Supporting Information

for

Tumor microenvironment responsive nanocarriers for efficient antisense DNA delivery and enhanced chemodynamic therapy

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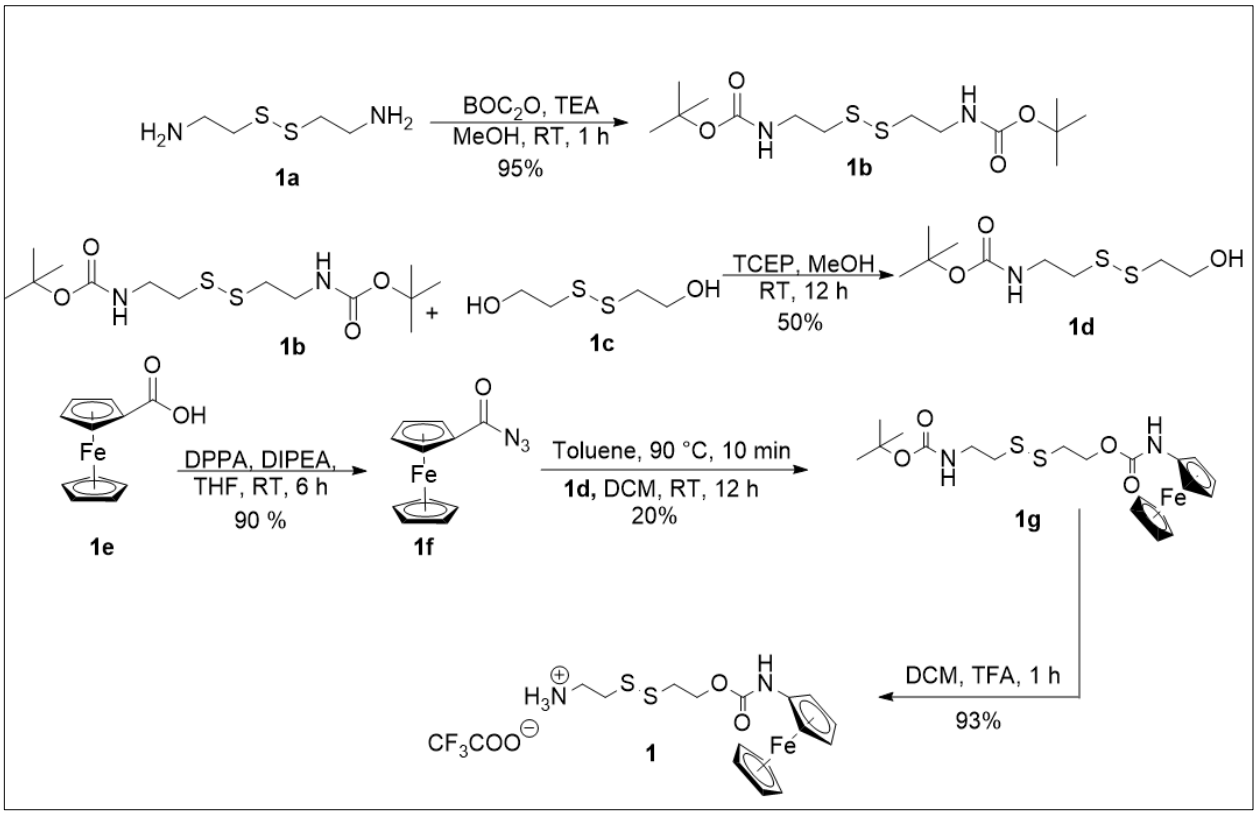
TABLE OF CONTENTS

Experimental methods

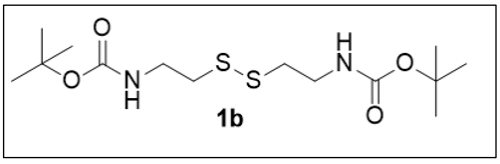
Ferrocene carboxylic acid for the synthesis was commercially purchased from Sigma Aldrich and artemisinin from TCI. All the DNAs used in this study were synthesized on K&A H-8 DNA synthesizer by following standard protocols. The morphology of the nanostructure was analyzed using various microscopic techniques like AFM and TEM analysis. AFM analyses were carried out on a Multimode BRUCKER AFM (Veeco Nanoscope V). TEM analyses were carried out on an FEI Tecnai G2 F20 (200 kV) high-resolution. Confocal imaging for cellular internalization, various localization studies, and ROS imaging were carried out on Nikon EclipseTi. MTT assay was used to study the cytotoxicity of the nanostructure using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) purchased from Sigma Aldrich. All the photophysical studies were carried out using quartz cuvette of 10 mm path length on a Shimadzu UV-3600 Vis-NIR Spectrophotometer. FACS analyses were carried out on a FACS LSR Fortessa flow cytometer (BD, USA). Cell lines were borrowed from NCCS, Pune (HeLa). The Annexin V-FITC apoptosis detection kit was purchased from BD Bioscience.

Synthesis of **1**

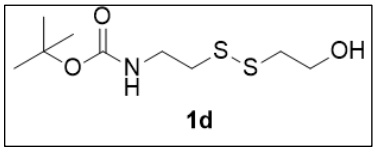


Scheme S1: Synthesis scheme for **1**.

Synthesis of 1b: To a solution of cystamine dihydrochloride (**1a**) (3.0 g, 13.76 mmol) in MeOH has added a solution of di-tert-butyl dicarbonate (12.3 g, 55 mmol) in MeOH and TEA (11.62 mL, 11.5 mmol) in a dropwise manner. The reaction mixture was stirred at room temperature for 8 h. The solvent was removed under vacuum, and the crude reaction mixture was extracted with DCM/H_2O and then washed with 10% citric acid, followed by 0.5N NaOH solution. The organic layer was collected, dried over anhydrous Na_2SO_4, and then concentrated under a vacuum to get desired product as off white solid (92%). TLC (PE:EA, 50:50), R_f = 0.51; ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm) = 6.97 (t, J= 5 Hz, 2H), 3.22 (t, J= 5 Hz, 4H), 2.76 (t, J= 5 Hz, 4H), 1.39 (s, 18H); ^{13}C NMR (125 MHz, DMSO-d_6), δ(ppm) = 156.0, 78.28, 38.14, 28.69; LC-MS (m/z): $[M+Na]^+$; $[C_{14}H_{22}N_2O_4S_2Na]^+$: 375.14 (cal.), 375.04 (expt.).

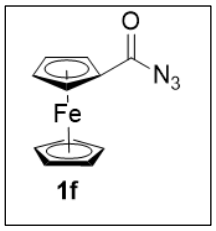


Synthesis of 1d: To a solution of compound **1b** (3.0 g, 8.5 mmol) and 2-hydroxyethyl disulfide (**1c**) (1.3 g, 8.4 mmol) in MeOH was added a catalytic amount of tris(2-carboxyethyl) phosphine hydrochloride and the reaction mixture was stirred at room temperature for 8 h. After completion of the reaction, the solvent was removed under vacuum, and the crude reaction mixture was extracted with DCM/H_2O. The organic layer was collected, dried over anhydrous Na_2SO_4, and then concentrated under a vacuum. The crude reaction mixture was purified using silica column chromatography to get desired product as a pale yellow oily liquid (53%). TLC (PE:EA, 70:30), R_f = 0.35; ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm) = 6.96 (s, 1H), 4.85 (t, J= 5 Hz, 1H), 3.63 (t, J= 10 Hz, 2H), 3.20 (t, J= 10 Hz, 2H), 2.80 (t, J= 10 Hz, 2H), 2.77 (t, J= 10 Hz, 2H) 1.37 (s, 9H); ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm)= 155.98, 78.24, 60.03, 41.56, 39.67, 38.16, 28.70; LC-MS (m/z): $[M+Na]^+$; $[C_9H_{19}NO_3S_2Na]^+$: 276.08 (cal.), 276.00 (expt.).



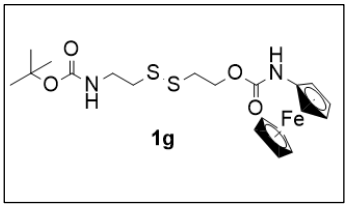
1d

Synthesis of 1f: To a solution of ferrocene carboxylic acid (2.07 g, 9 mmol) in THF (50 mL), DPPA (4.05 g, 15 mmol), and DIPEA (3.2 g, 30 mmol) was added and stirred at room temperature for overnight. After stirring at room temperature for 48 h. After completion of the reaction, the solvent was removed under vacuum, and the crude reaction mixture was extracted with DCM/H_2O. The organic layer was collected, dried over anhydrous Na_2SO_4, and then concentrated under a vacuum. The crude mixture was then purified by column chromatography on silica gel using EtOAc/hexane (v:v = 1:2) as the eluent (90%). TLC (PE: EA, 60:40), R_f = 0.45; ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm) = 4.754 (t, J= 2 Hz, 2H), 4.446 (t, J= 1.5 Hz, 2H), 4.189 (S, 5H); ^{13}C NMR (125 MHz, DMSO-d_6), δ (ppm)= 176.75,73.43, 72.16, 70.65; GC-MS-MS (m/z): $[C_{11}H_9FeN_3O]$: 255.0 (cal.), 227.0 (expt.). The experimental mass is 28 mass unit less and this might be attributed to the loss of a N_2 molecule.



1f

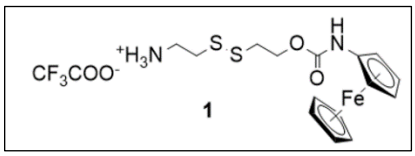
Synthesis of 1g: Compound (1.28 g, 5 mmol) was dissolved in toluene (5 mL) and degassed the solution with argon for 45 min. Then the solution was heated to reflux for 5 min at 100°C. Then the solution was immediately cooled with ice to room temperature and added compound **1f** (1.00 g, 5 mmol) in DCM (10 mL) and stirred for overnight. After completion of the reaction, the solvent was removed under vacuum and the crude reaction mixture was extracted with DCM/H_2O. The organic layer was collected, dried over anhydrous Na_2SO_4. The crude mixture



1g

was then purified by column chromatography on silica gel using EtOAc/hexane (v:v = 1:2) as the eluent (20%). TLC (PE: EA, 7:3), R_f = 0.85; ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm) = 8.89 (s, 1H), 7.002 (t, J= 5 Hz, 1H), 4.476 (s, 2H), 4.279 (s, 2H), 4.116 (s, 5H), 3.935 (t, J= 2 Hz, 2H), 3.258-3.220 (m, 2H), 3.010 (S, 1H), 2.799 (t, J= 7 Hz, 2H) 1.39 (s, 9H); LC-MS (m/z): $[C_{20}H_{28}FeN_2O_4S_2Na]^+$: 480.42 (cal.), 480.15 (expt.).

