

Supporting Information

***In Situ* Construction of Self-assembly AIE Probe for Tumor Hypoxia Imaging**

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Materials and Methods

(4-aminophenyl)methanol, 4-nitrophenyl chloroformate, Poly (ethylene glycol) methyl ether methacrylate (OEGMA, $M_n \approx 468$) were purchased from HEOWNS Technologies, LLC. Tianjin. 1-bromo-2-(2-(2-methoxyethoxy)ethoxy)ethane was purchased from TCI (Shanghai) Development Co., Ltd. 2-aminoethyl methacrylate hydrochloride, 4,4'-dihydroxybenzophenone were purchased from J&K Chemical. The phosphate buffer (pH=7.2-7.4) was acquired from Beijing Solarbio Co., Ltd. Human cervical cancer cells line HeLa were obtained from China Infrastructure of Cell Line Resource and cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) supplemented with 10% heat inactivated FBS (Biolind), 1% penicillin/streptomycin (Beyotime) at 37°C and 5% CO₂. If it was not mentioned specifically, other reagents and solvents were purchased commercially. And all the reactants and solvents were used without further purification. Ultrapure water was obtained from a Milli-Q water purification system (resistivity > 18 MΩ · cm).

¹H NMR spectra were measured by a JEOL JNM-ECA 600 NMR spectrometer. The UV-vis spectra were characterized with an Agilent Cary 300 UV-vis spectrophotometer. The fluorescence spectra were recorded on a HITACHI F-7000 fluorescence spectrophotometer. The measurements of nano particle size were performed on ALV/DLS/SLS-5022F Laser Light Scattering System with a solid-state 17 mW He-Ne laser ($\lambda = 633$ nm). And TEM observation was carried out with a JEM-2010 High Resolution TEM. Cellular imaging was measured by Nikon A1RMP multiphoton microscope and Olympus fluorescence microscope.

Experimental Procedures

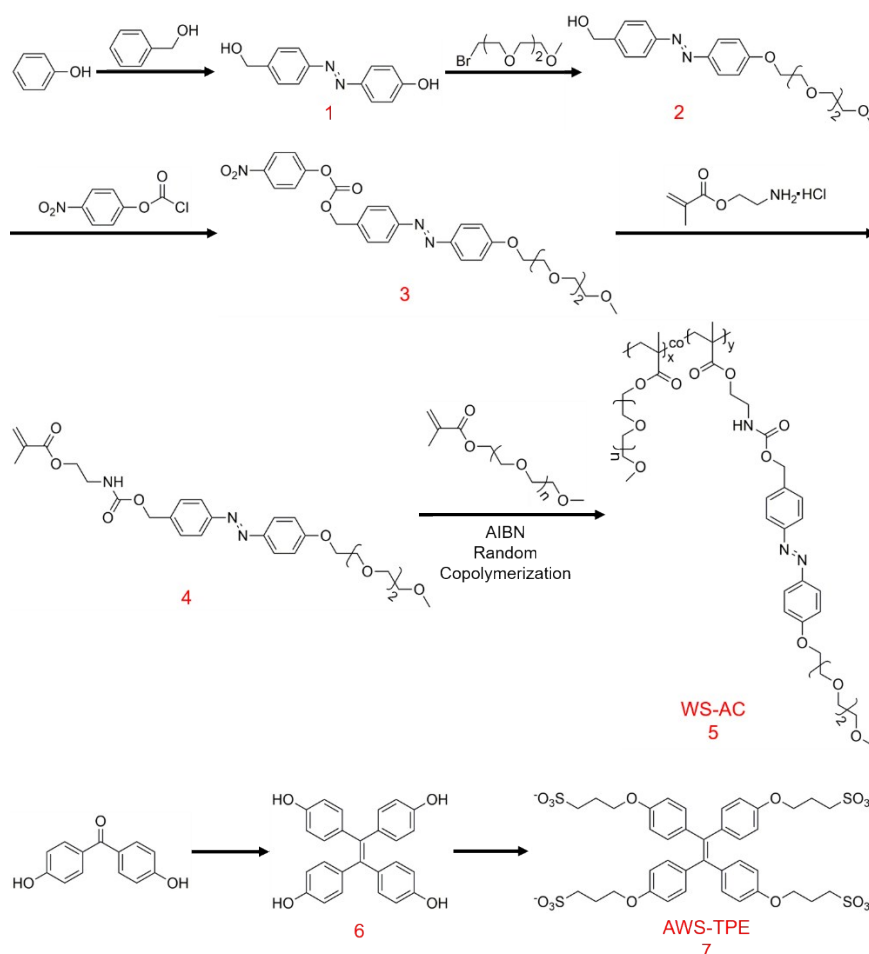


Fig. S1 The synthesis route of the WS-AC (**5**) and AWS-TPE (**7**).

Synthesis of 4-((4-(hydroxymethyl)phenyl)diazenyl)phenol (1)

Phenol (5.1 g, 55 mmol) was dissolved in a solution of NaOH (5 g, 0.125 mol in 70 mL of water). Then the solution of phenol was kept in ice-water bath with violently stirring. A diazonium salt of (4-aminophenyl)methanol was prepared by adding an aqueous solution of sodium nitrite (4 g, 58 mmol in 20 mL of water) into a mixture of (4-aminophenyl)methanol (6.65 g, 55 mmol), HCl (36%, 16 mL) and H₂O 32 mL. The mixture was stirred at 0 °C for 15 min and then was added dropwise into the aqueous solution of phenol. After addition, the reaction mixture was then kept at room temperature for 12 h to afford the crude product. Then the crude product was recrystallized in methanol for 2 times to afford the dark yellow solid product (1). (8 g, 35 mmol). Yield: 80%. ¹H NMR (600 MHz, DMSO-d₆) δ (TMS, ppm): 4.55(d, 2H), 5.31(t, 1H), 6.90(d, 2H), 7.45(d, 2H), 7.75(dd, 4H), 10.25(s, 1H).

