Electronic Supplementary Material (ESI) for ChemComm.

Supplementary data

Single Component Self-Assembled Thermally Activated Delayed Fluorescence Nanoprobe

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Contents

1. Abbreviations

DCM = Dichloromethane

DIPEA = *N*,*N*-Diisopropylethylamine

DMF = Dimethylformamide

DMSO = Dimethyl Sulfoxide

EA = Ethyl Acetate

FBS = Fetal Bovine Serum

FLIM = Fluorescence Lifetime Imaging Microscopy

HATU = 2-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium Hexafluorophosphate

HRMS = High Resolution Mass Spectrometry

MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

NMR = Nuclear Magnetic Resonance

PBS = Phosphate Buffer Saline

PE = Petroleum Ether

rt = Room Temperature

TADF = Thermally Activated Delayed Fluorescence

TFA = Trifluoroacetic Acid

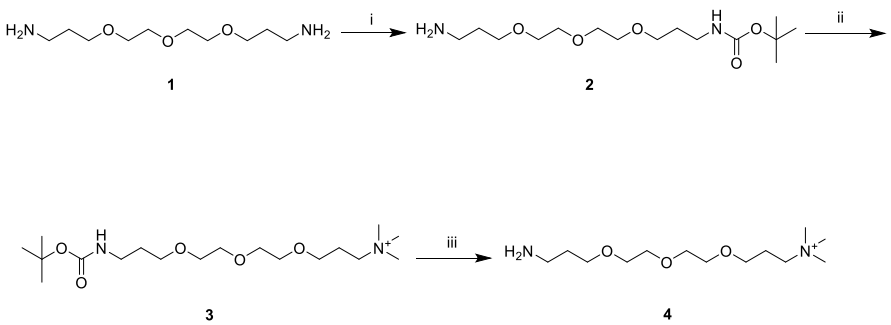
THF = Tetrahydrofuran

2. Experimental Procedures

2.1 General methods

All the chemicals were purchased from J&K, Energy Chemical or Innochem. Commercially available reagents were used without further purification. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin−streptomycin solution (100×) were purchased from Corning. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega. Fluorescence emission spectra and full wavelength absorption spectra were performed on Tecan Spark™ 10M Multimode Microplate Reader. CMC (Critical Micelle Concentration) were performed on Fluorescence spectrophotometer (HITACHI, F7000) by use fluorescence probe Pyrene. Absolute fluorescence quantum yield, fluorescence emission decays and corresponding fluorescence lifetimes of **AI-Cz-AM** and **AI-Cz-NP** under diffident conditions were measured with Edinburgh photonics FLS980 (laser model: VPL-375, the pulse width 500 ns). Confocal laser scanning microscope imaging were conducted with Leica TCS SP8 X Confocal Microscope. Fluorescence lifetime imaging were taken by ISS Q2 confocal laser scanning system coupled to a Nikon TE2000 microscope with the 60×/1.2 NA WI objective lens. TEM imaging in cells were conducted with Tecnai Spirit transmission electron microscope. All the nanoparticles were characterized on Scanning Electron Microscope (SEM, SU-8020, HITACHI, Inc.), Dynamic Light Scattering (DLS) and zeta potential (Malvern Nano ZS90, Malvern, Inc.), Transmission Electron Microscope (TEM, HITACHI H-7650B) after preparation. Transmission electron microscopy (TEM) images were obtained by a Hitachi 7650B microscope operated at 80 kV, and the TEM specimens of **AI-Cz-NP** were obtained by placing a drop of the nanoparticle suspension on a carbon-coated copper grid without further stain. TEM cell specimens with or without **AI-Cz-NP** treatment were stained with 2% uranium acetate and 2-4% lead citrate of double staining. All ^{1}H NMR spectra were recorded at 400 MHz or 500 MHz, respectively. ^{13}C NMR spectra were recorded at 100 MHz or 125 MHz, respectively. ^{19}F and ^{31}P spectra were recorded at 376 MHz and 162 MHz. HRMS was measured with Thermo Exactive Plus mass spectrometer for ESI.

2.2 Synthetic procedures and characterized data



Scheme S1. Synthesis of Compound **4**. Reagents and conditions: (i) Di-tert-butyl dicarbonate, DCM, 0 °C→ rt, reflux, 3 h; (ii) CH_3I, DIPEA, MeOH, 85 °C, overnight; (iii) TFA, DCM, rt, 0.5 h.

Compound **2**[1]

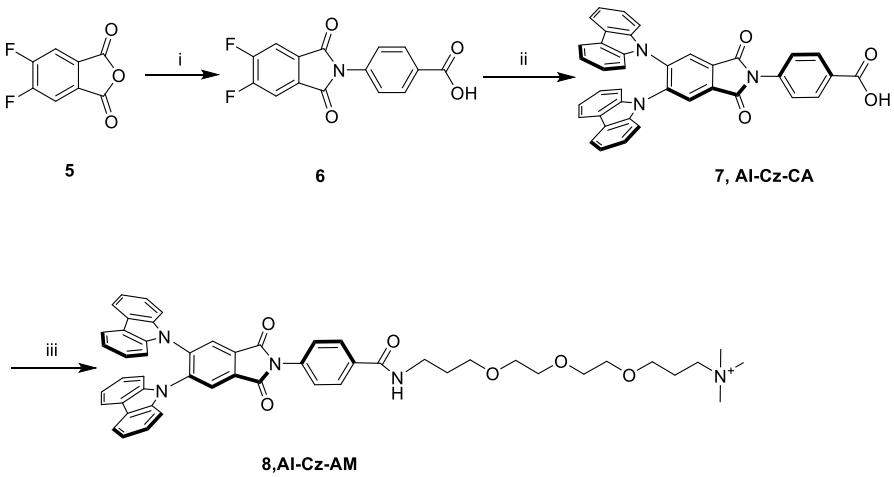
To a solution Compound **1** (1 g, 4.54 mmol) in DCM (3 mL) was added a solution of Boc_2O (0.099 g, 0.45 mmol) in DCM (2 mL) dropwise at 0 °C. After the addition was complete, the reaction mixture was allowed to warm to rt and after 3 h, it was quenched with H_2O (3 mL). The two layers were separated and the organic phase was washed with H_2O (3 × 3 mL) and brine (3 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give colorless liquid 0.083 g, yield 60 %. HRMS (*m*/*z*) $[M+H]^+$: calcd. for $C_{15}H_{33}O_5N_2$, 321.2384; found 321.2379.

Compound **3**

Compound **2** (0.16 g, 0.5 mmol), CH_3I (1.065 g, 7.5 mmol), DIPEA (388 mg, 3 mmol) was stirred in MeOH (5 mL) at 85 °C overnight. Reaction mixture was stirred in Et_2O, extraction filtration to give yellow solid 0.264 g (unable to purify, impurity: Compound **3** = 1:1, there is no production rate). HRMS (*m*/*z*) $[M]^+$: calcd. for $C_{18}H_{39}O_5N_2^+$, 363.2853; found 363.2850.

Compound **4**

A mixture of Compound **3** (263 mg, 0.72mmol) was stirred in TFA : DCM=1 : 1 (3 mL : 3 mL) at rt for 30 min. Washed with DCM (6 × 3 mL) and added DIPEA to adjust pH to 7.0. Direct next step. HRMS (*m*/*z*) $[M]^+$: calcd. for $C_{13}H_{31}O_3N_2^+$, 263.2329; found 263.2320.



Scheme S2. Synthesis of **AI-Cz-AM**. Reagents and conditions: (i) 4-aminobenzoic acid, acetic acid, reflux, 4 h; (ii) NaH, carbazole, DMF, 0 °C, 2 h; (iii) Compound **4** , HATU, DIPEA, DMF, rt, 1 h.

