Supporting Information

for

Multifunctional siRNA/ferrocene/cyclodextrin nanoparticles for enhanced chemodynamic cancer therapy

Gowtham Raj,[a] Vasudev D. S.,[a] Sarah Christopher,[a] Anupama Babulal,[a] Harsha P.,[a] Soumakanya Ram,[a] Mehul Tiwari,[a] Markus Sauer[b] and Reji Varghese[a]*

[a]Indian Institute of Science Education and Research (IISER) Thiruvananthapuram
Thiruvananthapuram-695551, India; Email: reji@iisertvm.ac.in

[b]Department of Biotechnology and Biophysics, Biocenter
Julius Maximilian University of Würzburg, Würzburg, Germany

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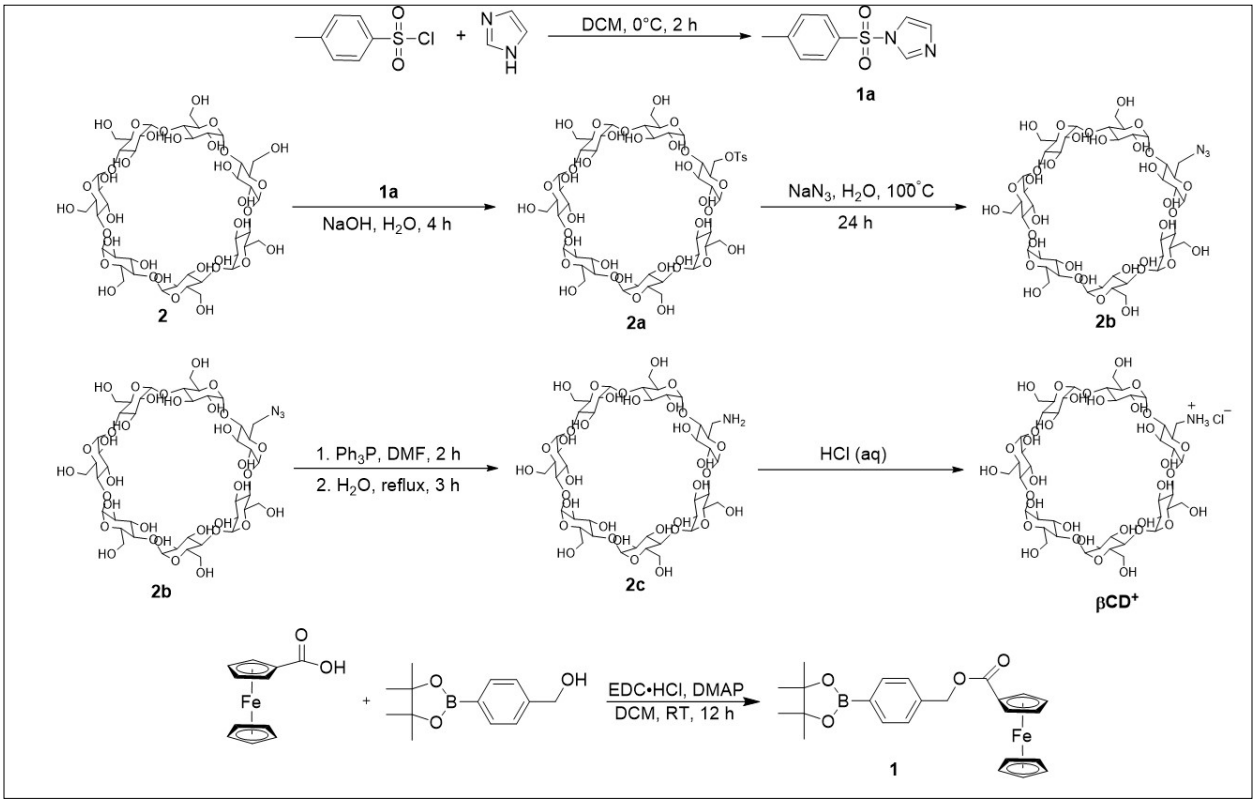
Experimental methods