Synthesis of 4-((4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)diazenyl)phenyl)methanol (2)

1-bromo-2-(2-(2-methoxyethoxy)ethoxy)ethane (3 g, 13.2 mmol), (1) (2.7 g, 11.8 mmol), K₂CO₃ (3 g, 21.7 mmol) and KI (0.23 g, 1.4 mmol) was dissolved in DMF (80 mL) and refluxed at 85 °C for 20 h. The solvent was removed under reduced pressure, and washed the crude product with water for 3 times. Then the above solution was extracted with dichloromethane. Further purification was carried out on column chromatography (silica gel, dichloromethane/methanol = 25/1) to afford the orange solid product (2) (3.7 g, 10 mmol). Yield: 65%. ¹H NMR (600 MHz, DMSO-d₆) δ (TMS, ppm): 3.19 (s, 3H), 3.37-3.40 (m, 2H), 3.46-3.51 (m, 4H), 3.54-3.57 (m, 2H), 3.70-3.76 (m, 2H), 4.13-4.18 (m, 2H), 4.56 (d, 2H), 5.34 (t, 1H), 7.09 (d, 2H), 7.47 (d, 2H), 7.79 (d, 2H), 7.84 (d, 2H).

Synthesis of 4-((4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)phenyl)diazenyl)benzyl (4-nitrophenyl) carbonate (3)

(2) (3.2 g, 8.5 mmol) was dissolved in tetrahydrofuran (THF, 80 mL) and stirred violently in ice-water bath. 4-nitrophenyl chloroformate (2.6 g, 13 mmol) was dissolved in 10 mL THF and added dropwise into above THF solution and kept reaction at 0 °C for 8 h. The solvent was removed under reduced pressure, and washed the crude product with saturated NaCl aqueous solution for 3 times, then dried over anhydrous MgSO₄. Then the above solution was extracted with dichloromethane. Further purification was carried out on column chromatography (silica gel, dichloromethane/methanol = 80/1) to afford the orange solid product (3) (4.6 g, 8.5 mmol). Yield: 80%. ¹H NMR (600 MHz, DMSO-d₆) δ (TMS, ppm): 3.19 (s, 3H), 3.36-3.40 (m, 2H), 3.49 (dt, 4H), 3.54-3.58 (m, 2H), 3.71-3.76 (m, 2H), 4.15-4.20 (m, 2H), 5.36 (s, 2H), 7.11 (d, 2H), 7.56 (d, 2H), 7.63 (d, 2H), 7.86 (dd, 4H), 8.29 (d, 2H).

Synthesis of Azo monomer (4)

(3) (3.2 g, 6 mmol), TEA (1.2 g, 12 mmol) and DMAP (1.4 g, 11 mmol) was dissolved in dichloromethane (DCM, 90 mL) and kept in ice-water bath. 2-aminoethyl methacrylate hydrochloride (2 g, 12 mmol) was dissolved in 5 mL dichloromethane and added dropwise into above dichloromethane solution and kept reaction at 0 °C for 2 h. Then the reaction was kept at r.t. for 8 h. The crude product was washed with saturated sodium carbonate aqueous solution for 2 times and dried over anhydrous MgSO₄. Then the above solution was extracted with dichloromethane. Further purification was carried out on column chromatography (silica gel, dichloromethane/methanol = 100:1 to 80/1) to afford the orange solid (3.6 g, 6.8 mmol). Yield: 70%. ¹H NMR (600 MHz, DMSO-d₆) δ (TMS, ppm): 1.83 (s, 3H), 3.20 (s, 3H), 3.28 (q, 2H), 3.39 (dd, 2H), 3.47-3.52 (m, 4H), 3.57 (dd, 2H), 3.72-3.76 (m, 2H), 4.07 (t, 2H), 4.17-4.20 (m, 2H), 5.08 (s, 2H), 5.60-5.66 (m, 1H), 6.02 (s, 1H), 7.11 (d, 2H), 7.49 (d, 2H), 7.80 (d, 2H), 7.85 (d, 2H).

Synthesis of WS-AC (5)

Azo monomer (4) (133 mg, 0.25 mmol), OEGMA (100 mg, 0.05 mmol) and AIBN (0.82 mg, 0.005 mmol) was dissolved in DMF (2 mL) and kept the reaction at 70 °C for 12h with violently stirring. Then the crude product was precipitated in 30 mL diethyl ether for 2 times to afford the orange viscous liquid (200 mg). Yield: 85%. Mn = 28000, Mw=36000, PDI = 1.3. ¹H NMR (600 MHz, DMSO-d₆) δ (TMS,

ppm): 3.30-3.70 (m, oligoethylene glycol chain), 7.04-7.90 (br, Azo benzene ring). ¹H NMR (600 MHz, D₂O) δ (TMS, ppm): 3.33-3.87 (m, oligoethylene glycol chain), 6.66-7.98 (br, Azo benzene ring).

Synthesis of AWS-TPE (7)

Synthesis route of TPE-4OH (6) have already been reported before.^[1] TPE-4OH (500 mg, 0.96 mmol) was dissolved in 20 mL anhydrous ethanol and kept in ice-water bath. Sodium ethoxide (400 mg, 5.88 mmol) dissolved in 2 mL ethanol was added dropwise in the above solution for 1 h. And then, 1, 3-propane sultone (660 mg, 5.4 mmol) in 5 mL of ethanol was added. Then the reaction was kept at r.t. for 8 h. The crude product was collected by filtration and washed with ethanol and acetone respectively to afford the white solid. (800 mg, 0.80 mmol) Yield: 69%. ¹H NMR (600 MHz, DMSO-d₆) δ (TMS, ppm): 1.93 (d, 8H), 2.49-2.54 (m, 8H), 3.91-3.93 (m, 8H), 6.63 (d, 8H), 6.79 (d, 8H).

Enzyme Treatment.

