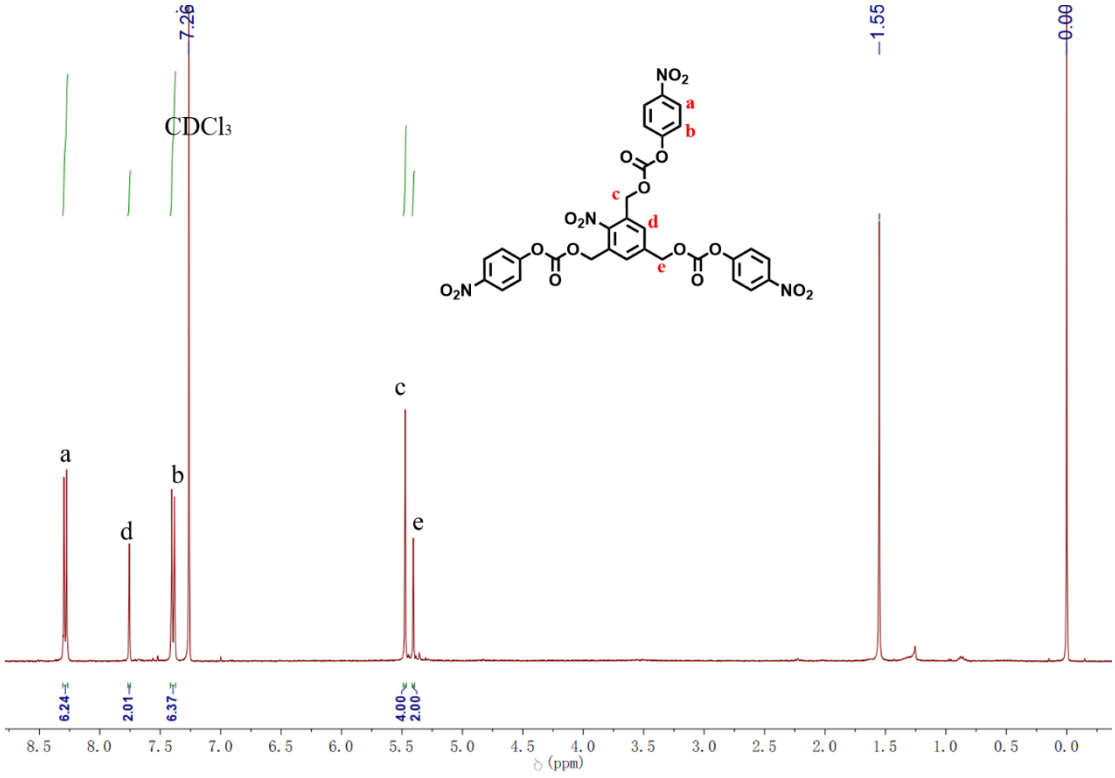

Fig. S3. ^{1}H NMR spectra of L ($CDCl_3$).