Ferrocene carboxylic acid and β-CD were commercially purchased from Sigma Aldrich. The siRNA (**siRNA**) and FAM-labelled siRNA (**siRNA-FAM**) were purchased from Sigma Aldrich and used as obtained. Nuclease-free water was used for all the experiments. The morphology of the nanostructure was analysed using various microscopic techniques including AFM, TEM and CLSM analyses. AFM analyses were carried out on a Multimode BRUCKER AFM (Veeco Nanoscope V). TEM analyses were carried out on FEI Tecnai G2 F20 (200 kV) microscope. Confocal imaging was done using Nikon EclipseTi. MTT assay was used to study the cytotoxicity of the nanoformulation 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 lysosomes and mitochondria were stained using lysotracker deep red and mitotracker deep red. The nucleus of the cell was stained using Hoechst. The TMRM dye was used to analyse the integrity of mitochondria after administration of nanoparticle formulation. The Annexin V-FITC apoptosis detection kit was purchased from BD Bioscience. The live/dead assay was done using calcein-AM/PT staining following reported protocols. Dynamic Light Scattering (DLS) was performed on Malvern Zetasizer Nano ZS equipped with 655 nm laser. Experiments were done at 20℃ at a back-scattering angle of 173˚.

Synthesis of β-CD$^+$ and 1



Scheme S1: Synthesis scheme for **β-CD$^+$** and **1**.

Synthesis of **β-CD$^+$** was achieved by following reported procedures.[1,2]

Synthesis of 1: Ferrocene carboxylic acid (0.50 g, 1.86 mmol) was suspended in anhydrous dichloromethane (30 mL). Then, EDC (0.86 g, 4.46 mmol) was added, followed by DMAP (0.27 g, 2.23 mmol). Then anhydrous DCM solution of [4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]methanol (1.86 mmol) was slowly added to the reaction mixture. The mixture was stirred at room temperature for 12 h. The solvent was removed on a rotary evaporator and purified by silica column chromatography using EtOAc/hexane (v:v = 1:2) as the eluent (Yield: 92%). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 1.275 (s, 12H), 4.063 (s, 5H), 4.33 (t, J = 1.5 Hz, 2H), 4.76 (t, J = 1.5 Hz, 2H), 5.21 (s, 2H), 7.39 (d, J = 8 Hz, 2H), 7.77 (d, J = 8, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 20.13, 24.88, 60.93, 64.99, 65.46, 66.62, 69.73, 70.21, 72.03, 72.28, 72.53, 76.77, 77.02, 77.27, 79.13, 122.52, 127.27, 130.28, 134.93, 135.03; ^{11}B NMR (160 MHz, $CDCl_3$, δ ppm): 31.36. MALDI-TOF MS-m/z of $C_{23}H_{24}BFeO_4$: $[M+H]^+$ = 447.13 (calc); 447.06 (expt.)

Synthesis of **β-CD$^+$/1/siRNA** NPs

The previously synthesized **β-CD$^+$** was redissolved in deionized water at pH 2.0 (pH was adjusted using 0.01N HCl solution) to keep all the amino end groups protonated at high concentrations. In order to fabricate **β-CD$^+$/1** NPs, **β-CD$^+$** in nuclease-free water (1 μM) was added to five equivalences of **1** in THF (5 μM) and annealed at 90 °C for 5 minutes, followed by slow cooling to room temperature. The excess guest molecules were removed by repeated (3 times) centrifugation (4000 rpm for 3 minutes), and supernatant was collected and used for further experiments. **β-CD$^+$/1/siRNA** NPs were synthesized by adding **siRNA** at 25°C to the purified **β-CD$^+$/1** NPs solution and shaken for 30 minutes.

Microscopic analyses

For TEM analyses, **β-CD$^+$/1** and **β-CD$^+$/1/siRNA (**10 μM **β-CD$^+$**, 10 μM **1** and **siRNA** 20 nM) were 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 cast onto a freshly cleaved mica surface and dried overnight. CLSM analyses were done by drop-casting samples over pre-detergent washed glass slides mounted with the coverslip. The samples were then viewed under Nikon EclipseTi inverted confocal microscope.

Catalytic activity

The peroxidase-like catalytic activity of **β-CD$^+$/1** NPs (1 mM) were carried out using methylene blue degradation assay. The degradation of methylene blue (15 μM) was used as a marker for monitoring ROS generation. Briefly **β-CD$^+$/1** NPs in sodium acetate buffer at pH 5.0 and H_2O_2 (10 mM) was added to this solution. Subsequently, the absorbance of methylene blue at 660 nm was monitored during the course of the reaction.