Freshly prepared **WS-AC** suspension (0.1 mg/mL, 0.5 mL) and **AWS-TPE** ((0.05 mg/mL, 0.5 mL) was added into a 2 mL centrifuge tube. rat liver microsomes solution (0.2 mL, 1 mg/mL, pH=7.2-7.4 in phosphate buffer) was added into above solution. Then the centrifuge tube was put onto the Thermo-Shaker to keep the temperature at 37 °C. After that, the reduction reaction was initiated by the addition of NADPH solution (0.2 mL, 2 mg/mL, pH 7.2~7.4 in phosphate buffer). The reaction mixture was kept at 37 °C overnight under argon atmosphere.

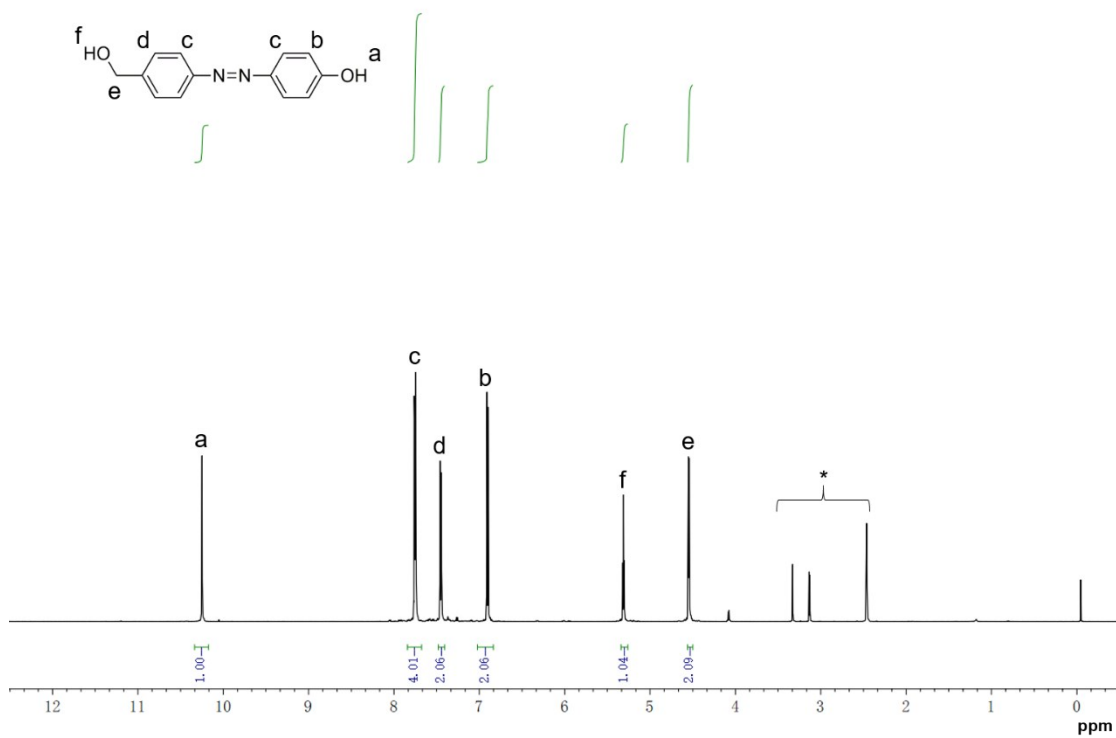
3D multicellular spheroid formation.

3D multicellular spheroids were formed in agarose-coated 96-well plates as previously described.^[2] Two hundred microliters of a 2.5×10⁴ cells/mL single cell suspension was plated onto agarose-coated (sterile, 1.5% (w/v) in DMEM) 96-well microplates. Plates were centrifuged for 15 min at 1500 rcf. The cells were allowed to aggregate for 96 h without agitation, resulting in the formation of single spheroids.

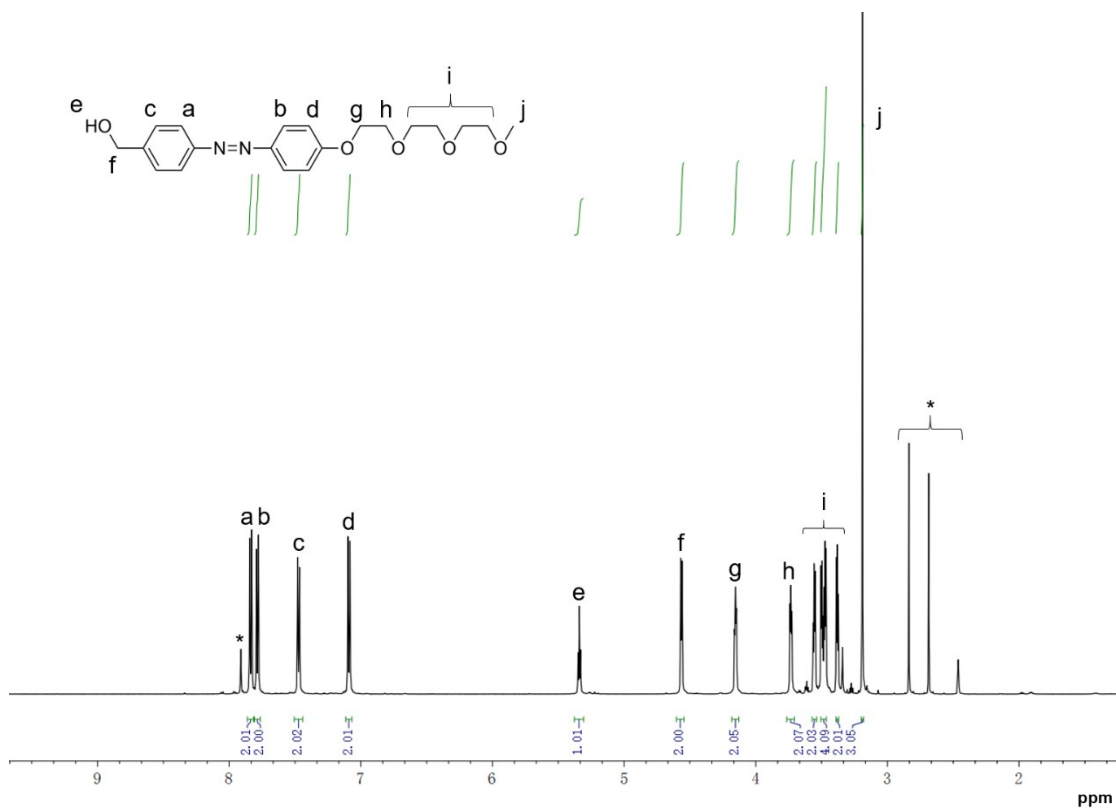
Cellular imaging by fluorescence microscope.