Synthesis of 1: To a solution of compound **1g** (0.1 g, 0.14 mmol) in dry DCM was added TFA (0.5 mL, 2.00 mmol) and the reaction mixture was stirred at room temperature for 0.5 h. After



completion of the reaction monitored by TLC, the crude reaction mixture was extracted with DCM/H_2O. The organic layer was collected, dried over anhydrous Na_2SO_4 and concentrated to obtain the desired product as an orange solid (90% yield). TLC (DCM:MeOH, 90:10), δ (ppm) = 8.931 (s, 1H), 7.786 (t, J= 5 Hz, 1H), 4.382 (s, 2H), 4.022 (s, 2H), 3.846 (s, 5H), 3.041 (t, J= 7Hz, 2H), 2.943 (s, 2H), 2.846 (t, J= 7 Hz, 2H), 2.799 (t, J= 7 Hz, 2H) 1.39 (s, 9H); LC-MS (m/z): $[C_{15}H_{21}FeN_2O_2S_2]^+$: 381.31 (cal.), 381.04 (expt.).

Synthesis of DNA1@1-ART NPs

The previously synthesized **1** was redissolved in deionized water at pH 2.0 (pH was adjusted using 0.01N HCl solution) to keep the amino end groups protonated at high concentrations and adjusted to a concentration of 2 mM as the stock solution. Next, for the fabrication of the nanoparticle, **DNA1** (0.1 μM), **1** (2 μM) in PBS at pH 5.5 was taken and vortexed using a thermomixer for 10 min and then annealed at 90°C for 10 min, followed by cooling to room temperature. During the cooling process, artemisinin (15 μM) was added to the reaction mixture and kept undisturbed for 1 h. After that, the NPs were centrifuged using a molecular weight cut-off the filter at 4000 rpm for 10 min and redispersed in PBS buffer at pH 5.5 for further studies.

Microscopic analyses

DNA1@1 NPs were prepared according to the procedure described above, keeping the ratio of **DNA1**:**1** as 1:20. Samples were then drop casted onto a 400-mesh carbon-coated copper grid (Ted Pella, Inc.). The samples were kept on the grid for 2 min, and the excess sample was wiped off using tissue paper. After repeating the process 2-3 times, the samples were kept under a desiccator for drying and were used for the TEM analyses. For the AFM analyses, samples were drop casted onto a freshly cleaved mica surface and dried overnight.

Catalytic activity

The peroxidase mimicking catalytic behaviour studies were carried out using **DNA1@1** NPs **(**50 μM w. r. t. to **DNA1**, **1** (1 mM), using methylene blue (15 μM) as an indicator for ROS generation. The catalysis experiment was performed in sodium acetate buffer with pH 5.0 with GSH (0.5 mM) and H_2O_2 (100 mM) as other substrates for the reaction. The degradation rate of methylene blue was monitored at 665 nm using a UV spectrophotometer to assess the catalytic activity of the **DNA1@1** NPs. The radical generation was further determined by EPR analysis of **DNA1@1** NPs treated H_2O_2 (1 mM), and GSH (1 mM) using DMPO as the spin trap in dibasic sodium phosphate-citric acid buffer pH 5.

Monitoring Fe^{3+} release

DNA1@1 NPs (5 μM **DNA1**, 100 μM **1)** were incubated with GSH (10 mM**)** for 1 h, then incubated with H_2O_2 (9.8 mM) for 30 min in PBS buffer at pH 5.0. Then the reaction was added with sodium dithionite, which converts Fe^{3+} into Fe^{2+}. Finally, 2,2′-bipyridine solution (3 μL, 100 mM in DMSO) was added to form a dark red $[Fe(2,2'\text{-bipyridine})_3]^{2+}$ complex (λ_{max} = 519 nm). $FeCl_2.4H_2O$ (100 μM) was used as a positive control.

Detection of GSH consumption

For this purpose DTNB (10 μM) probe was used. DTNB, which upon reacting with GSH (25 μM) will give a chromogenic product, with absorption maxima shifted from 322 nm to 412 nm compared to GSH untreated DTNB. The DTNB solution was treated with different sets of the sample, including DTNB alone, GSH alone, **1** alone, DTNB +**1**, DTNB + GSH, DTNB + GSH+ **1**. The pH of the PBS buffer solution used in the experiment was 7.4.

Detection of ROS generation by ART–Fe^{2+} reaction

The ROS generation from the **ART–Fe^{2+}** based reaction was monitored by the change in absorption of DPBF in an ethanol solution containing artemisinin (ART) and $FeCl_2.4H_2O$ (Fe^{2+}source). Artemisinin (1 mM) and labile Fe^{2+} (500 μM), DPBF (50 μM) were used for the experiment.

Confocal laser scanning microscopic (CLSM) analyses

The HeLa cells were plated in μ-Slide 8 Well slides at a seeding density of 1000 cells, kept at 5 % CO_2 incubator at 37 °C in DMEM culture media for 24 h. Subsequently, the cells were treated

with **DNA2@1** NPs with Cy3.5 labelled **DNA1** (**DNA2**) and were used at desired concentration incubated according to the experiment. The nucleus of cells was stained using hoechst, lysosome by lysotracker deep red, and mitochondria by mitotracker deep red and washed off three times with PBS and imaged under Nikon Eclipse Ti with a 20X objective.

Fluorescence-activated cell sorting (FACS) analyses

For cellular internalization studies **DNA2@1** NPs, HeLa cells were seeded at a cell density of 0.8 × 10^6 cells in a 6 well plate and grown in a 5 % CO_2 incubator at 37 °C in DMEM cell culture media for 24 h. Once the cells has reached 50 % confluency, **DNA2@1** NPs were diluted with DMEM into different concentrations (**DNA2** equivalent 400 nM). The **DNA2@1** NPs were added to the wells and were incubated for 6 h. Once the incubation time was over, cells were collected by trypsinization and washed three times with 1X PBS. Cells were then collected in 300 µl PBS for flow cytometry analyses.

MTT assay

For evaluating the cytotoxicity of **DNA1@1-ART** NPs, HeLa cells were seeded to 96-well with a density of 1 × 10^3 cells per well and were grown in DMEM cell culture media for 24 h. Then, the cells were incubated with **DNA3** (1000 nM**)**, **DNA1 (**1000 nm**)**, **1** (50 µM) **DNA3@1** NPs, **DNA3@1-ART** NPs, **DNA1@1-ART NPs** (**ART** 15, 30 µM), for 24 h, respectively. After the incubation period was over, cytotoxicity was analysed by MTT assay. MTT ((3-[4,5-dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide)) reagent in fresh media was added and kept for 4 h incubation at 37 °C in 5 % CO_2 incubator. 100 µL DMSO was then added to solubilize the formazan crystals, and absorbance was measured at 565 nm using a microplate reader to evaluate the cytotoxicity.

ROS generation studies

The HeLa cells were plated in µ-Slide 8 Well slides at a seeding density of 1000 cells, kept at 5 % CO_2 incubator at 37 °C in DMEM culture media for 24 h. Subsequently, the cells were treated with **ART**, **DNA1@1** NPs, **DNA1@1-ART** NPs incubated for 12 h. Cells were stained with ROS-sensitive dye DCFHDA (10 µM) in an FBS-free medium as the staining for 45 min and washed off three times with PBS, and imaged under Nikon Eclipse Ti with a 20 X objective.

Annexin V-FITC apoptosis assay

The Annexin V-FITC apoptosis assay was performed on HeLa cells using an Annexin V-FITC apoptosis detection kit (BD Bioscience). The HeLa cells were plated in µ-Slide 8 Well slides at a seeding density of 1000 cells, kept at 5 % CO_2 incubator at 37 °C in DMEM culture media for 24 h. Subsequently, the cells were treated with **DNA1@1-ART** NPs and incubated for 24 h. Once the incubation time was over, cells were washed with PBS. The Annexin V-FITC (2.5 µL), an Annexin V-FITC binding buffer (195 µL), and a PI solution (10 µL from 10 µg/mL) at room temperature for 20 min. After that, imaged under Nikon Eclipse Ti with a 20 X objective.

Live/dead cell staining assay

The Live/dead cell staining assay was performed on HeLa cells using Calcein AM/PI costaining technique. The HeLa cells were plated in µ-Slide 8 Well slides at a seeding density of 1000 cells, kept at 5 % CO_2 incubator at 37 °C in DMEM culture media for 24 h. Subsequently, the cells were treated with **DNA1@1-ART** NPs and incubated for 24 h. Once the incubation time was over, cells were washed with PBS. In fresh medium, Calcein AM (1 µM) and propidium iodide (10 µLfrom10 µg/mL) were added and incubated for 30 min. After that, imaged under Nikon Eclipse Ti with a 20 X objective.

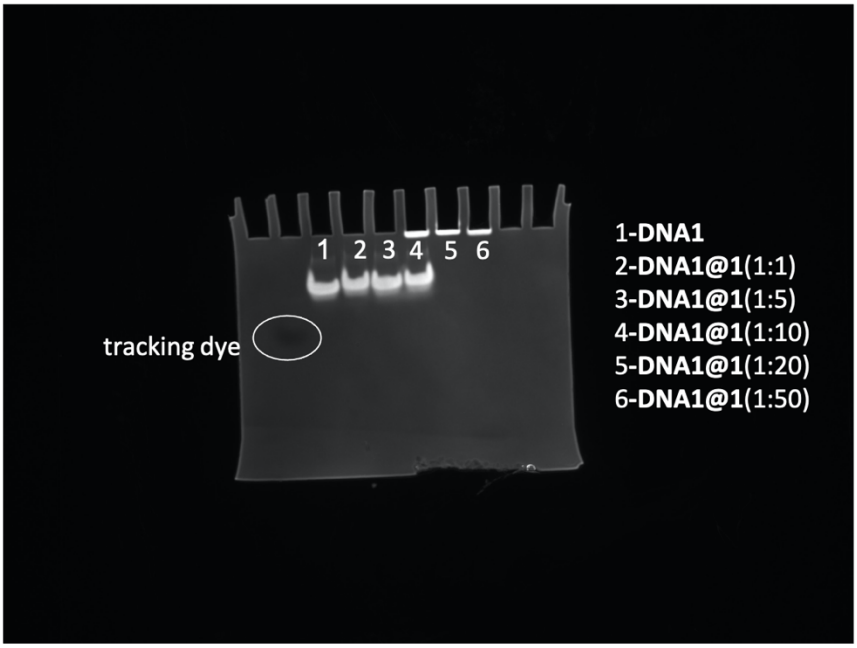


Figure S1. Native PAGE (20 %) analysis showing the formation of **DNA1@1** NPs at different molar ratios of **DNA1** and **1**.