Compound **6**[2]

Compound **5** (1 g, 5.43 mmol) and 4-aminophenylacetic acid (1.12 g, 8.17 mmol) were added to acetic acid (30 mL) and the mixture was stirred and refluxed for 4 h. The solution was cooled down to 4 °C overnight, extraction filtration to give white solid 1.32 g, yield 80 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.14 (s, 1H, -COO*H*), 8.22 (t, *J* = 7.6 Hz, 2H, -Ar), 8.11 – 8.08 (m, 2H, -Ar), 7.61 – 7.59 (m, 2H, -Ar). ^{13}C NMR

(100 MHz, DMSO-d_6) δ 167.11, 165.33, 155.04, 154.94, 153.33, 153.23, 135.98, 130.33, 129.40, 127.35, 114.34, 114.30, 114.24, 114.20. HRMS (*m*/*z*) $[M+H]^+$: calcd. for $C_{15}H_8O_4NF_2$, 304.0416; found 304.0411.

Compound **7** (**AI-Cz-CA**)

Compound **6** (1 g, 3.30 mmol) was stirred in DMF (10 mL) at 0 °C and NaH (330 mg, 8.25 mmol) stage addition in an hour, then mixture with carbazole (1.65 g, 9.90 mmol) for 1 h. Extracted with EA (2 × 50 mL) and dried over Na_2SO_4. The residue was purified by silica gel column chromatography (PE : EA = 3 : 1) to give yellow solid 1.47 g, yield 75.6 %. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.48 (s, 2H, -Ar), 8.33 (d, *J* = 8.0 Hz 2H, -Ar), 7.80 – 7.78 (m, 4H, -Ar), 7.74 (d, *J* = 8.0 Hz 2H, -Ar), 7.15 (d, *J* = 8.0 Hz, 4H, -Ar), 7.11 – 7.05 (m, 8H, -Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 165.4, 139.7, 138.7, 136.5, 131.3, 130.6, 128.7, 126.1, 126.0, 125.9, 124.0, 121.1, 120.2, 109.3. HRMS (*m*/*z*) $[M+H]^+$: calcd. for $C_{39}H_{24}O_4N_3$, 598.1761; found 598.1755.

Compound **8 (AI-Cz-AM)**

Compound **7** (516 mg, 0.86 mmol), HATU (326 mg, 0.86 mmol), DIPEA (186.12 mg, 1.44 mmol) were stirred in DMF (6 mL) at rt for 30 min. Then added Compound **4** (189.36 mg, 0.72 mmol) for 0.5 h. Extracted with EA (2 × 50 mL) and dried over Na_2SO_4. The residue was purified by recrystallization in DCM, to give yellow solid 95 mg, yield 15 %. ^{1}H NMR (500 MHz, Acetone-d_6) δ 8.51 (s, 2H, -Ar), 8.11 (d, *J* = 7.0 Hz, 2H, -Ar), 7.91 (d, *J* = 3.5 Hz, 4H, -Ar), 7.74 (d, *J* = 6.5 Hz, 2H, -Ar), 7.34 (d, *J* = 5.0Hz, 4H, -Ar), 7.11 (d, *J* = 3.5 Hz, 8H, -Ar), 3.65-3.61 (m, 12H, -CH_2-), 3.59-3.55 (m, 4H, -CH_2-), 3.36 (s, 9H, -CH_2-), 2.21-2.15 (m, 2H, -CH_2-), 1.91 (t, *J* = 5.0 Hz, 2H, -CH_2-). ^{19}F NMR (376 MHz, DMSO-d_6) δ -69.17, -71.06. ^{31}P NMR (162 MHz, DMSO-d_6) δ -131.02, -135.41, -139.80, -144.19, -148.59, -152.98, -157.37. ^{13}C NMR (125 MHz, Acetone-d_6) δ166.58, 166.40, 140.34, 140.18, 135.52, 132.79, 128.68, 127.58, 126.65, 124.71, 121.63, 120.98, 110.57, 71.09, 71.08, 70.92, 70.88, 69.97, 68.07, 65.54, 53.66, 38.58, 30.60, 24.28. HRMS (*m*/*z*) $[M]^+$: calcd. for $C_{52}H_{52}N_5O_6^+$, 842.3912; found 842.3872.

3. Results and Discussion

3.1 Preparation of the AI-Cz-NP

To 5 mL of THF solution was added 500 μL of the **AI-Cz-AM** in THF (1.0 mM); then, under ultrasonic condition, the solution was rapidly injected into 12 mL of water without any surfactants. After the ultrasound was sustained for 3 min, the solution was bubbled by Ar for about 2 h to remove the THF. Then, by continual bubbling, the obtained solution was heated to 110 °C until 5 mL of water was remained. Thus, the uniformly distributed **AI-Cz-NP** were obtained.

3.2 Stability studies of AI-Cz-NP

Nanoparticles were stored at 4 °C, and their particle sizes were measured at 1, 3, 5, 8, 10, 14 and 28 days.

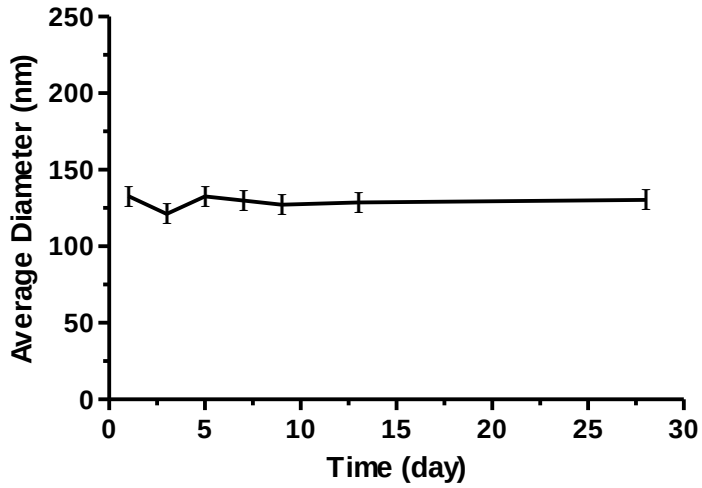


Fig. S1 The stability results indicated that **AI-Cz-NP** remained stable in particle size for at least 28 days.

3.3 Zeta potential of AI-Cz-NP

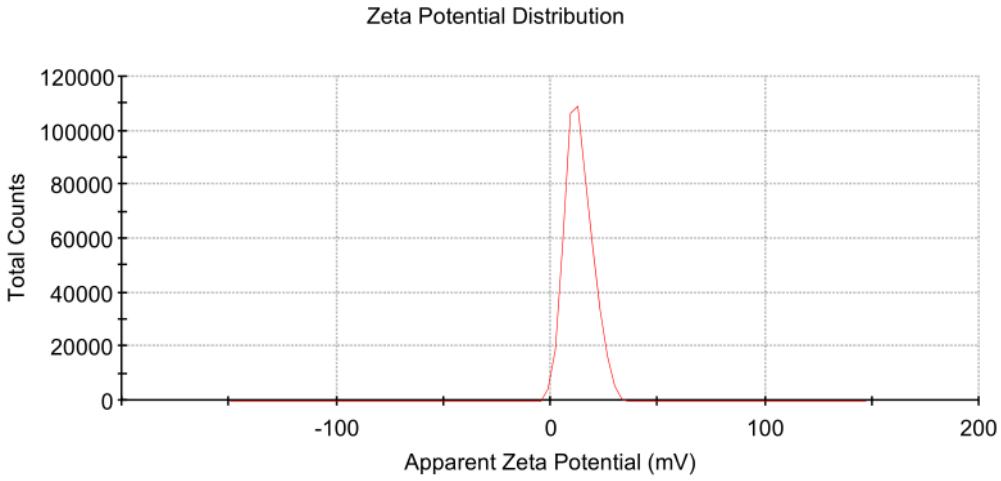


Fig. S2 Zeta potential analysis of **AI-Cz-NP**.

3.4 Molecular modeling of AI-Cz-AM

Molecular geometry of **AI-Cz-AM** was investigated using time-dependent density functional theory (TD-DFT) plus a PBE/DZP basis set with the Amsterdam Density Functional (ADF) 2018 program. The length of structural optimized **AI-Cz-AM** is about 3 nm.