Human cervical cancer cells line HeLa was chosen as cell model. 3D multicellular spheroids were harvested after approximately 4 days of growth. Test compounds (**WS-AC** and **AWS-TPE**) were then added to the wells to achieve final concentrations of 0.1 mg/mL and 0.05 mg/mL respectively. The fluorescence images were captured by nikon A1RMP multiphoton microscope (10×).

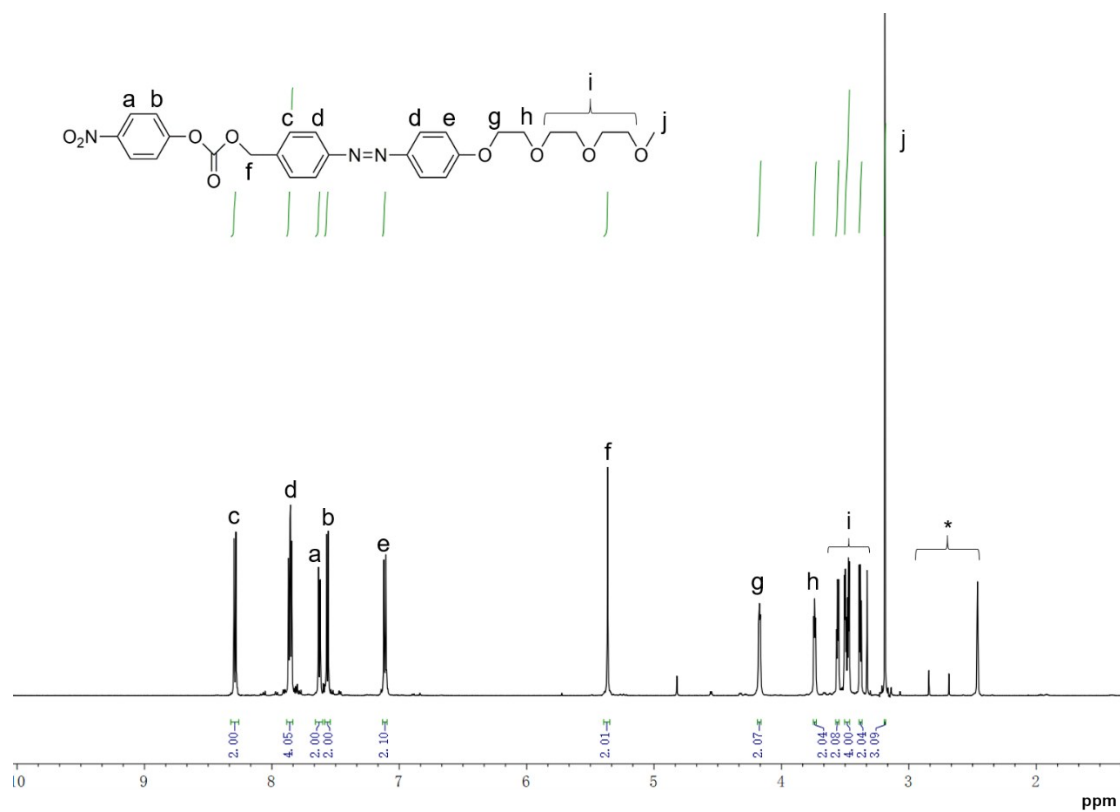
Characterization



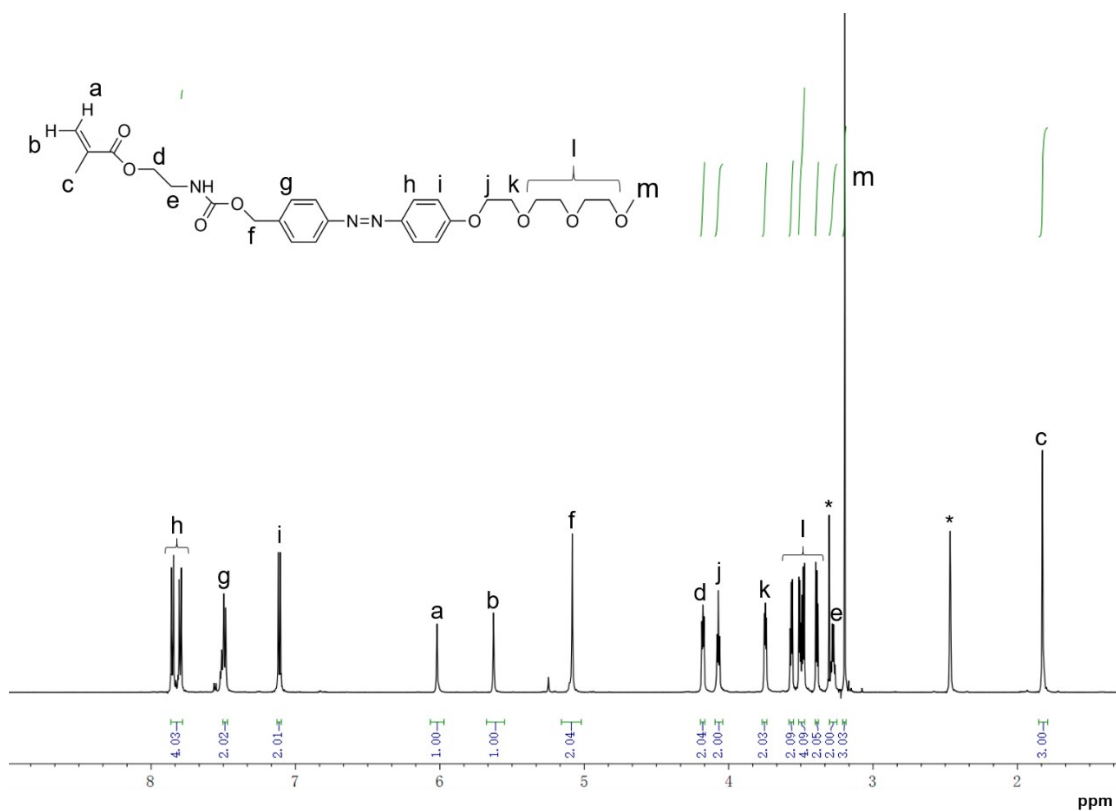
(a). ¹H NMR spectra of **1** in DMSO-d₆.