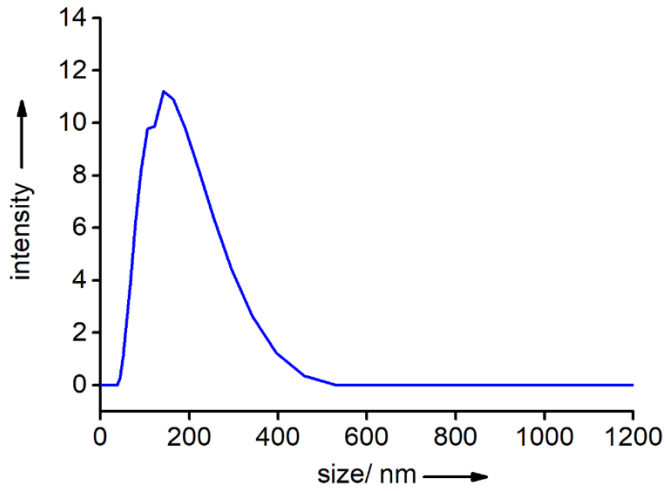


Figure S2. DLS size distribution of **DNA3@1** NPs .

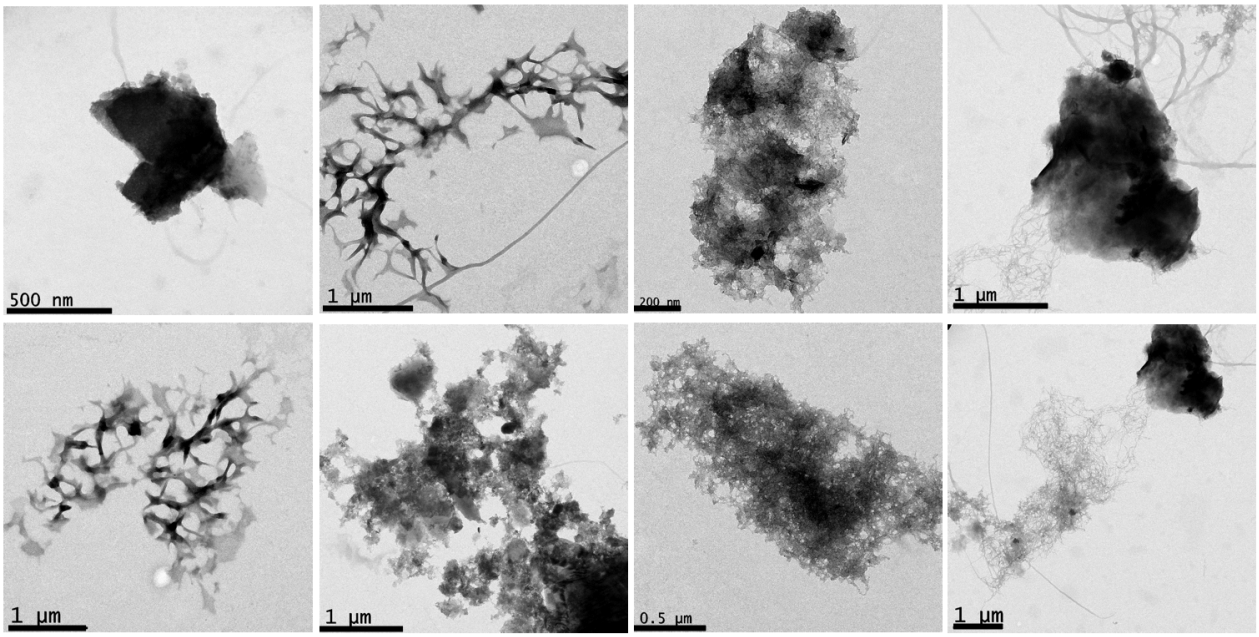


Figure S3. TEM images of **1** alone showing the formation of ill-defined aggregates.

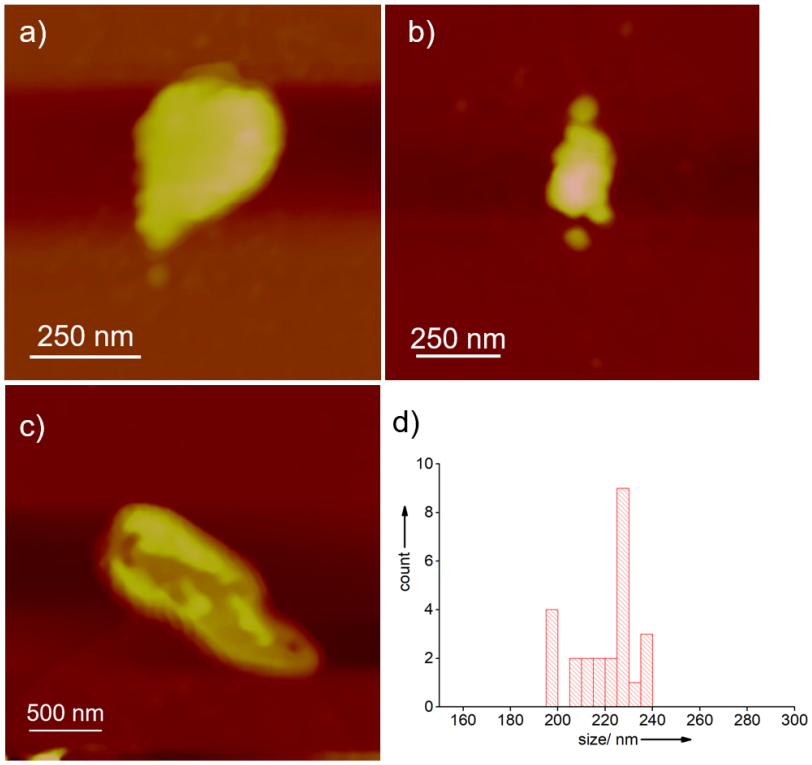


Figure S4. (a-c) AFM height images of **1** showing the formation of ill-defined aggregates and (d) the corresponding histogram showing the size distribution .

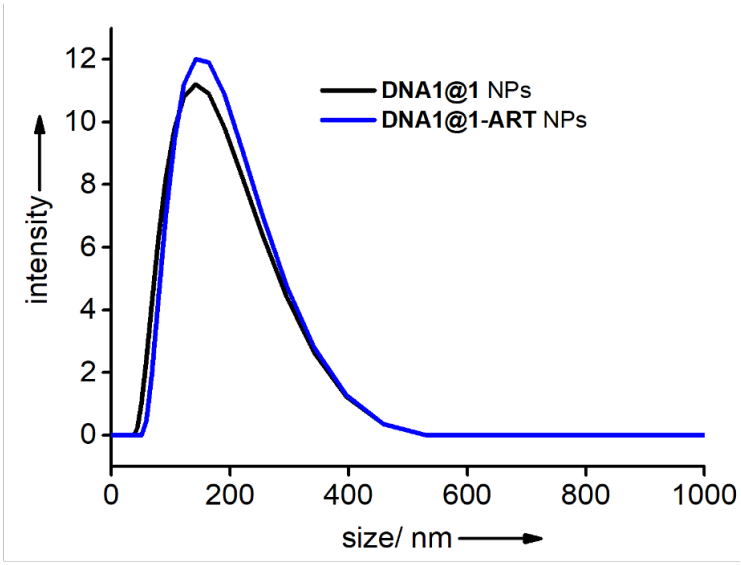
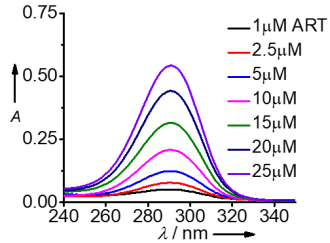
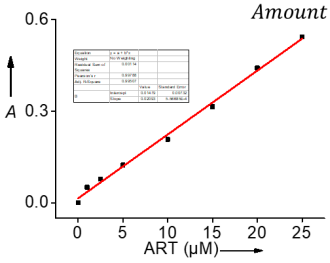


Figure S5. DLS size distribution of **DNA1@1** NPs and **DNA1@1-ART** NPs.





Concentration of DNANPs (wrt to DNA) = 0.1μM

Amount of initial ART concentration = 5 μM

Amount of ART collected after centrifugation = 0.98μM

$$Amount\ of\ ART\ encapsualted = \frac{Initia\ ART - Free\ ART\ after\ centrifugation}{Initial\ conc\ of\ ART} \times 100$$

$$= \frac{5\mu M - 0.98\mu M}{5\ \mu M} \times 100$$

$$= 80.314\ \%$$

Figure S6. Details for the calculations of loading of **ART** into **DNA1@1** NPs.

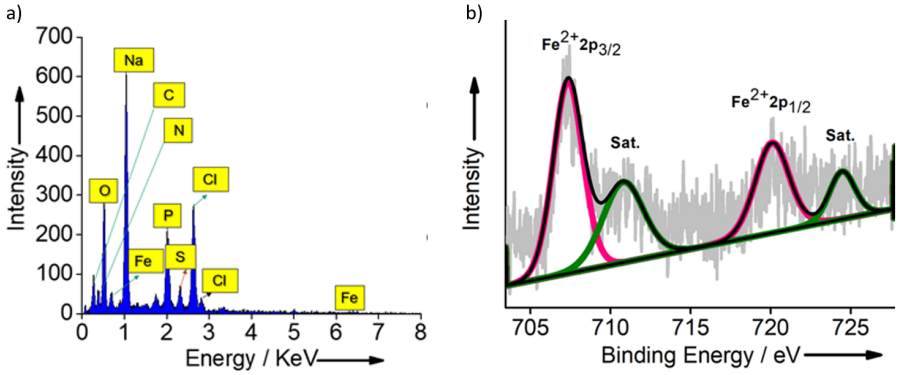


Figure S7. (a) Energy-dispersive X-ray spectroscopy (EDS) analyses of **DNA1@1** NPs.(b) X-ray photoelectron spectroscopy (XPS) analyses of **DNA1@1** NPs.