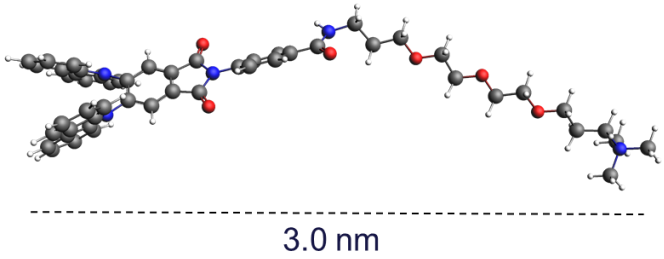


Fig. S3 The optimized structure of **AI-Cz-AM**, calculated by TD-DFT.

To obtain the configurations of self-assembled **AI-Cz-NP**, Molecular Dynamics (MD) Simulations were performed using the GROMACS (version 2018.4). Initially, 20 optimized **AI-Cz-AM** molecules were placed into a 10 nm ×10 nm ×10 nm simulation cubic box with TIP3P water model[3]. The system was placed under the NVT ensemble (T = 300 K) and balanced at 50 ps. Subsequently, the system was placed in the NPT ensemble (1 atmosphere, 300K) for 50 ns molecular dynamics simulation. The MD Simulations showed **AI-Cz-AM** could aggregate in water with the TADF skeleton placed inside and the hydrophilic chains outside. In addition, the particle size was getting larger with the increase of MD simulations running time.

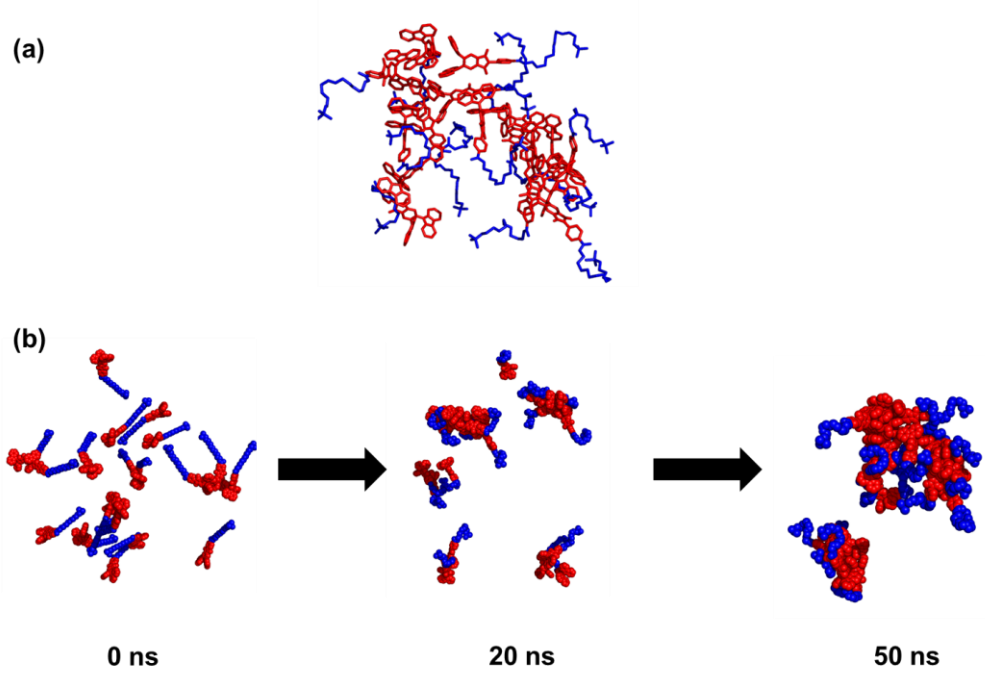


Fig. S4 (a) Molecular dynamics simulation at 50 ns. (b) MD simulations at 0 ns, 20 ns, 50 ns, respectively.

3.5 TADF characterized data of AI-Cz-AM and AI-Cz-NP

Table S1. The maximum fluorescence and phosphorescence wavelengths, S_1 and T_1 energy levels, ΔE_{ST} of **AI-Cz-AM** in THF and **AI-Cz-NP** in water at 77 K. Φ_F of **AI-Cz-AM** in THF and **AI-Cz-NP** in water at 298K.

Compound	Solvent	λ_{FL}[a] (nm)	E_{S1}(eV)	λ_{Phos}[b] (nm)	E_{T1}(eV)	ΔE_{ST}[c] (eV)	Φ_F[d](%)
AI-Cz-AM	THF	517	2.40	560	2.30	0.10	0.94
AI-Cz-NP	water	514	2.42	540	2.30	0.12	1.36

[a] Fluorescence emission peaks ($c_{\text{AI-Cz-AM}}$ = 10.0 μM, $c_{\text{AI-Cz-NP}}$ = 30.0 μM). [b] Phosphorescence emission peaks ($c_{\text{AI-Cz-AM}}$ = 10.0 μM, $c_{\text{AI-Cz-NP}}$ = 30.0 μM). [c] $\Delta E_{ST} = E_{S1} - E_{T1}$. [d] Absolute fluorescence quantum yield in THF.

Table S2. Fluorescence lifetime compositions of delayed components of **AI-Cz-AM** (10.0 μM) in THF after deoxygenating and **AI-Cz-NP** (30 μM) in water exposed to air. Excited at 380 nm and monitored at 585 nm.

Compound	τ_1[a] (μs)	n_1[b] %	τ_2[a] (μs)	n_2[b] %	τ[c] (μs)
AI-Cz-AM	0.835	2.020	6.093	97.98	6.078
AI-Cz-NP	2.103	13.97	10.950	86.03	10.683

[a] Obtained from the double-exponential fitting of transient decay curves on a 100 μs scale; [b] The contribution of each component to average lifetime; [c] The average fluorescence lifetime of delayed component.

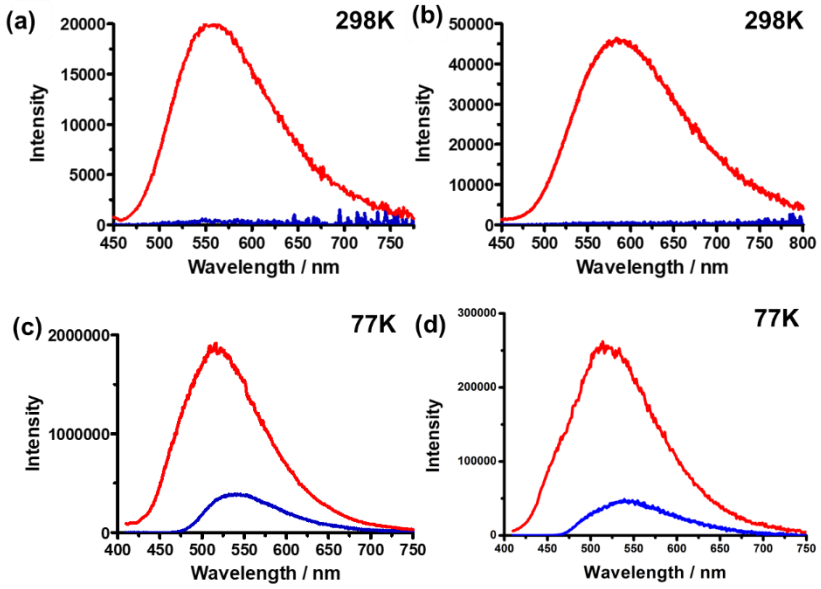


Fig. S5 Fluorescence (red) and phosphorescence (blue) spectra of **AI-Cz-NP** (a, c) in aqueous solution and **AI-Cz-AM** (b, d) in degassed THF solution at 298K (a, b) and 77K (c, d), excited at 390 nm.