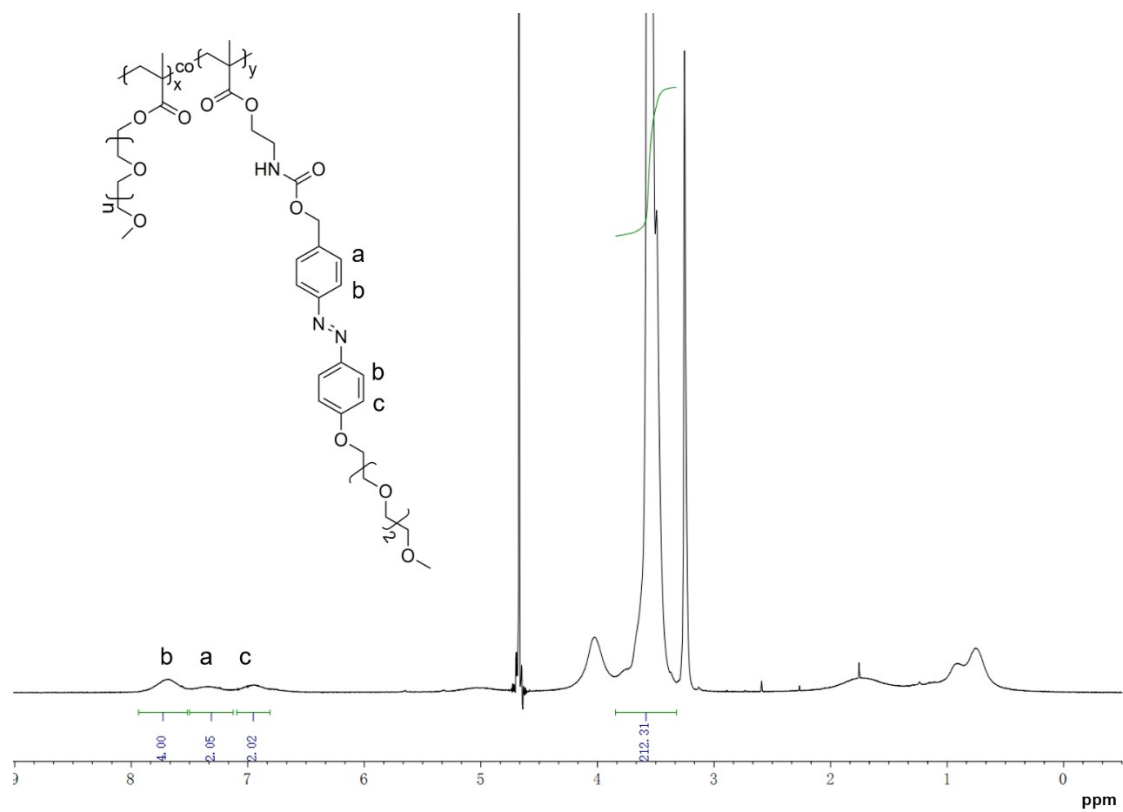
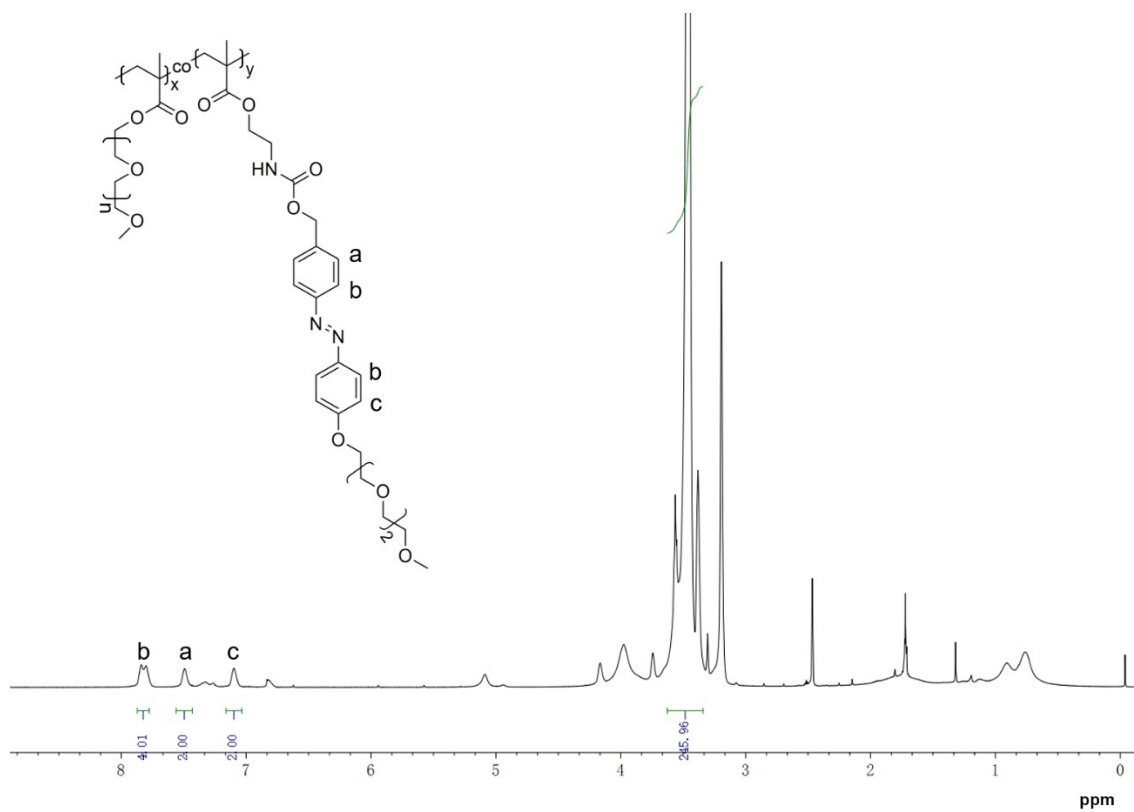
(b). ¹H NMR spectra of **2** in DMSO-d₆.