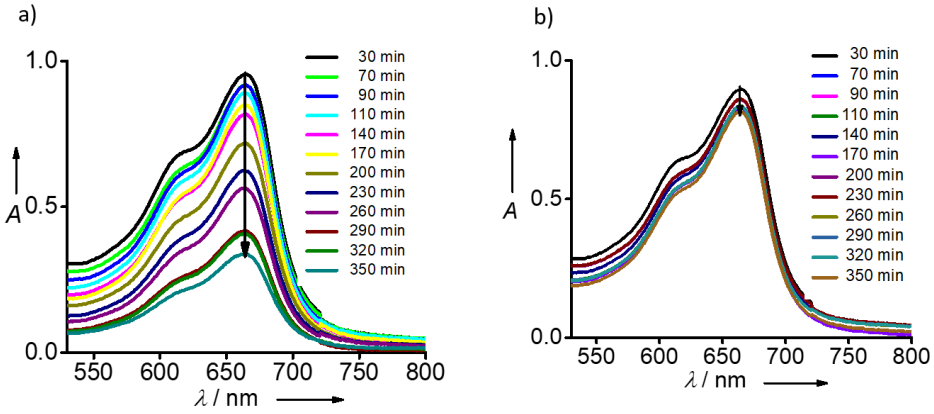


Figure S8. Time-dependent methylene blue degradation assay of (a) **DNA1@1** NPs and (b) in the absence of **DNA1@1** NPs.

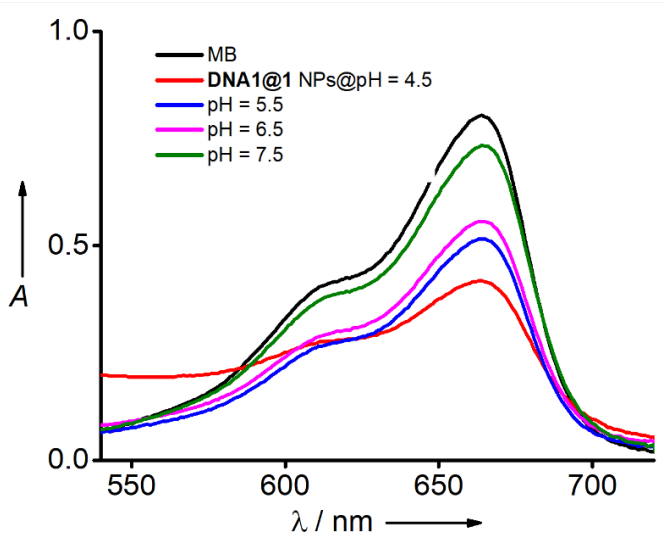


Figure S9. pH-dependent methylene blue degradation assay of **DNA1@1** NPs.

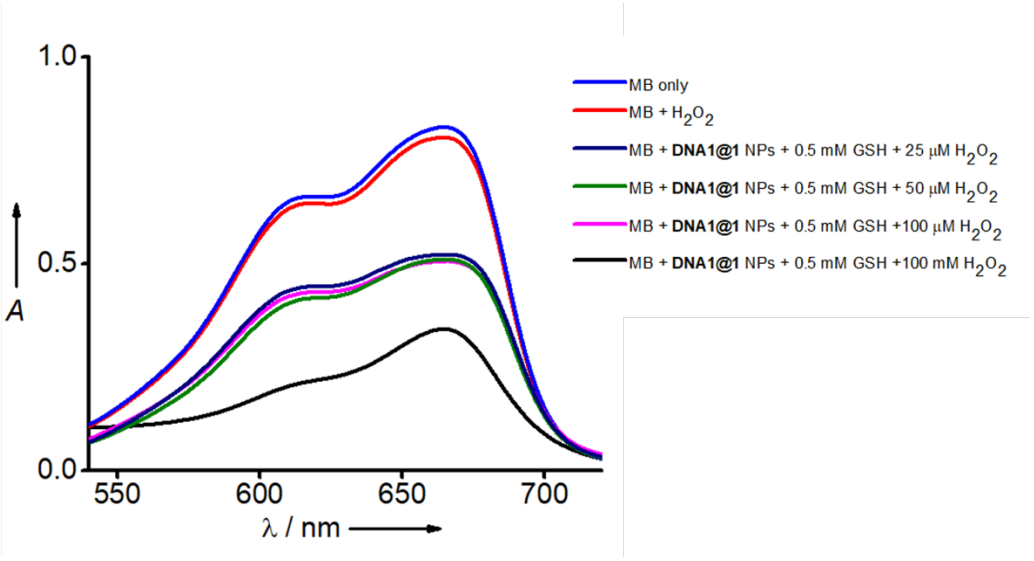


Figure S10. H_2O_2-dependent methylene blue degradation assay of **DNA1@1** NPs.

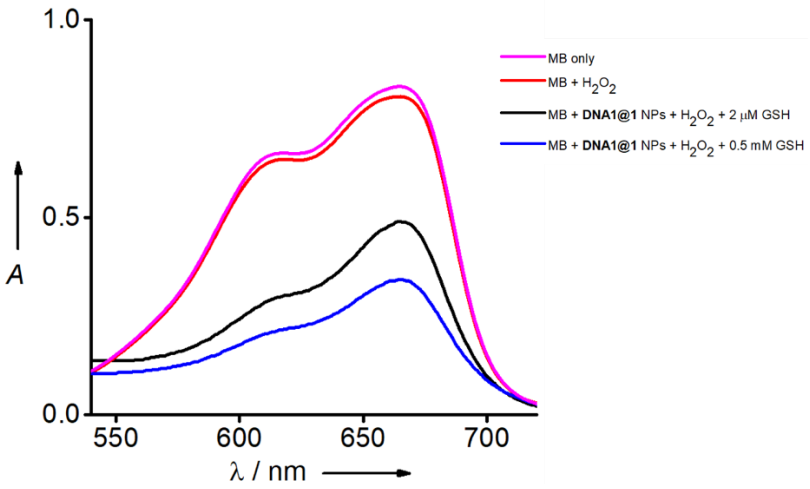


Figure S11. GSH-dependent methylene blue degradation assay of **DNA1@1** NPs.

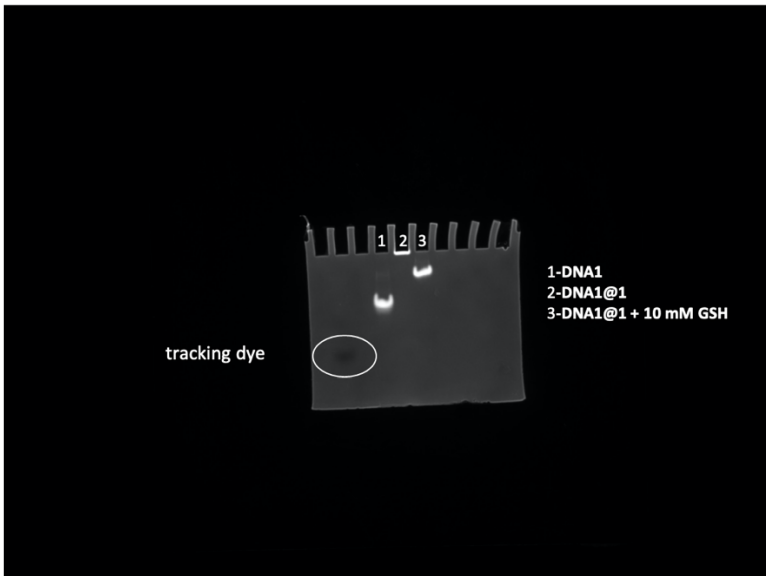


Figure S12. GSH-triggered release of **DNA1** from **DNA1@1** NPs.

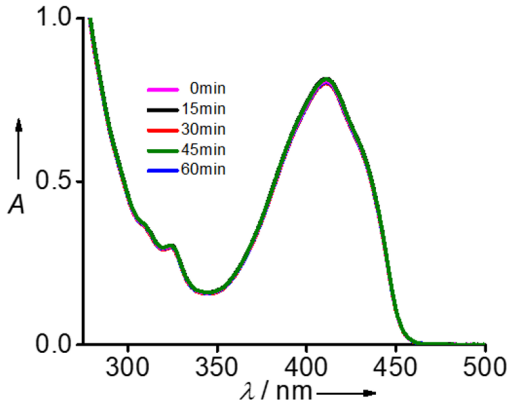


Figure S13. Time-dependent degradation of DPBF with **ART**.

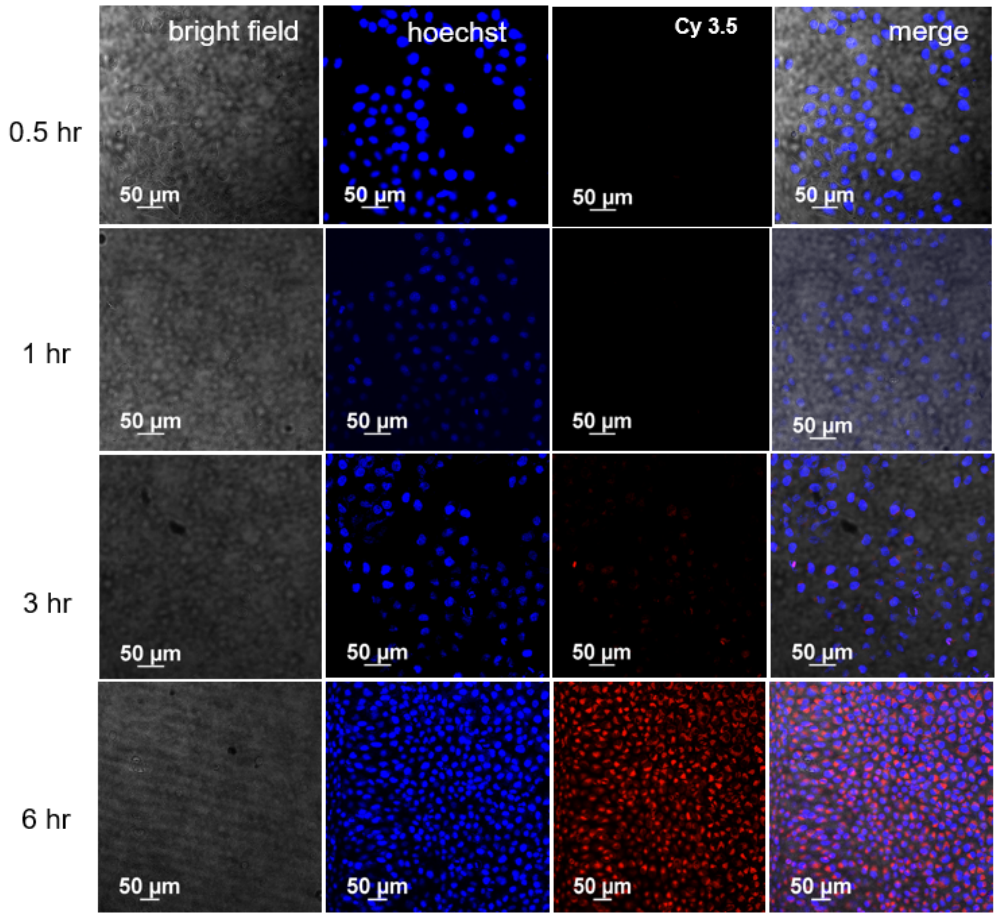


Figure S14. Time-dependent cellular internalization of **DNA2@1** NPs.