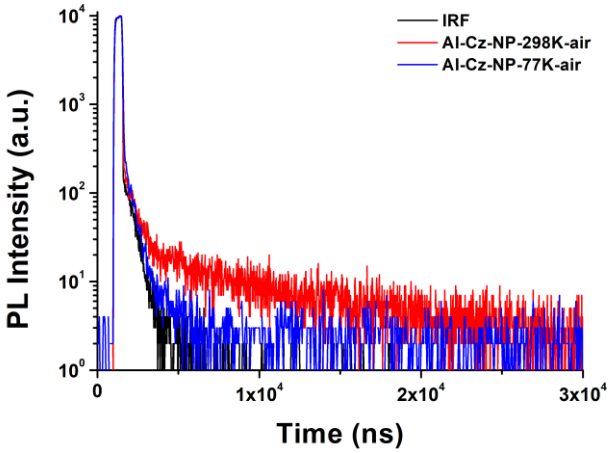


Fig. S6 Fluorescence emission decays of **AI-Cz-NP** (30 μM) in the water at different temperature. Red curve represents the profile at 298K under air, blue curve represents the profile at 77K under air and black curve represents instrument response function (IRF). Excited at 390 nm and monitored at 556 nm.

Table S3. Fluorescence lifetime compositions of delayed components of **AI-Cz-NP** (30 μM) in water in different condition. Excited at 390 nm and monitored at 556 nm.

Compound	τ_1[a] **(ns)**	n_1[b] **%**	τ_2[a] **(ns)**	n_2[b] **%**	τ[c] **(ns)**
298K-air	6.29	94.71	7253.54	5.29	7142.74
77K-air	18.06	97.34	327.39	2.66	120.53

[a] Obtained from the double-exponential fitting of transient decay curves on a 40000 ns scale; [b] The contribution of each component to average lifetime; [c] The average fluorescence lifetime of delayed component.

3.6 CMC and CAC measurements of AI-Cz-AM

Critical micelle concentration (CMC): First, the stock solution (1 mM in THF) was diluted to different concentrations with water. In each solution, 5 μL pyrene in THF solution (2×10^{-4} M) was added to 2 mL **AI-Cz-AM** solution to produce the final pyrene concentration at 5×10^{-7} M. The fluorescence spectra were recorded with the excitation wavelength of 334 nm. The I_1 and I_3 values were measured as the maximum emission intensity at 373 and 383 nm, respectively. Plot of the pyrene I_1/I_3 ratio against log value of **AI-Cz-AM** concentration. Critical aggregation concentration (CAC): CAC values obtained through emission intensity of **AI-Cz-AM** at different log value of **AI-Cz-AM** concentration when the water fraction is 99%. λ_{ex} = 390 nm and λ_{em} = 540 nm.

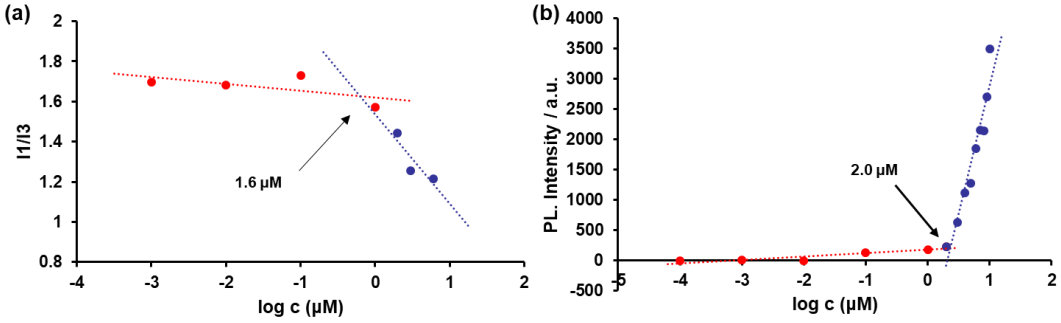


Fig. S7 (a) Plot of the pyrene I_1/I_3 ratio against log value of **AI-Cz-AM** concentration. (b) CAC values obtained through emission spectra of **AI-Cz-AM** at different log value of **AI-Cz-AM** concentration.

3.7 HepG 2 cell line and culture conditions

HepG 2 cell line was purchased from American Type Culture Collection (ATCC), USA. HepG 2 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) (Corning) containing 10 % fetal bovine serum (Invitrogen) and 1 % penicillin−streptomycin (Corning). All cell lines were maintained at a humidified incubator with 5 % CO_2 at 37 °C.

3.8 Cytotoxicity assay

The cytotoxicity of **AI-Cz-AM** and **AI-Cz-NP** were determined by a standard MTS assay *in vitro*. HepG 2 cells were seeded at a density of 1×10^4 cells/mL per well in 96-well flat bottom microtitration plates with 100 μL per well, incubated in a humidified 5 % CO_2 atmosphere at 37 °C for 24 h, then exposed to different concentrations of **AI-Cz-AM** (0-1.6 μM) and **AI-Cz-NP** (0-25 μM) for 24 h, respectively. After treatment, 20 μL MTS solution was added to each well and continued to incubated for 3 h. After 3 h incubation at 37 °C, the absorbance was measurement at 490 nm with Tecan Spark™ 10M Multimode Microplate Reader. Cell survival rate (%) = $(A-A_0)\ /(A_s-A_0)\ \times100$, where A is the absorbance of the experimental group, A_s is the absorbance of the control group, and A_0 is the absorbance of the blank group (no cells). The experiment was repeated three times.

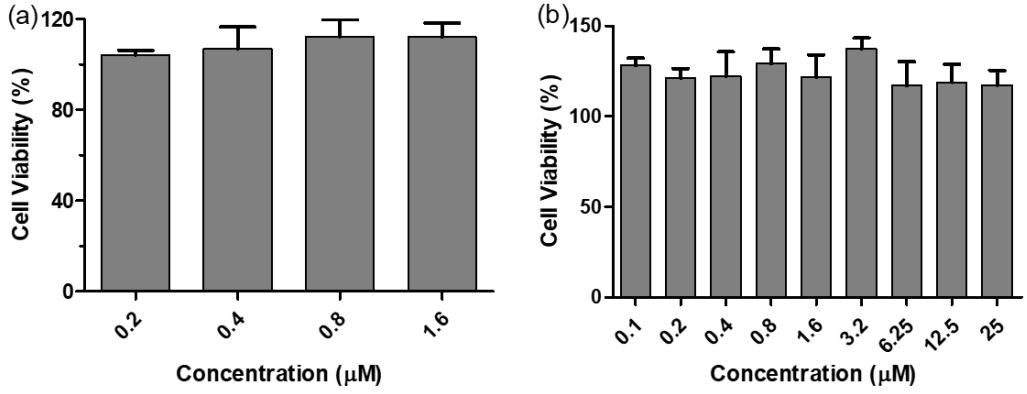


Fig. S8 Cytotoxicity of different concentrations of **AI-Cz-AM** and **AI-Cz-NP** to HepG 2 cells by a standard MTS assay. The experiment was repeated three times and the data are shown as mean ± SD.

3.9 Confocal imaging of HepG 2 cells treated with AI-Cz-AM and AI-Cz-NP

Upon reaching 80 % confluence, HepG 2 cells (300 μL, 4×10^4 cells/mL) were transferred into an 8-well chamber. On the following day, the media were removed and the cells were washed twice with PBS buffer, then incubated with **AI-Cz-AM** (1 μM) or **AI-Cz-NP** (10 μM) in DMEM medium, respectively. After incubation at 37 °C for 2 h, the cells were washed three times with PBS buffer and were observed with Leica TCS SP8 X Confocal Microscope using 63× magnification. All the probes were excited at 405 nm and the fluorescence was monitored at 500−600 nm.