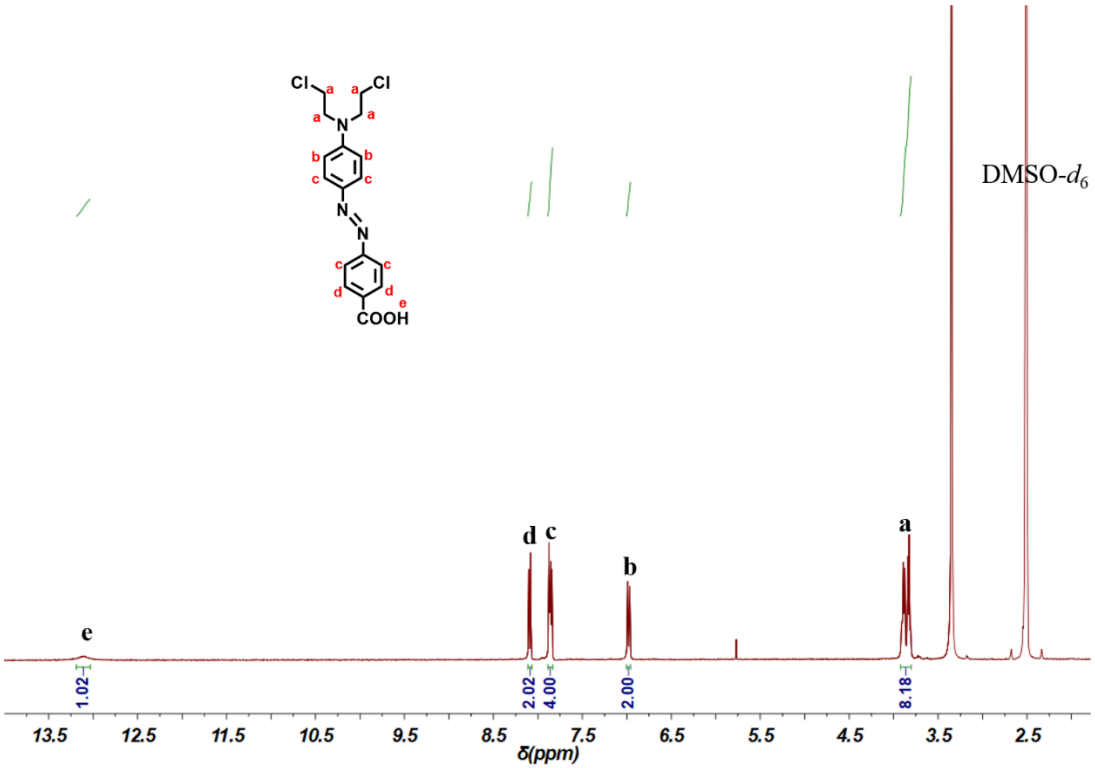

Fig. S4. ^{1}H NMR spectra of Azo-CB (DMSO-d_6).

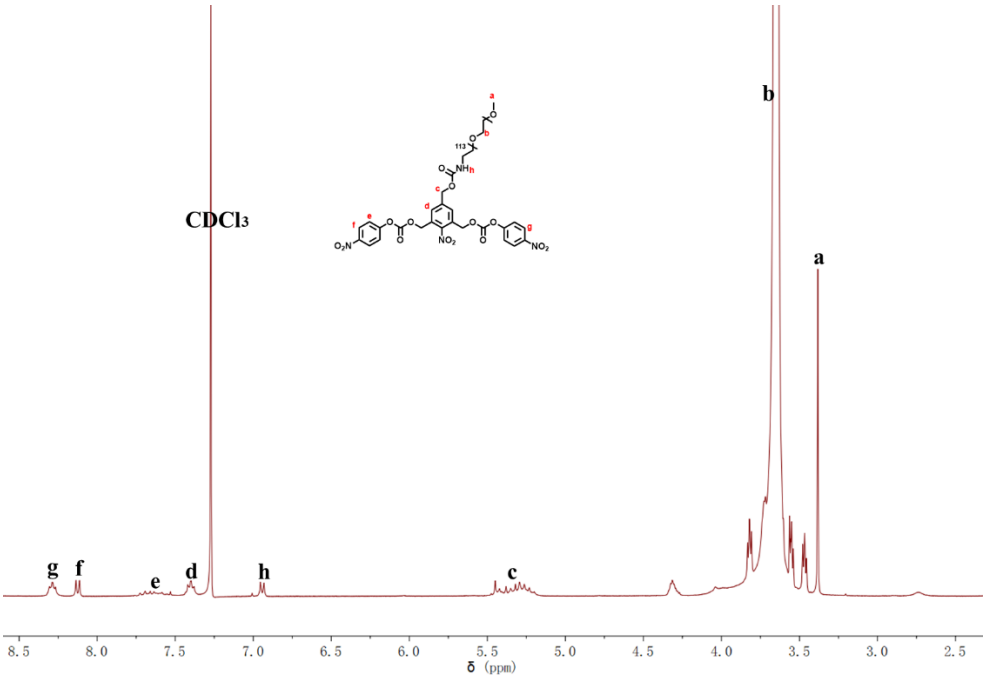

Fig. S5. ^{1}H NMR spectra of PEG-L ($CDCl_3$).