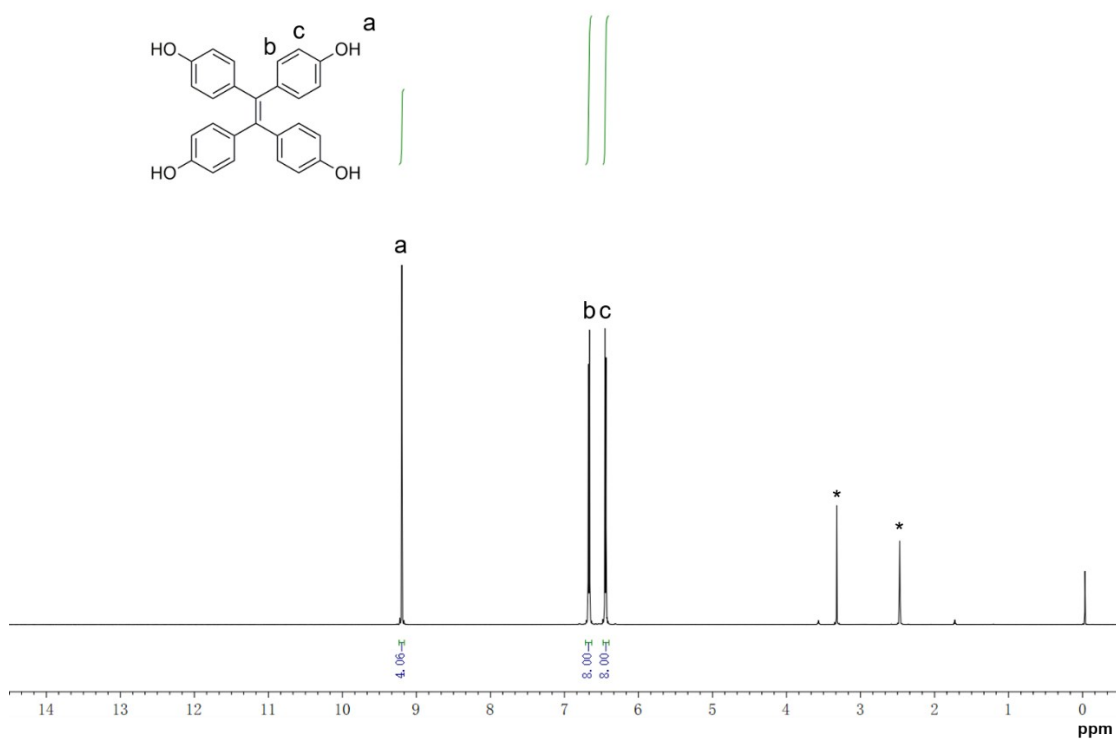


(c). ^1H NMR spectra of **3** in DMSO-d_6 .

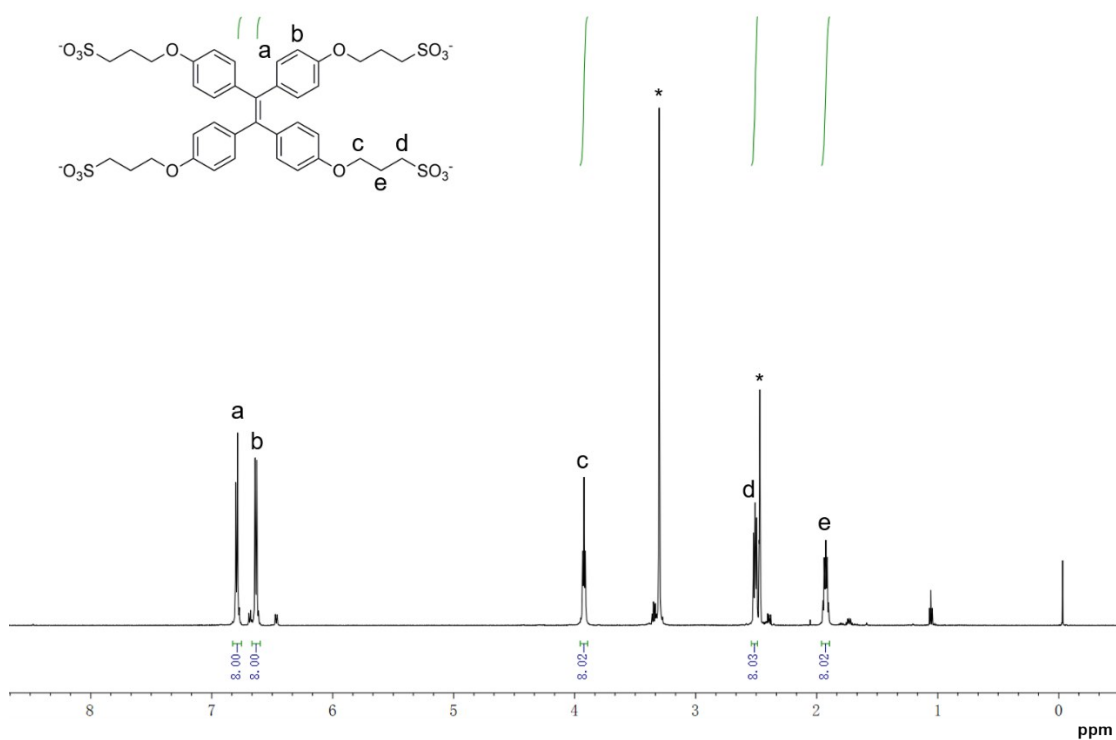


(d). ^1H NMR spectra of **4** in DMSO-d_6 .