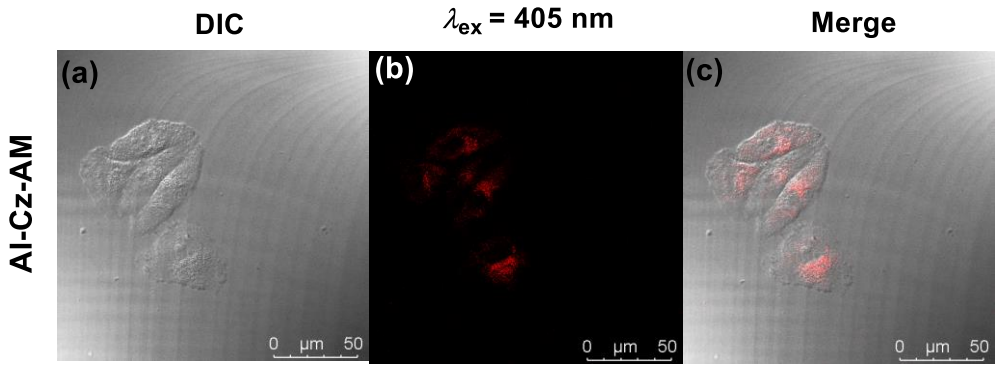


Fig. S9 CLSM images of HepG 2 cells incubated with 1 μM **AI-Cz-AM** (a-c). λ_{ex} = 405 nm and λ_{em} = 500−600 nm. Scale bar = 50 μm.

3.10 FLIM imaging of HepG 2 cells treated with AI-Cz-NP

HepG 2 cells were precultured in 24-well plates containing cell culture coverslips to achieve 80 % confluence. Then the medium was removed and the cells were incubated with **AI-Cz-NP** (10 μM) in fresh DMEM medium at 37 °C for 2 h, respectively. After labeling, the cells were washed twice with PBS buffer solution and then fixed by 4 % paraformaldehyde. Then, the cells were washed three times with PBS buffer and used for fluorescence lifetime imaging. Fluorescence lifetime images were taken by ISS Q2 confocal laser scanning system coupled to a Nikon TE2000 microscope with the 60×/1.2 NA WI objective lens. The excitation wavelength of the probe is 405 nm (20000Hz repetition rate), fluorescence emission and lifetime signals were collected through 500nm-550nm long-pass edge filter.

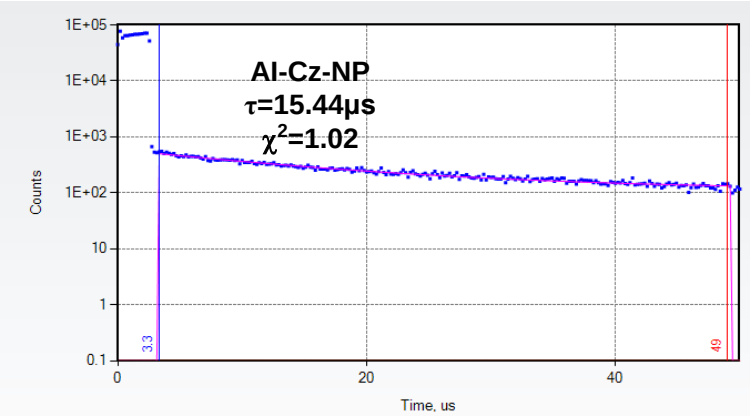


Fig. S10 Corresponding fluorescence lifetime decay curve with a reliable one exponential fitting of **AI-Cz-NP** in HepG 2 cells.

3.11 TEM imaging of HepG 2 cells treated with AI-Cz-NP

Cells in cell culture dish with or without **AI-Cz-NP** treatment were fixed with 2 % glutaraldehyde and 2 % paraformaldehyde in PBS buffer, pH 7.2, at 4 °C for 1 h. The samples were then washed in PBS followed by washing in 0.1M cacodylate buffer, pH 7.2, and post-fixed in 1 % osmium tetroxide in 0.1M cacodylate buffer for 1 hour at room temperature. The samples were then washed briefly in dH_2O and dehydrated through a graded ethanol series. Infiltrated and embedded with EPON epoxy resin. The embedding samples at 60 °C

for 24-48h. Make ultrathin slices and collected onto copper grids and stained with 2% uranium acetate and 2-4% lead citrate of double staining and images were collected using TEM (Tecnai Spirit).

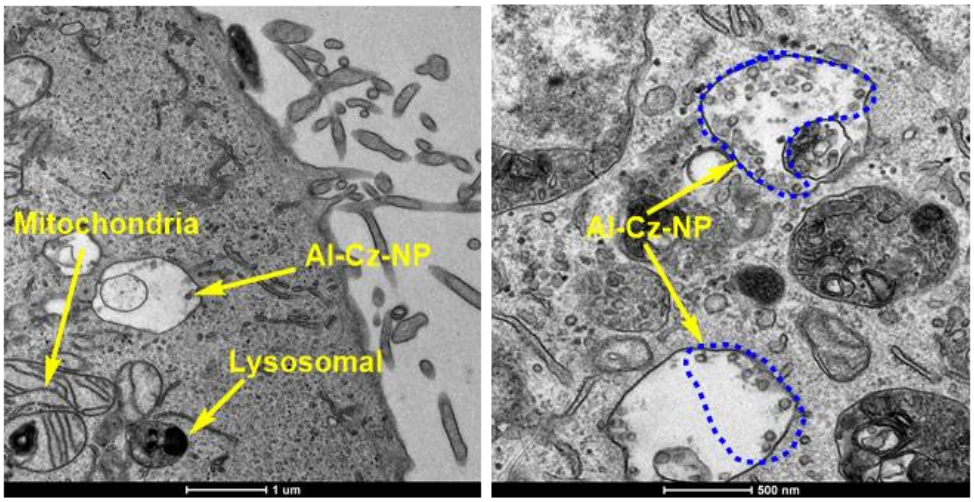


Fig. S11 TEM images of **AI-Cz-NP** in HepG 2 cells, the scale bar is 1 μm and 500 nm. Subcellular organelles, for example, mitochondria and lysosomal, were observed with **AI-Cz-NP** nearby.