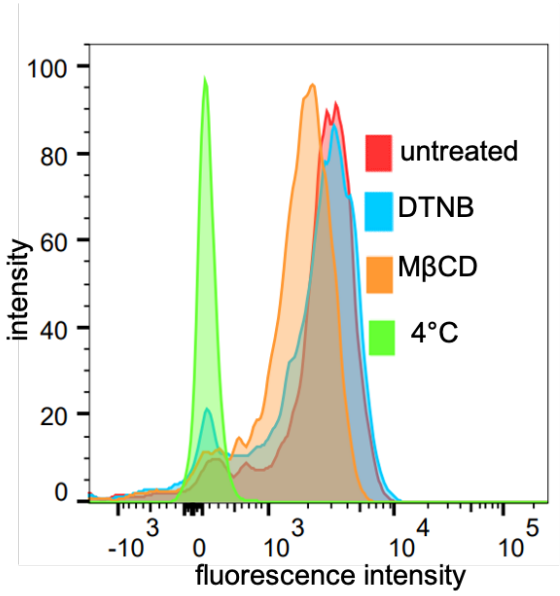


Figure S15. FACS analyses for the cellular internalization of **DNA1@1-ART** NPs-treated HeLa cells in the presence of different inhibitors.

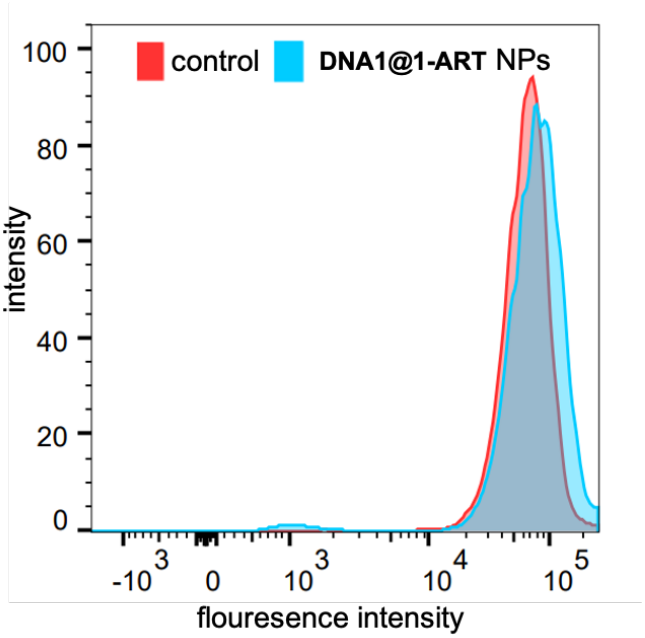


Figure S16. FACS analyses of Acridine Orange assay for **DNA1@1-ART** NPs-treated HeLa cells.

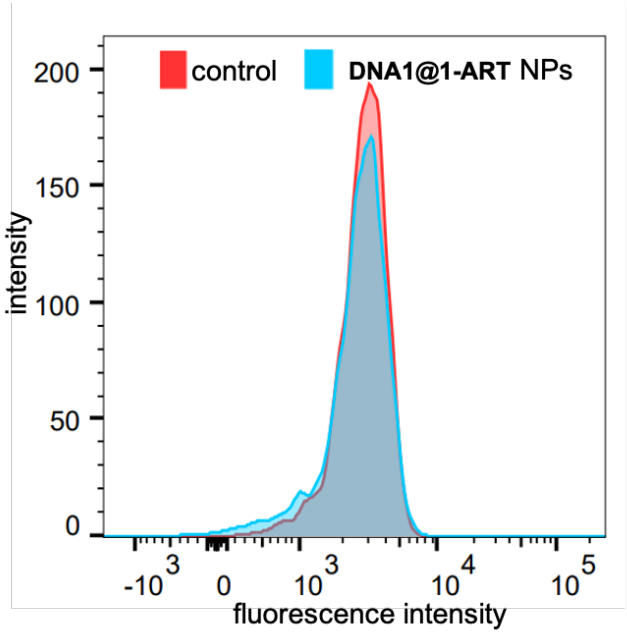


Figure S17. FACS analyses of TMRM assay for **DNA1@1-ART** NPs-treated HeLa cells.

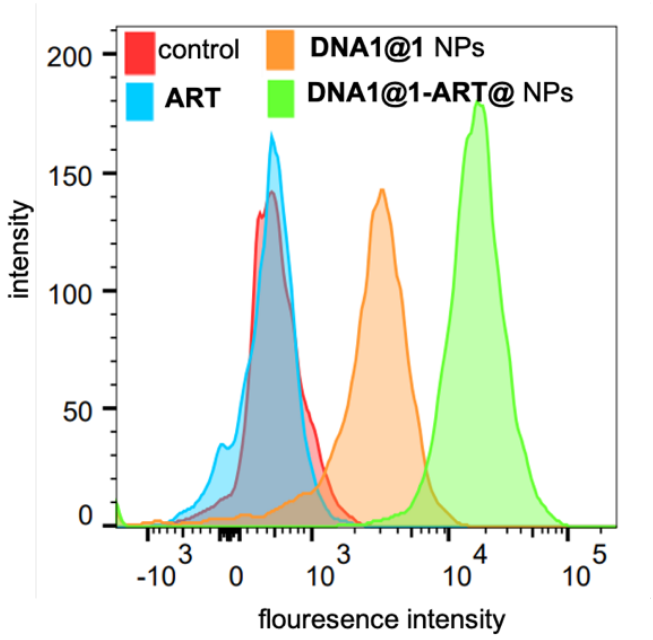


Figure S18. FACS analyses for ROS generation of **ART**, **DNA1@1** NPs, **DNA1@1-ART** NP-treated HeLa cells.

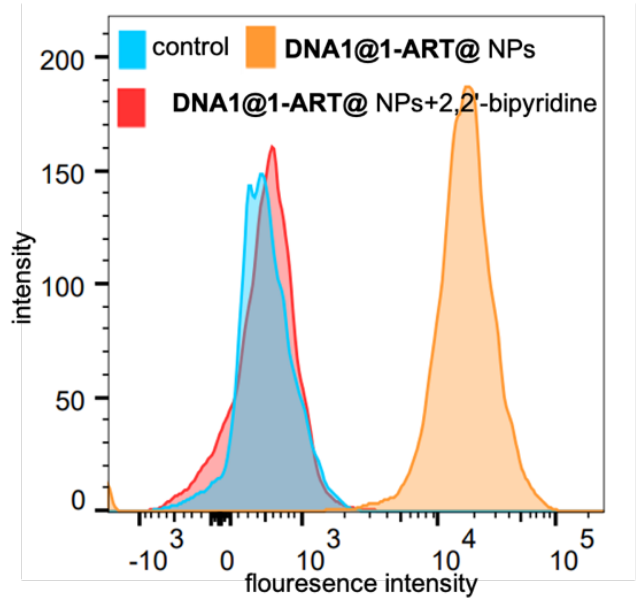


Figure S19. FACS analyses for the quenching of ROS of **DNA1@1-ART** NP-treated HeLa cells in presence of 2,2'-bipyridine .

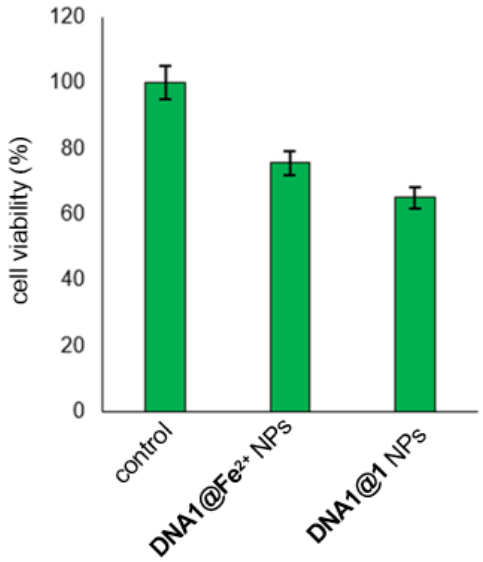


Figure S20. MTT assay Comparing the efficiency of **DNA1@Fe^{2+}** with **DNA1@1** NPs.

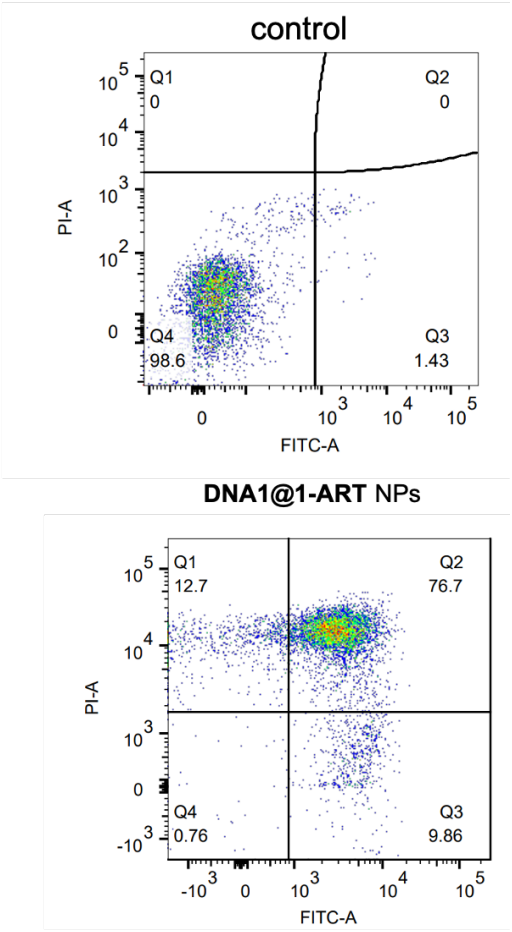


Figure S21. FACS analyses of Annexin V-FITC (AV)/propidium iodide (PI) assay of **DNA1@1-ART** NPs-treated HeLa cells .