(g). ^1H NMR spectra of **6** in DMSO-d_6 .



(h). ^1H NMR spectra of AWS-TPE (**7**) in DMSO-d_6 .

Fig. S2 ^1H NMR spectra of **1** (a), **2** (b), **3** (c), **4** (d), **WS-AC** (**5**) (e, in DMSO-d_6) and (f, in D_2O), **6** (g), **AWS-TPE** (**7**) (h). Black asterisk (*) indicates the solvent peak.

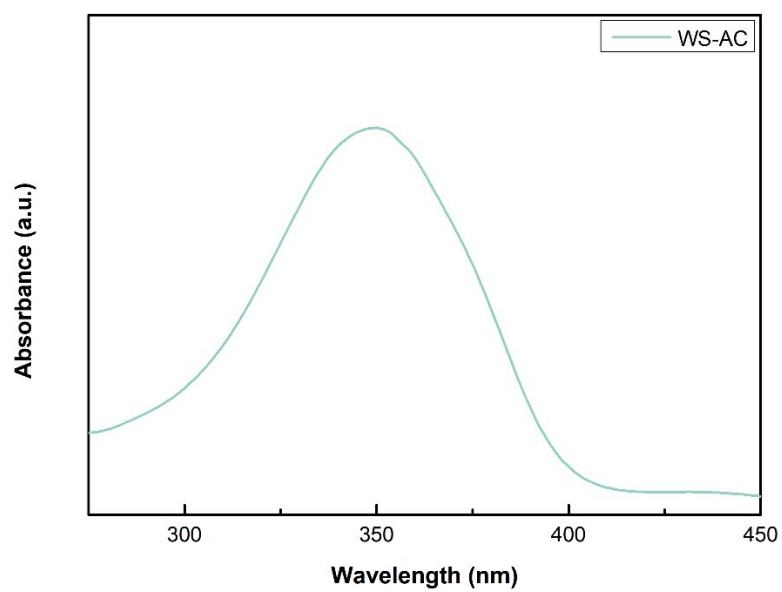


Fig. S3 UV-vis absorption spectra of the **WS-AC (5)** (0.1 mg/mL) in aqueous solution.

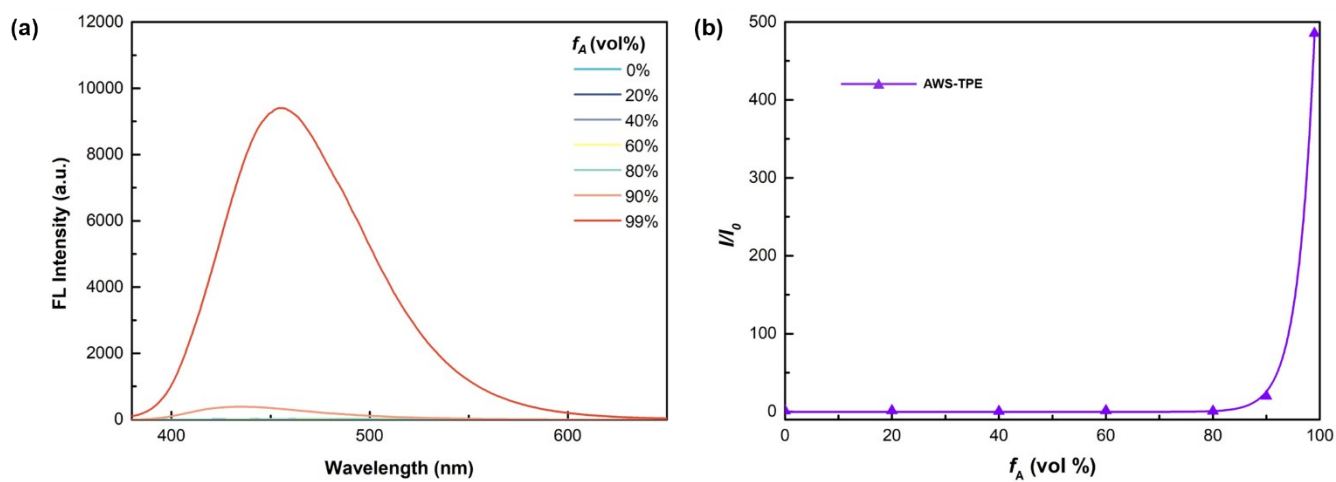


Fig. S4 Fluorescence spectra (a) and relative peak intensity (I/I_0) (b) of **AWS-TPE** in H₂O/acetone mixtures with different acetone fractions (f_A) (Excitation wavelength: 365 nm)