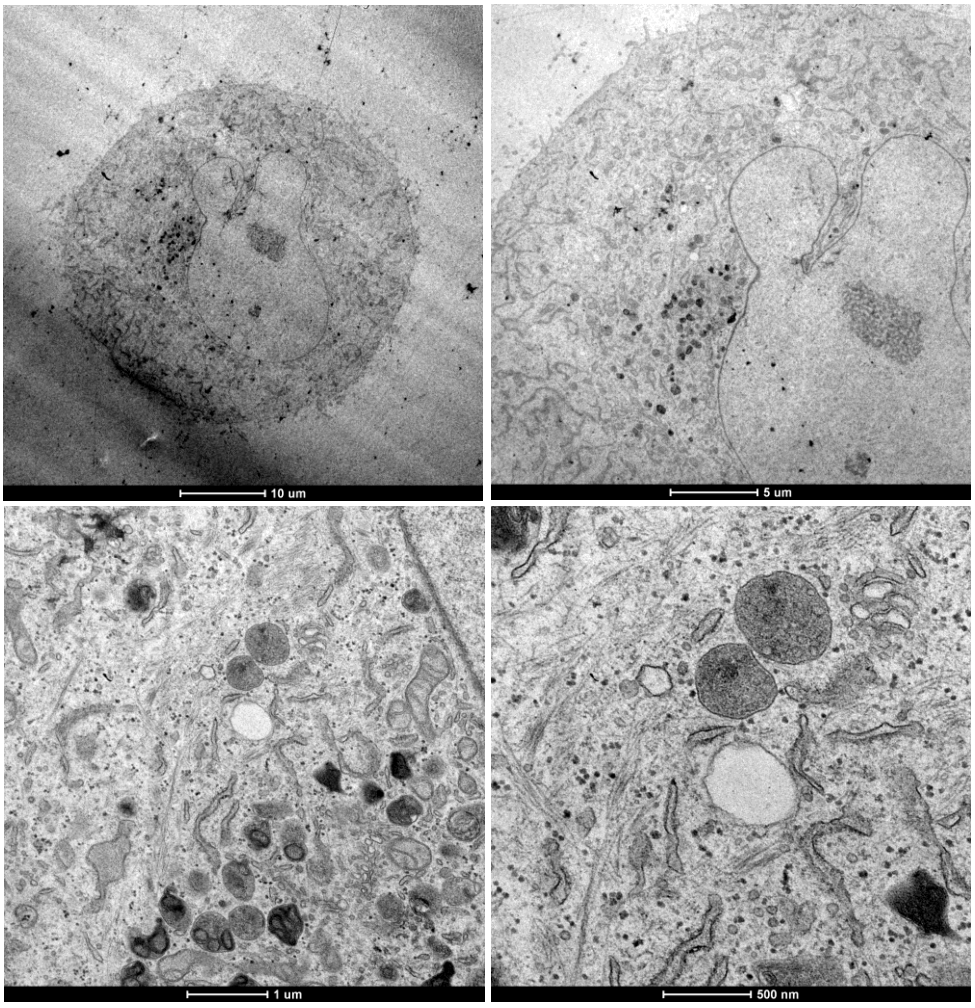
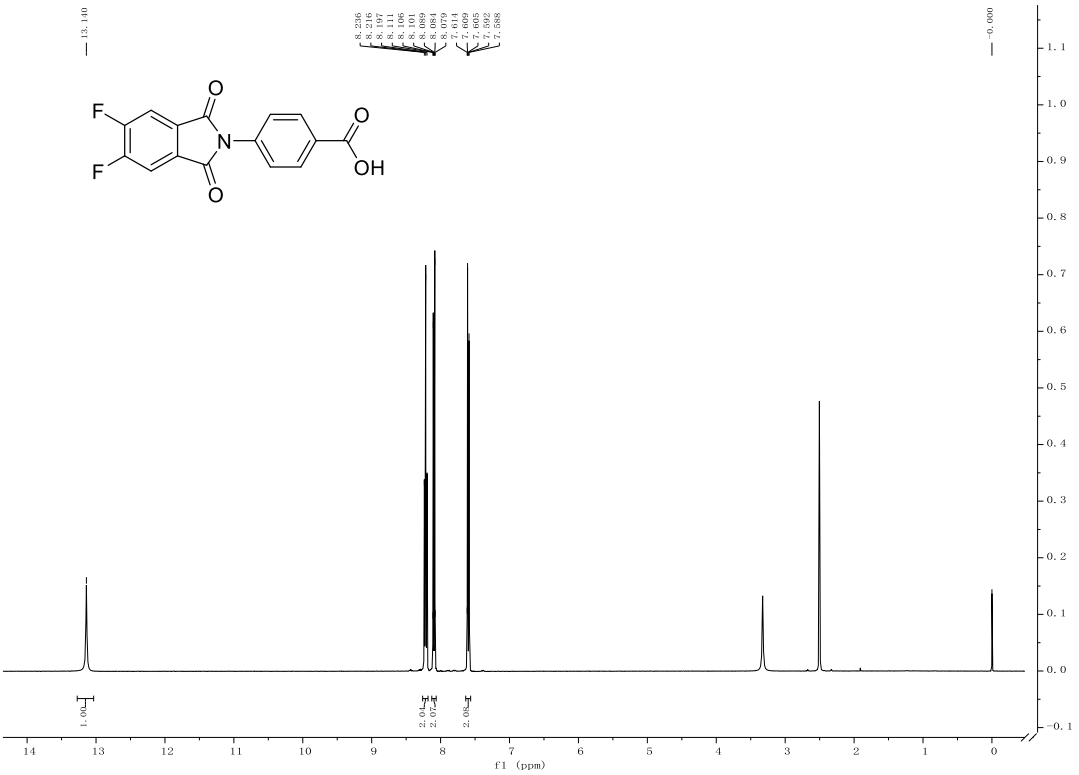
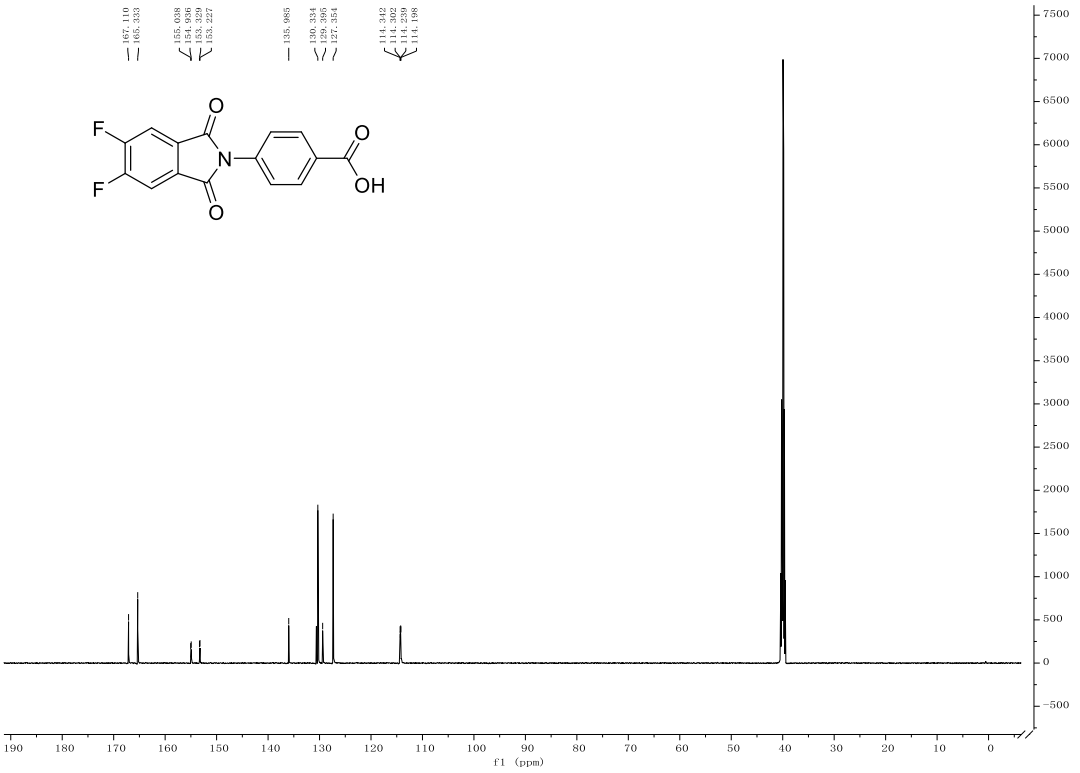


Fig. S12 The TEM image without **AI-Cz-NP** in HepG 2 cells at different magnifications.