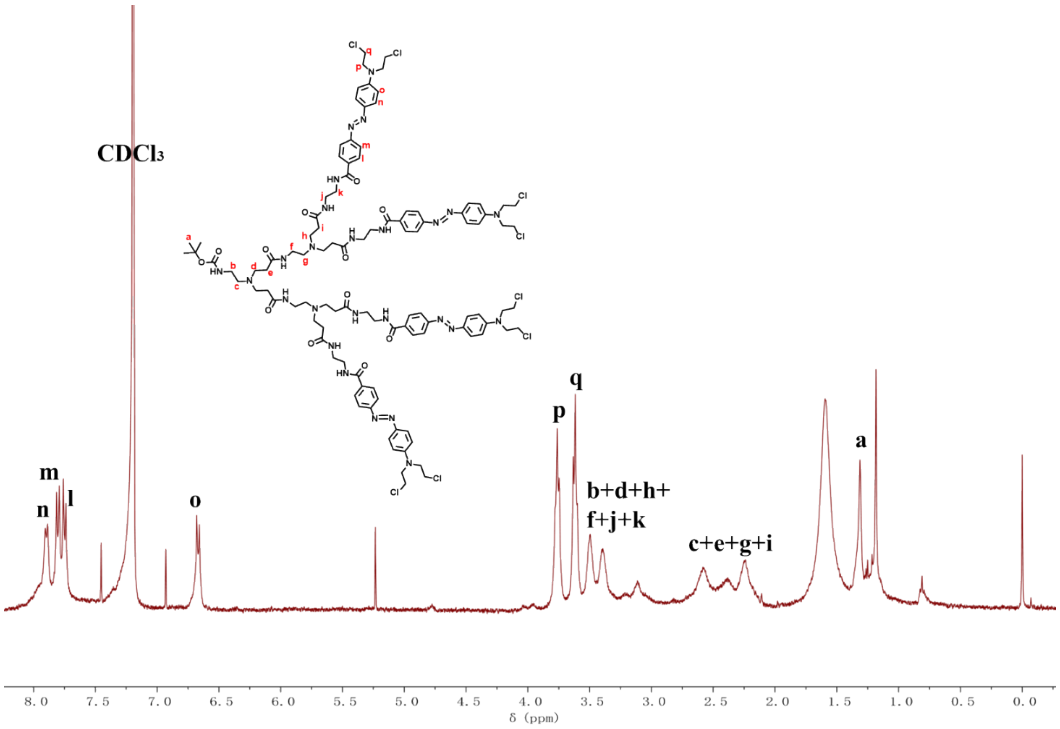

Fig. S6. ^{1}H NMR spectra of BOC-D2-(Azo-CB)$_4$ ($CDCl_3$).

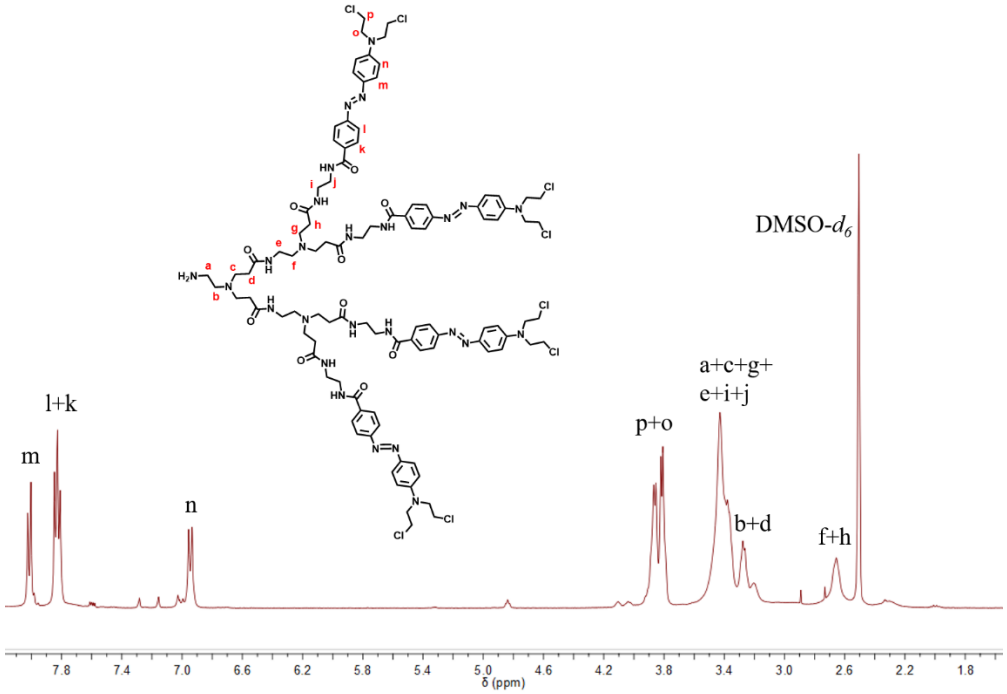

Fig. S7. ^{1}H NMR spectra of D2-(Azo-CB)$_4$ (DMSO-d_6).