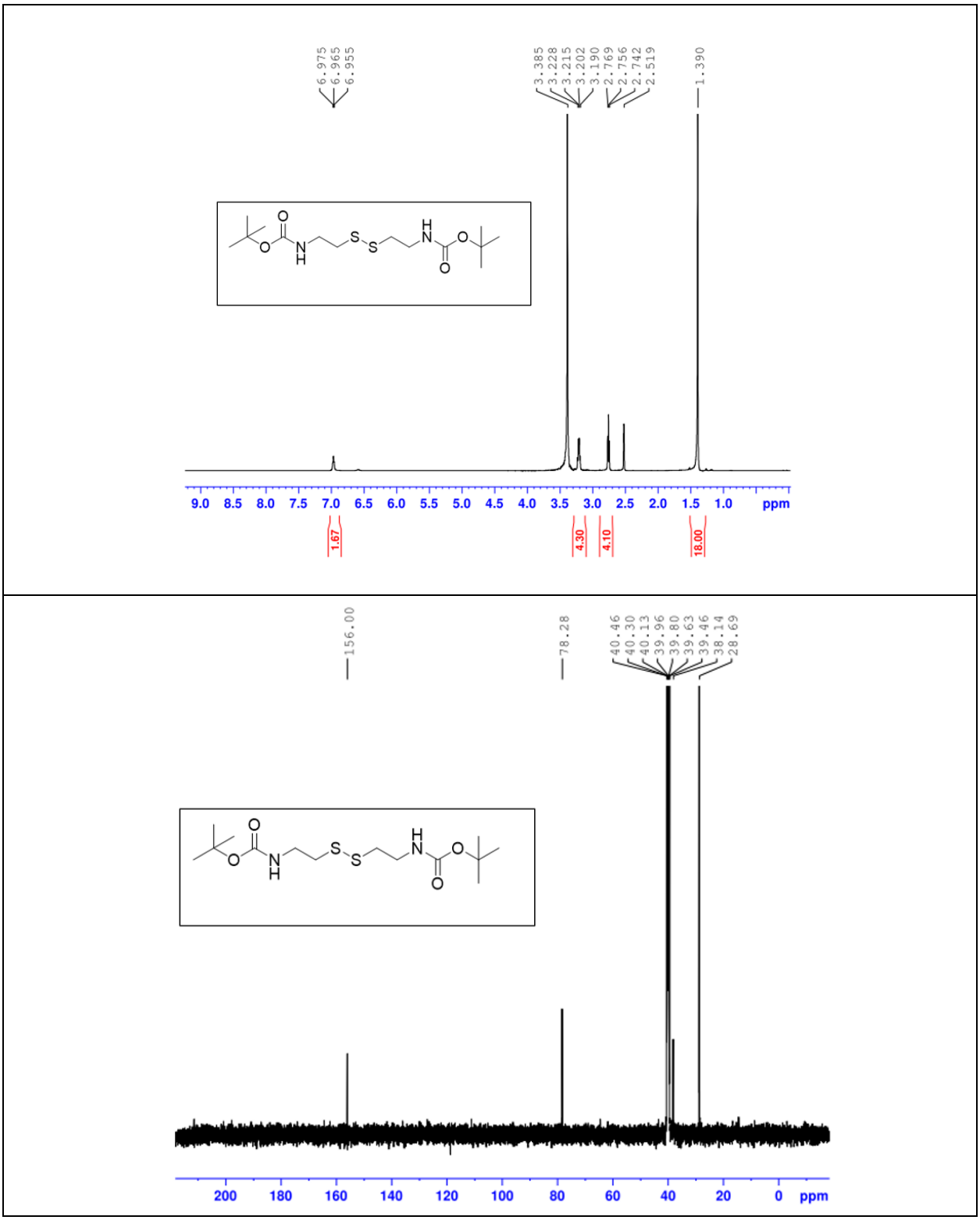


Figure S22. ^{1}H (above) and ^{13}C (below) NMR spectra of **1b**.

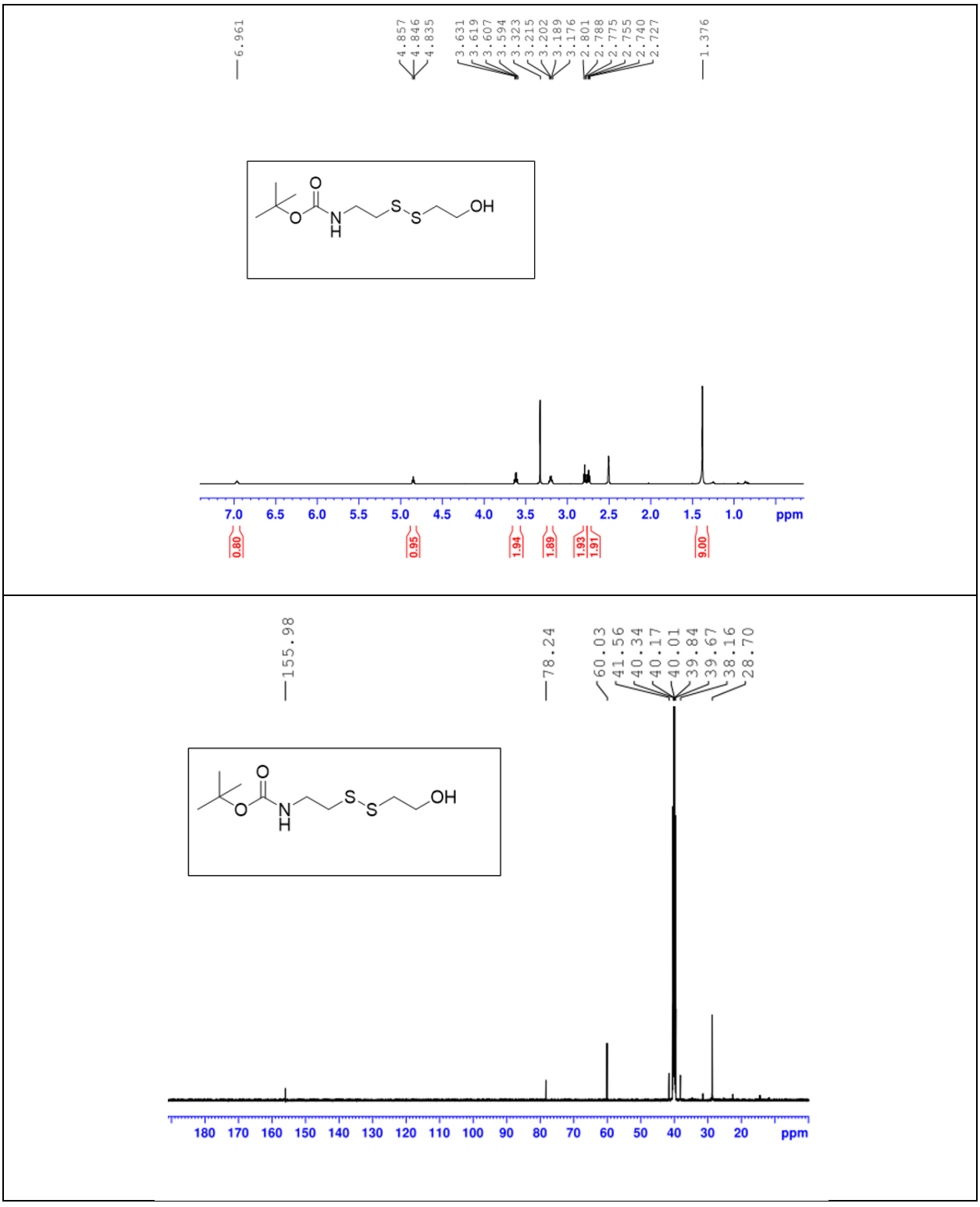


Figure S23. ^{1}H (above) and ^{13}C (below) NMR spectra of **1d**.