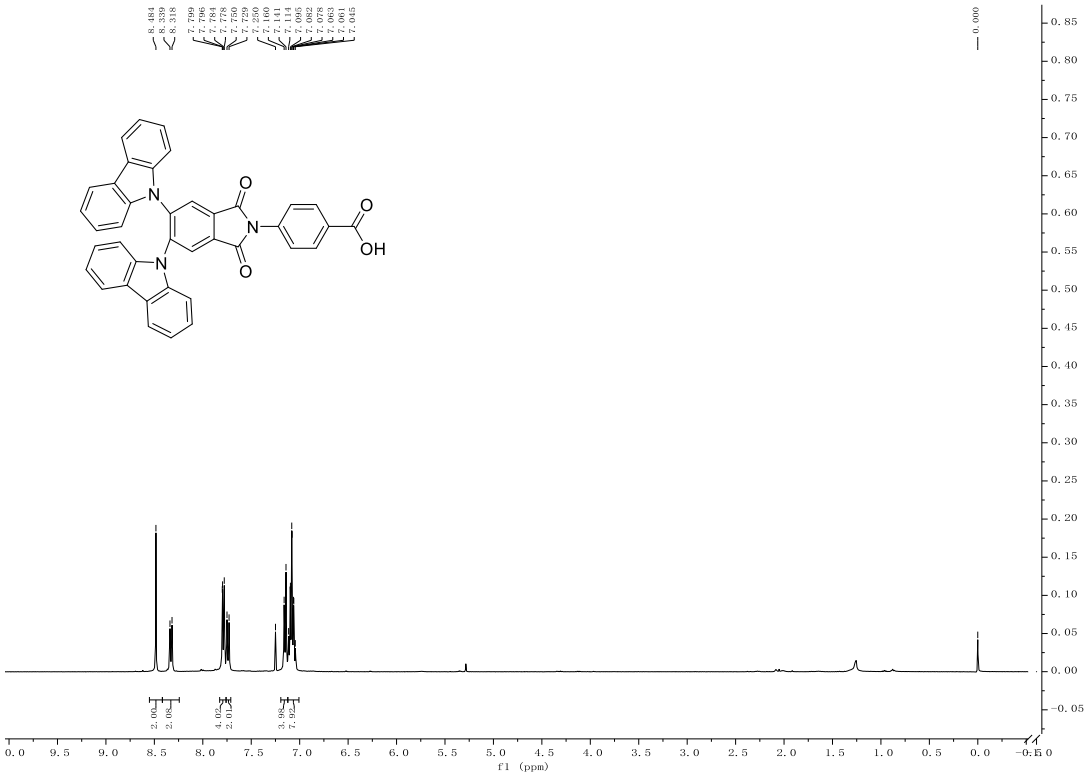
4. Copies of NMR spectrum of compounds



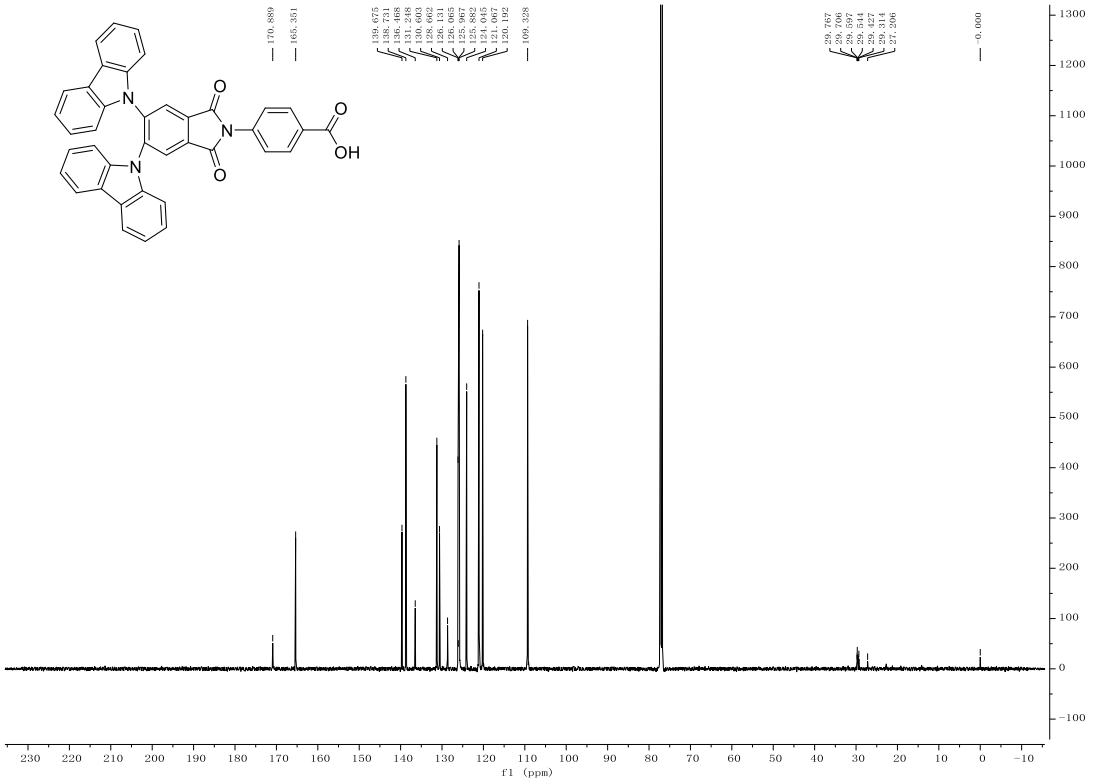
^{1}H NMR (400 MHz, DMSO-d_6) of compound **6**



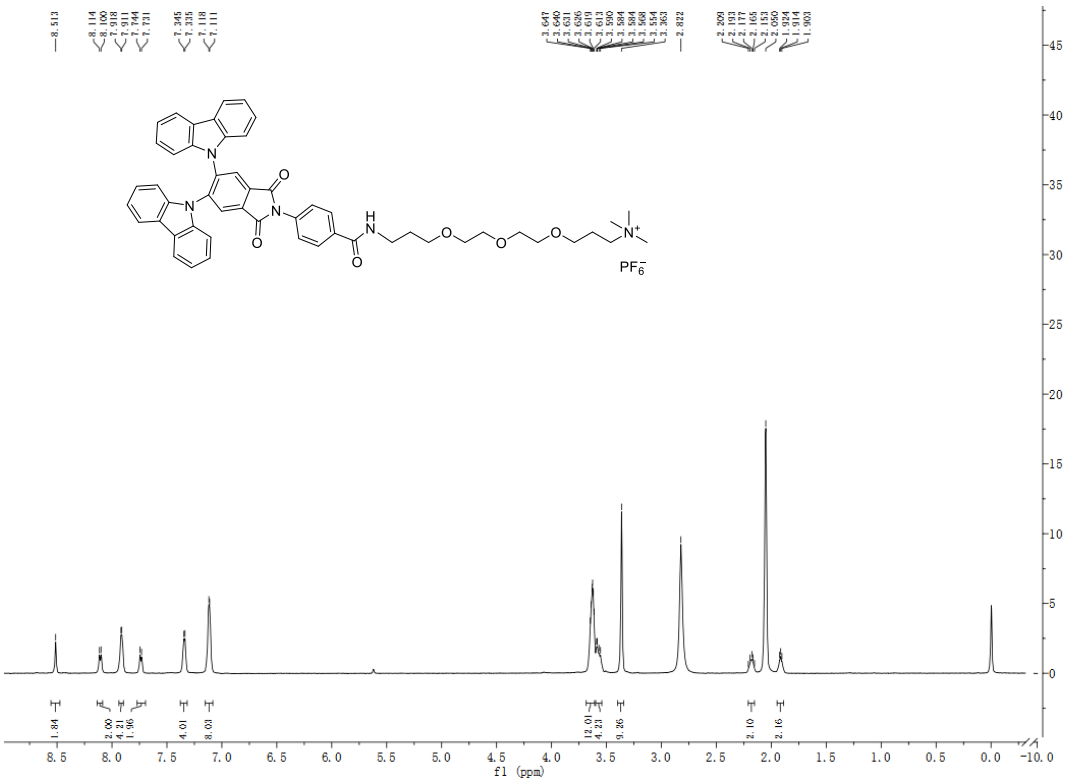
^{13}C NMR (100 MHz, DMSO-d_6) of compound **6**