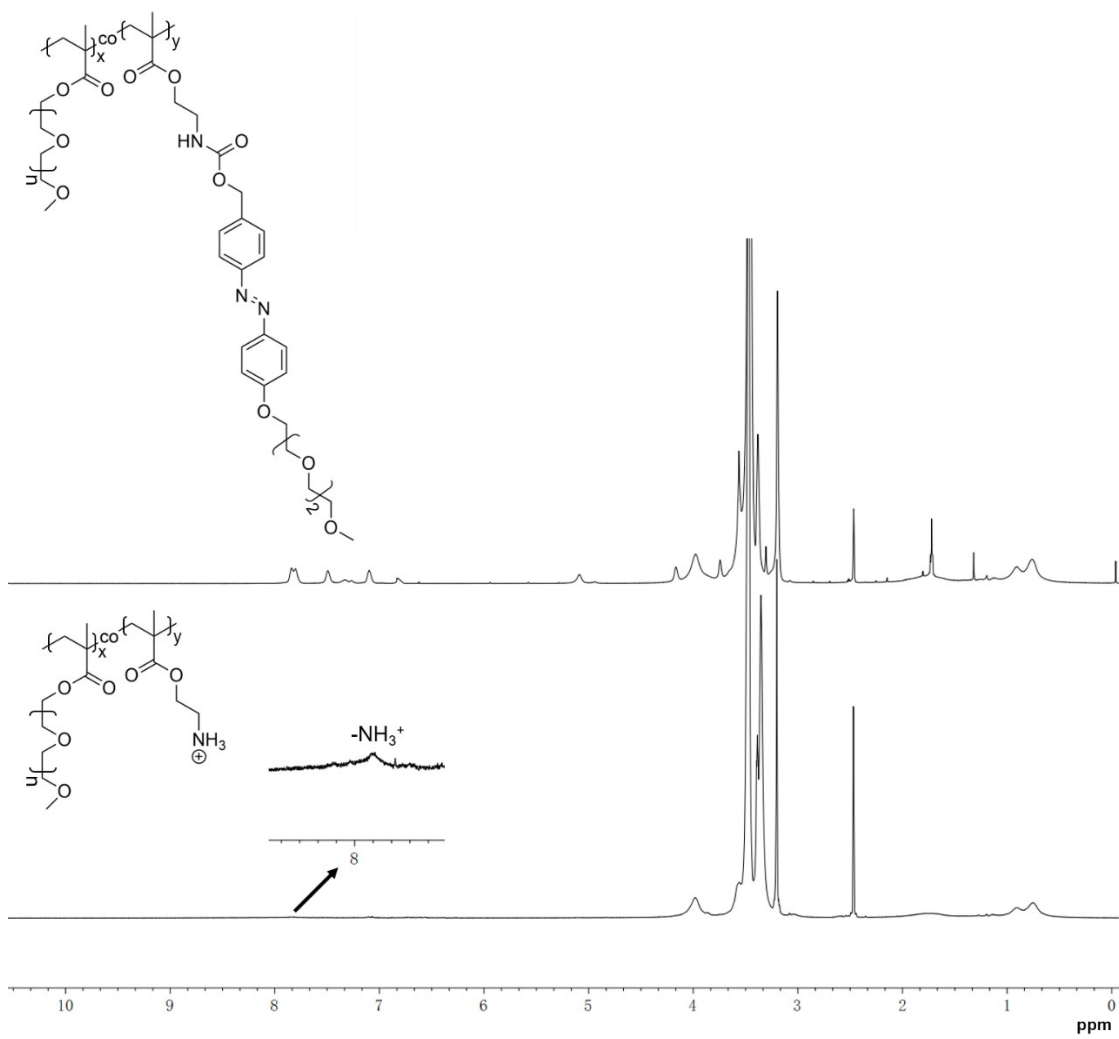


Fig. S5 ¹H NMR spectra of WS-AC (5) before and after reduction reaction in DMSO-d₆.

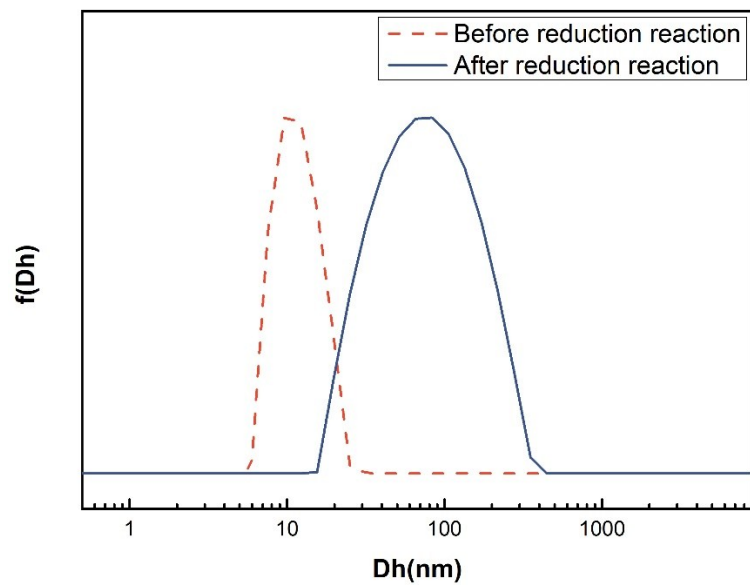


Fig. S6 The distribution curve of the hydrodynamic diameter (Dh) of the self-assembly aggregates (based on **WS-AC** (0.1 mg/mL) and **AWS-TPE** (0.05 mg/mL)) in the aqueous before and after reduction reaction. $\langle Dh \rangle = 75$ nm.

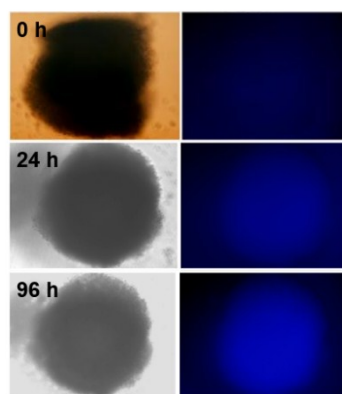


Fig. S7 Fluorescence microscopy images of Hela MCTS incubated with WS-AC/AWS-TPE complex. (based on **WS-AC** (0.1 mg/mL) and **AWS-TPE** (0.05 mg/mL))

Reference

[1] Li J, Shi K, Drechsler M, et al. A supramolecular fluorescent vesicle based on a coordinating aggregation induced emission amphiphile: insight into the role of electrical charge in cancer cell division[J]. *Chemical Communications*, 2016, 52(84): 12466-12469.

[2] Fischbach C, Chen R, Matsumoto T, et al. Engineering tumors with 3D scaffolds.[J]. *Nature Methods*, 2007, 4(10):855.