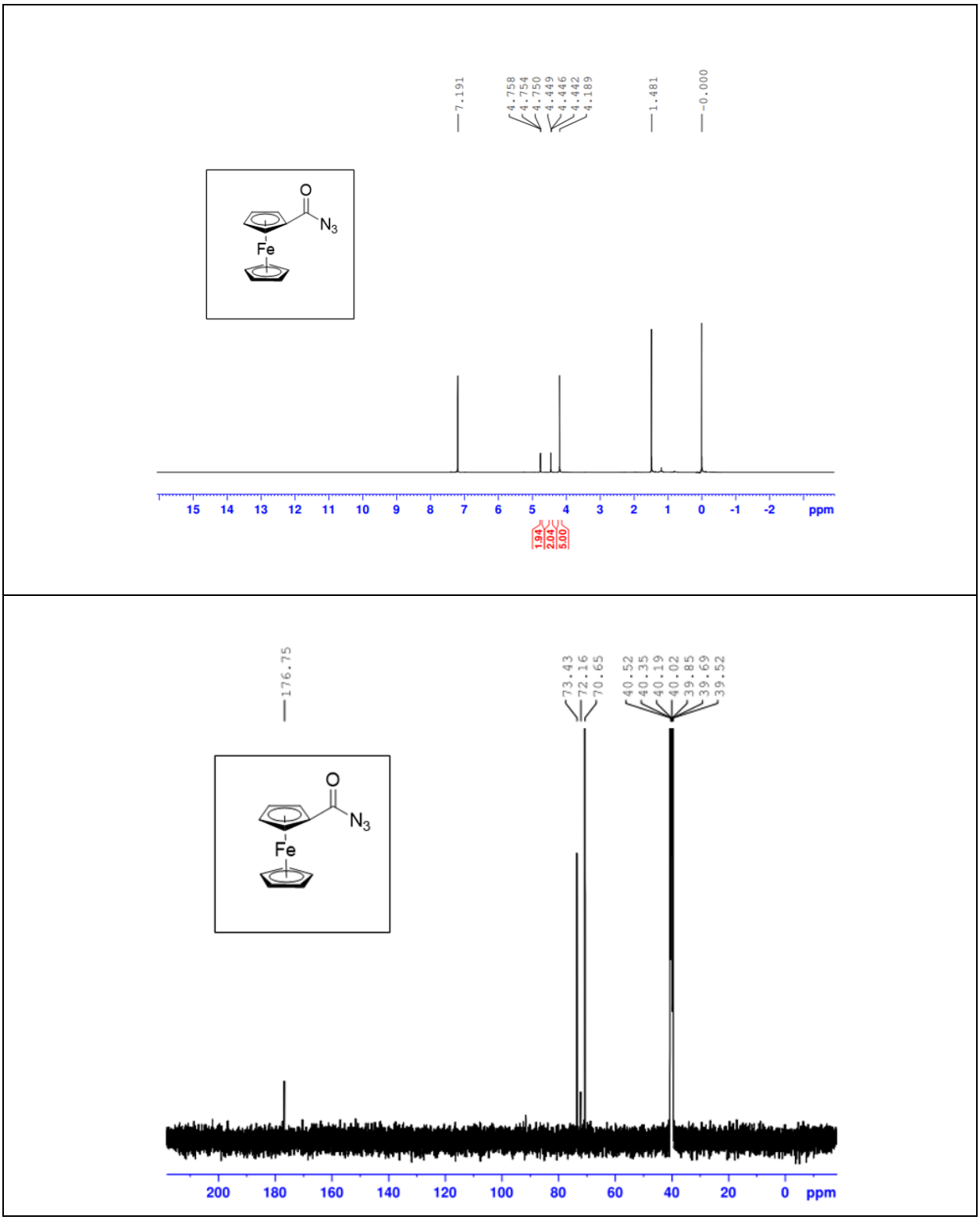


Figure S24. ^{1}H (above) and ^{13}C (below) NMR spectra of **1f**.

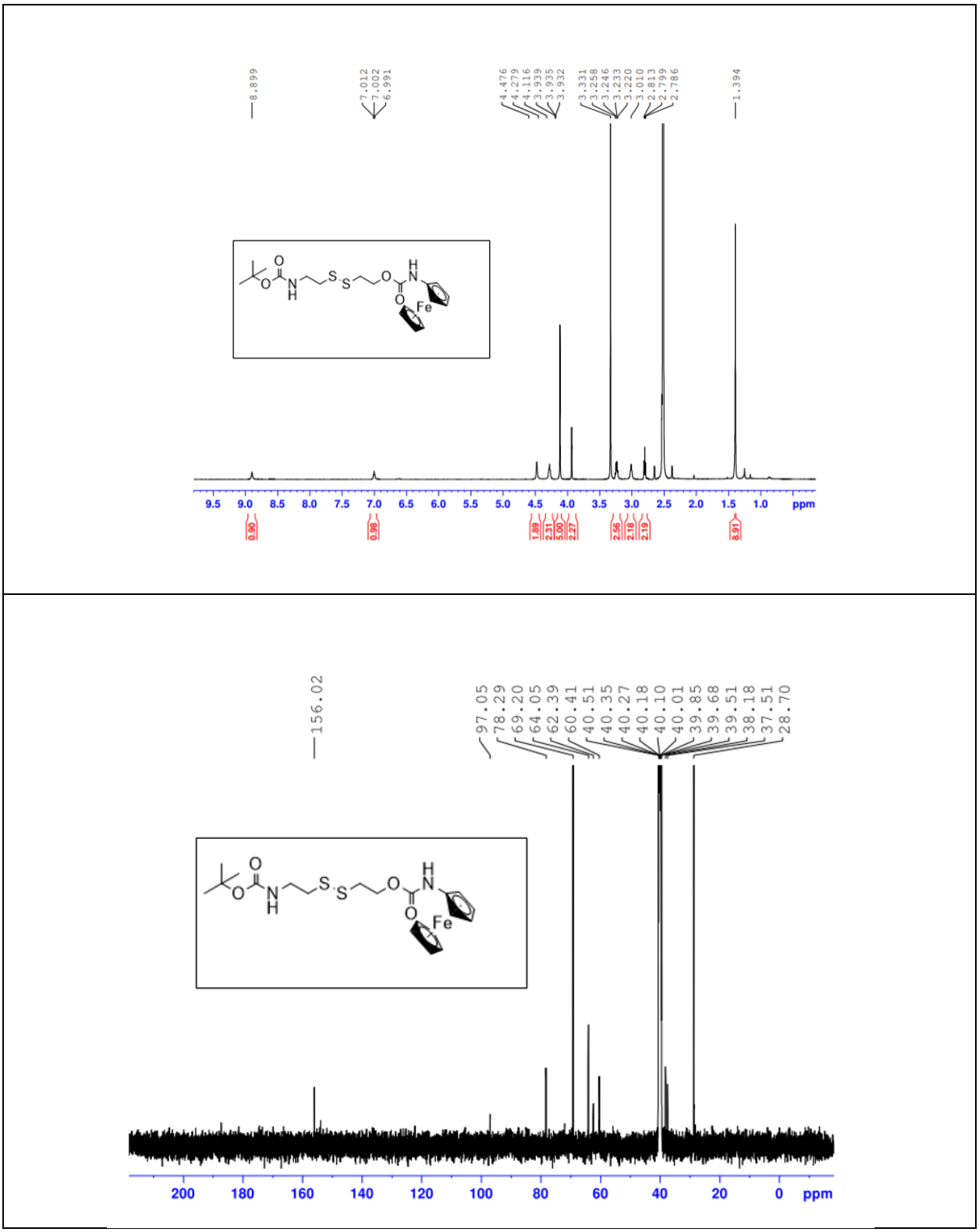


Figure S25. ^{1}H (above) and ^{13}C (below) NMR spectra of **1g**.

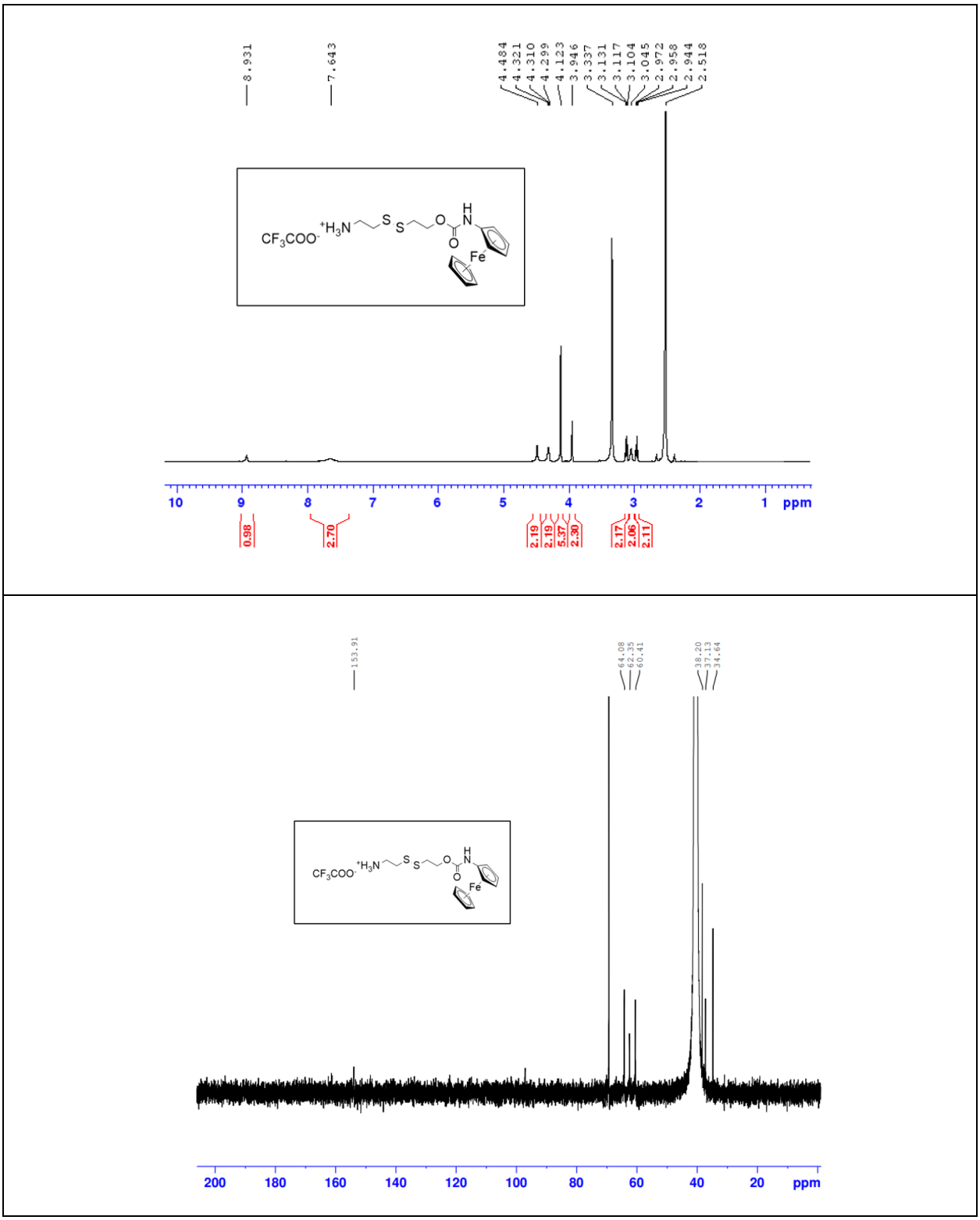


Figure S26. ^{1}H (above) and ^{13}C (below) NMR spectra of **1**.

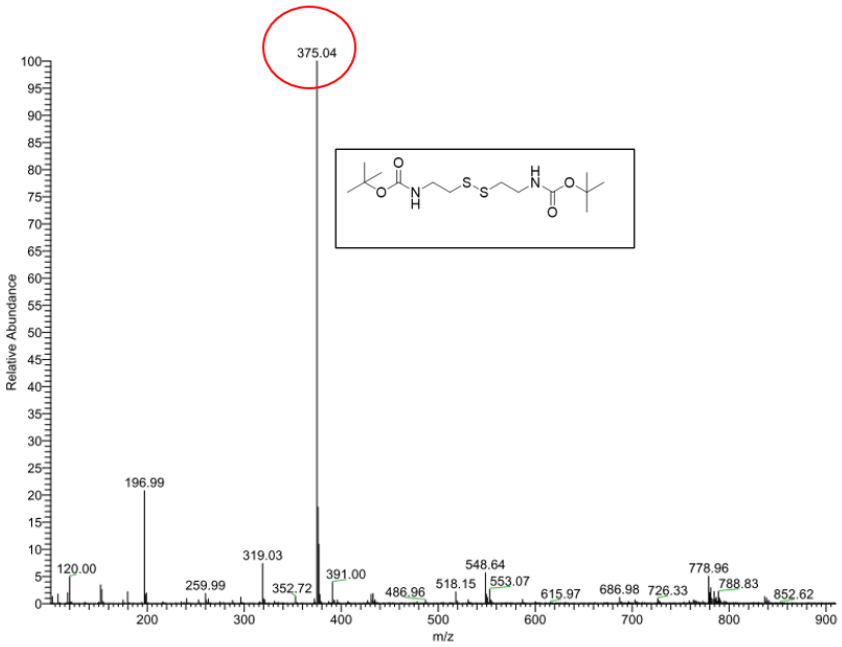


Figure S27. LC-MS spectrum of **1b**.