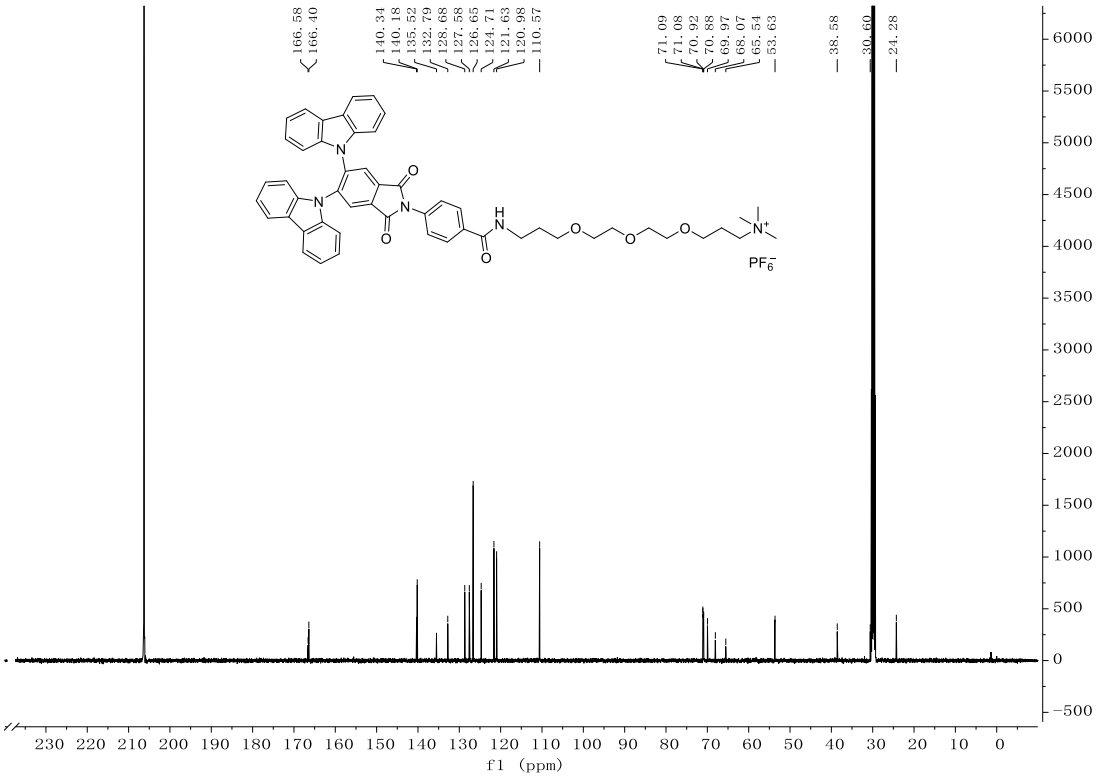
^{1}H NMR (400 MHz, $CDCl_3$) of compound **7**



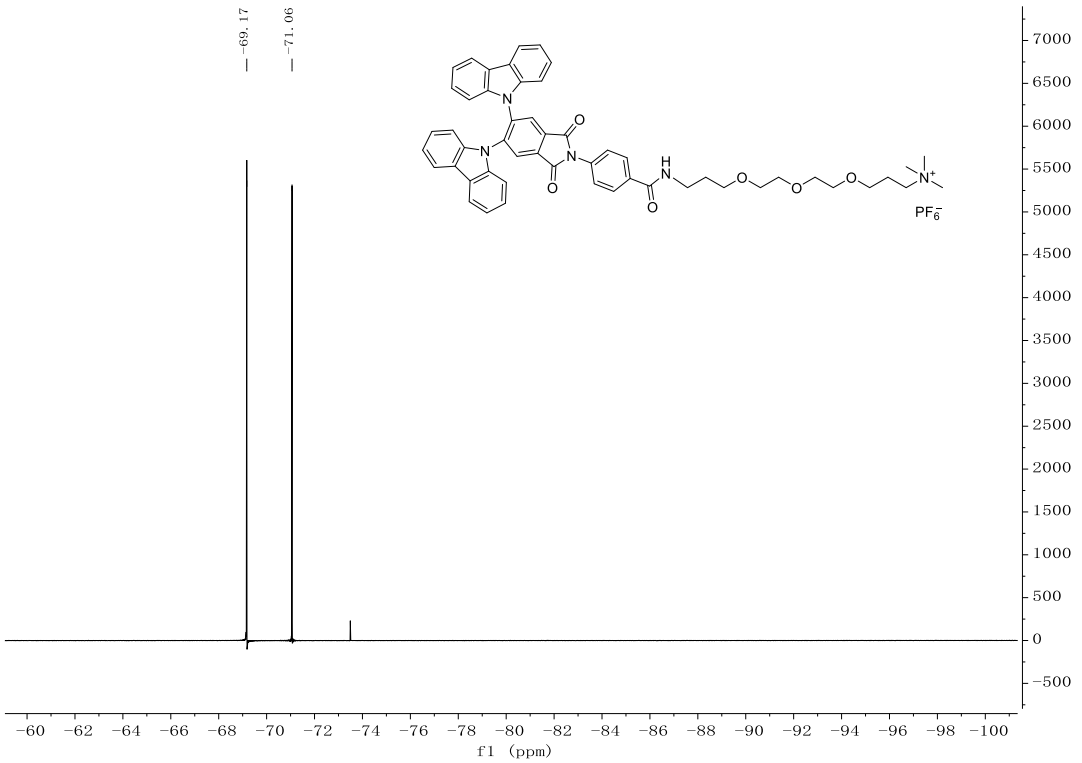
^{13}C NMR (100 MHz, $CDCl_3$) of compound **7**



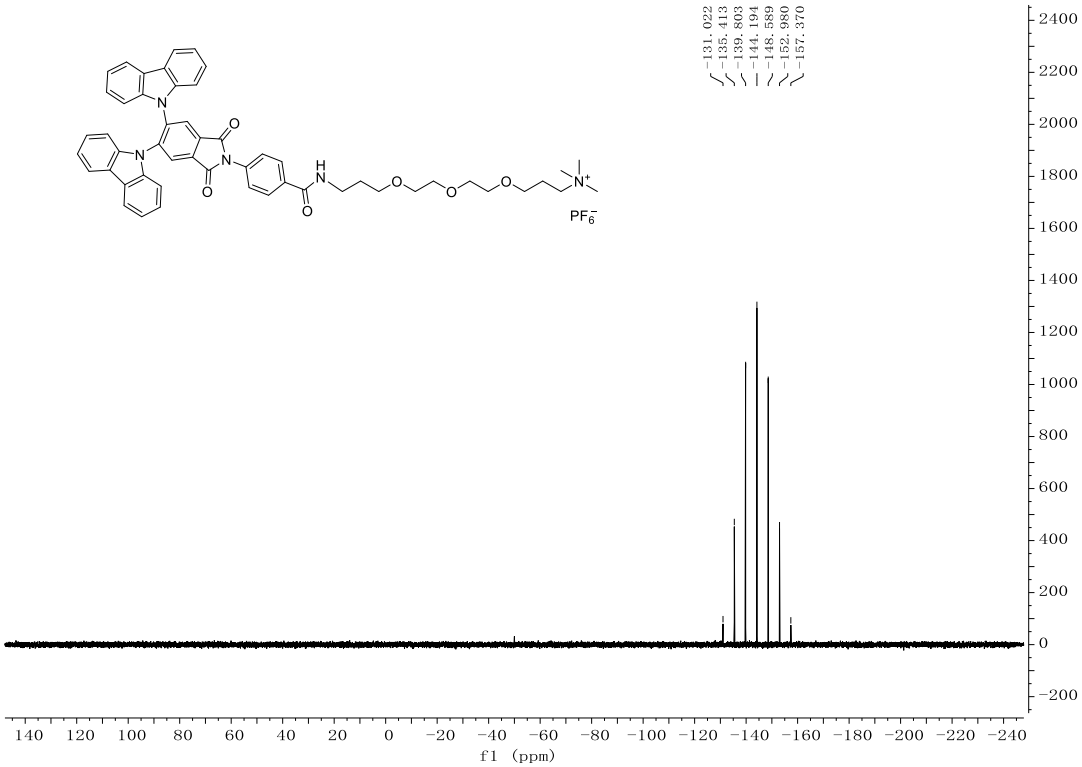
^{1}H NMR (500 MHz, Acetone-d_6) of **AI-Cz-AM**



^{13}C NMR (125 MHz, Acetone-d_6) of **AI-Cz-AM**



^{19}F NMR (376 MHz, DMSO-d_6) of **AI-Cz-AM**



^{31}P NMR (162 MHz, DMSO-d_6) of **AI-Cz-AM**

5. References

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[2] Q. Zhang, S. Xu, M. Li, Y. Wang, N. Zhang, Y. Guan, M. Chen, C. F. Chen, H. Y. Hu, Chem Commun (Camb) 2019, 55 (39), 5639.

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