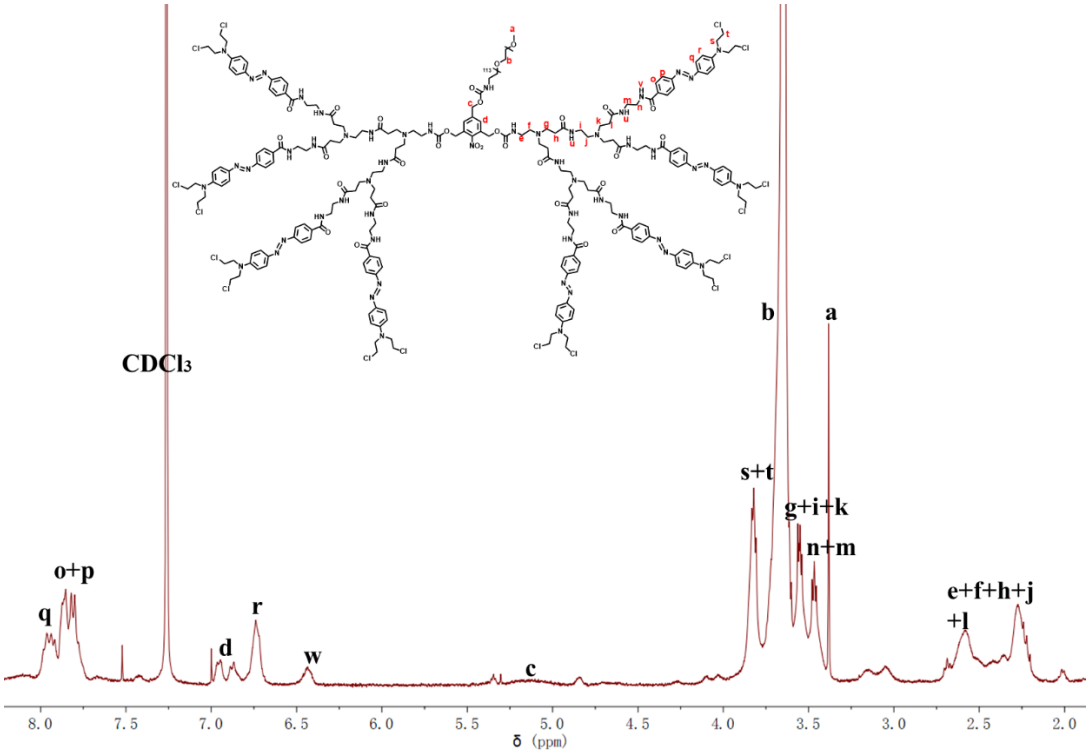

Fig. S8. ^{1}H NMR spectra of PEG-L-(Azo-CB)$_8$ ($CDCl_3$).

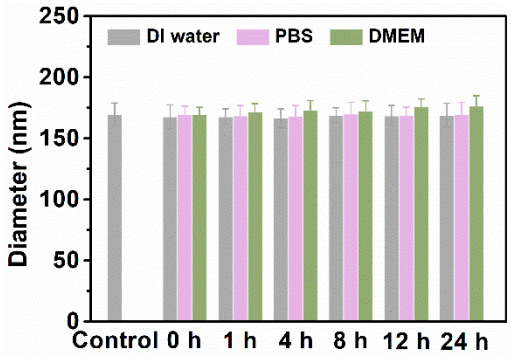

Fig. S9. Hydrodynamic diameter distribution of NM-CB/Ce6 incubated in DI water, PBS, and DMEM. Data are expressed as mean ± SD (n =3).

Table S1. The half maximal inhibitory concentrations (IC_{50}) of NM-CB/Ce6 against HepG2 cells under different conditions.

Conditions	Normoxia		Hypoxia	
	Dark	Light	Dark	Light
IC_{50} (μg/mL CB)	9.4	1.5	2.1	0.7

References

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2. H. Chen, Q. Guo, Y. Chu, et al. Smart hypoxia-responsive transformable and charge-reversible nanoparticles for the deep penetration and tumor microenvironment modulation of pancreatic cancer. Biomaterials, 2022, 287, 121599.
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