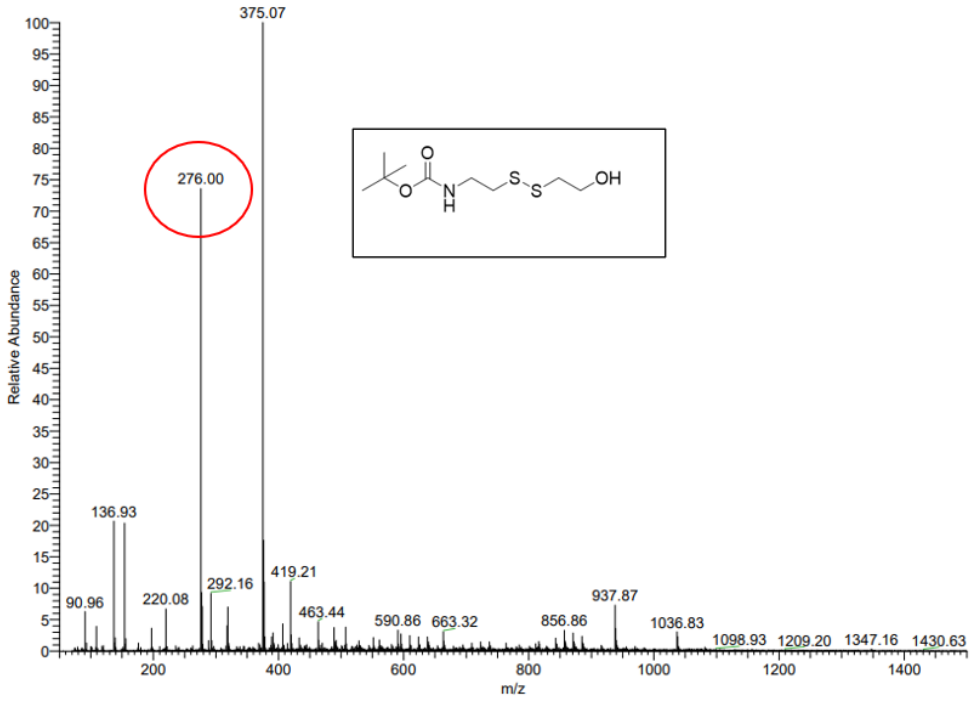


Figure S28. LC-MS spectrum of **1d**.

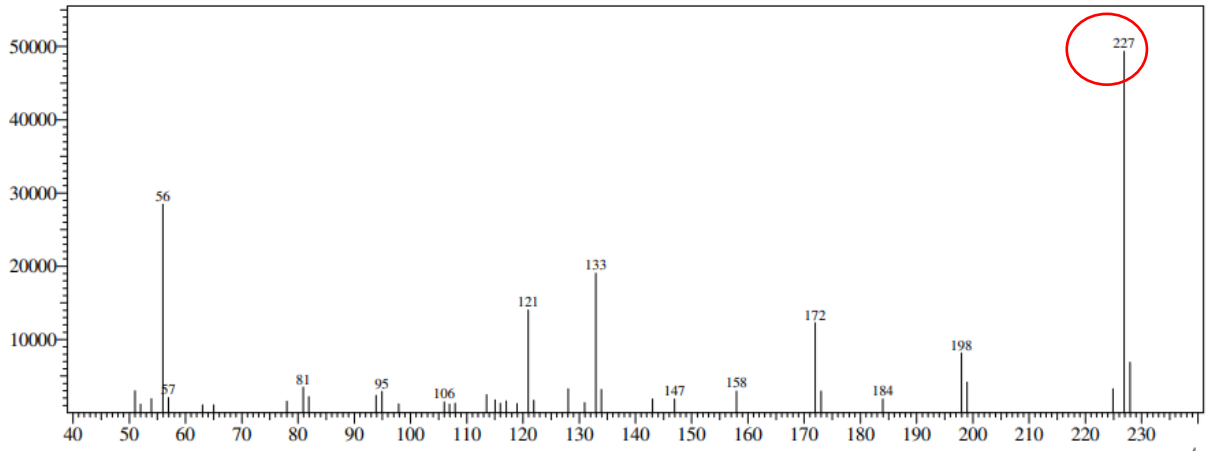


Figure S29. GC-MS spectrum of **1f**.

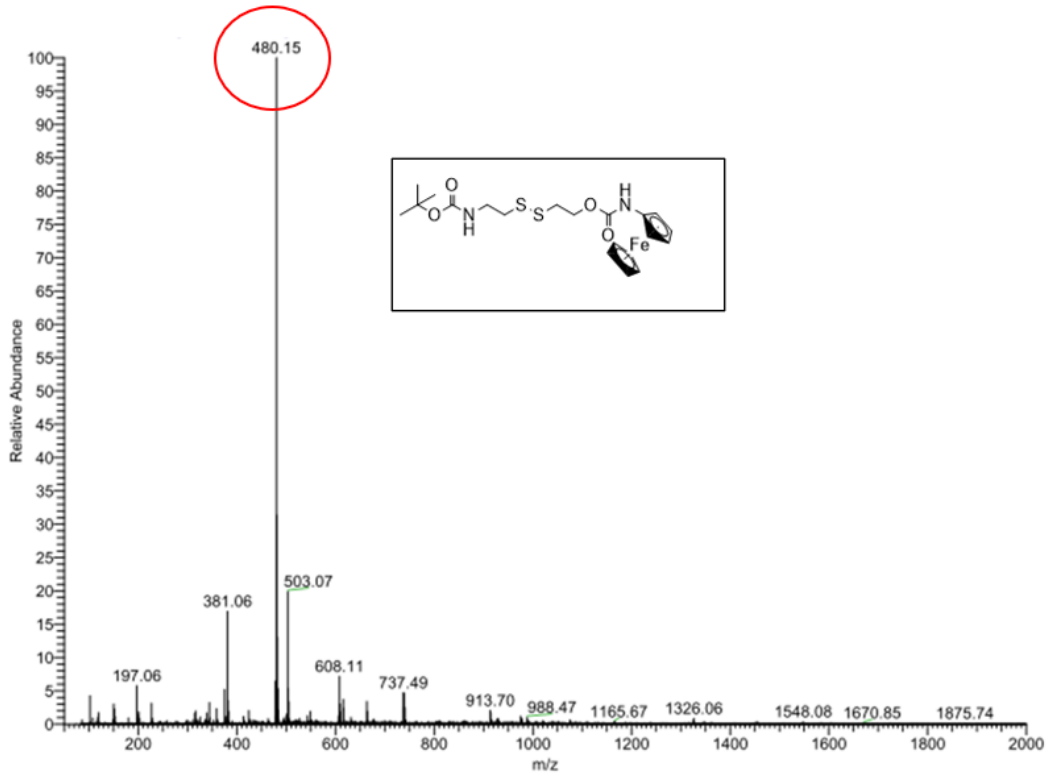


Figure S30. LC-MS spectrum of **1g**.

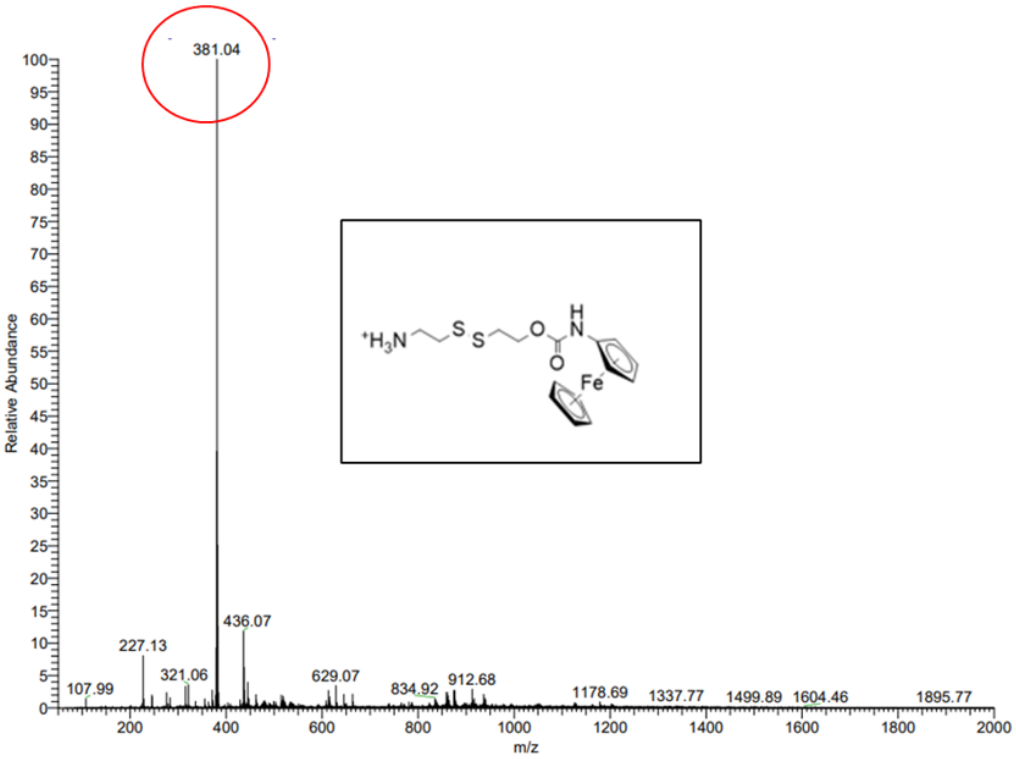


Figure S31. LC-MS spectrum of **1**.