Monitoring of Fe^{3+} release

β-CD$^+$/1 NPs (100 μM) were treated with H_2O_2 (9.8 mM) for 30 min in PBS buffer at pH 5.0. Then sodium dithionite (1M), was added to the reaction mixture, which reduces Fe^{3+} into Fe^{2+}. Finally, 2,2′-bipyridine solution (100 μM) was added to form a dark red $[Fe(2,2'\text{-bipy})_3]^{2+}$ complex (λ_{max} = 519 nm). $FeCl_2.4H_2O$ (100 μM) was used as a positive control.

Detection of GSH consumption

For the detection of GSH consumption, DTNB (10 μM) probe was used. DTNB, which upon reacting with GSH (25 μM) give a chromogenic product with absorption maxima centered at 412 nm compared to GSH untreated DTNB. The DTNB solution was treated with different sets of the sample, including (i) DTNB, (ii) GSH, (iii) **β-CD$^+$/1**, (iv) DTNB + **β-CD$^+$/1** + H_2O_2, (v) DTNB + GSH, (vi) DTNB + GSH+ **β-CD$^+$/1** + H_2O_2. These experiments were carried out in PBS buffer at pH 7.4. The concentration of H_2O_2 was (100 μM).

Confocal laser scanning microscopic (CLSM) analyses

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. Once the cells have reached 70 % confluency the media was changed and the cells were treated with **β-CD$^+$/1/siRNA-FAM** (**β-CD$^+$** and **1**: 10 μM and **siRNA-FAM:** 10 nM) for internalization studies. The lysosome was stained with lysotracker deep red, and mitochondria with mitotracker deep red. TMRM was used for monitoring the mitochondrial damage 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 of **β-CD$^+$/1/siRNA-FAM** NPs, HeLa cells were seeded at a cell density of 0.8×10^6 cells in 35 mm culture dish and grown in a 5 % CO_2 incubator at 37 °C in DMEM cell culture media for 24 h. Once the cells has reached 70 % confluency, **β-CD$^+$/1/siRNA-FAM** were diluted with DMEM with final **siRNA-FAM** concentration of 20 nM and kept for 6 h. Once the incubation time was over, the media was changed with fresh media and cells were collected by trypsinization and washed three times with 1X PBS. Cells were then collected in 300 μl PBS for flow cytometry analyses.

Measurement of intracellular GSH consumption

HeLa cells were seeded at a cell density of 0.8×10^6 cells in 35 mm culture dish and grown in a 5 % CO_2 incubator at 37 °C in DMEM cell culture media for 24 h. Once the cells has reached 70 % confluency, **β-CD$^+$/1/siRNA** NPs ([**siRNA**] = 1 μM and [**1**] = 500 μM) were diluted with DMEM and kept for 24 h. Once the incubation time was over, the media was changed with fresh

media and cells were collected by lysis using chaps lysis buffer and washed three times with 1X PBS (10000 RPM, 10 minutes, 4 °C). The GSH content was measured using GSH/GSSG detection kit (origin) according to the manufacturer's instructions.

MTT assay

HeLa cells were seeded to 96-well with a density of 1×10^2 cells per well and were grown in DMEM cell culture media for 24 h. After confirming the cellular confluency of about 70 % cells were incubated with different concentrations of **siRNA** (5-250 nM**)**, and different concentration of **β-CD$^+$/1, β-CD$^+$/1/siRNA** NPs (50-500 μM). 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 by replacing the old media and kept for 3 h incubation at 37 °C in 5 % CO_2 incubator. After 3 h the media was aspirated and 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

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 **β-CD$^+$/1/siRNA** NPs and 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. The cells were treated with **β-CD$^+$/1/siRNA** NP**s** and incubated for 24 h. Once the incubation time was over, cells were washed with PBS. The Annexin V-FITC (5 μL) in Annexin V-FITC binding buffer (200 μL), and a PI solution (10 μL from 10 μg/mL) at room temperature for 20 min. Washed with PBS 1X and after that, imaged under Nikon Eclipse Ti with a 20 X objective. For the FACS analysis HeLa cells were seeded at 24 well plates and grown in a 5 % CO_2 incubator at 37 °C in DMEM cell culture media for 24 h. Once the cells has reached 70 % confluency, **β-**

CD$^+$/1/siRNA (**β-CD$^+$** and **1**: 500 μM and **siRNA**: 1 μM) were diluted with DMEM and kept for 24 h. Once the incubation time was over, the media was changed with fresh media and cells were collected by trypsinization and washed three times with 1X PBS. Cells were then collected in Annexin V-FITC binding buffer and treated with Annexin V-FITC (5 μL) in Annexin V-FITC binding buffer and a PI solution (10 μL from 10 μg/mL) which is kept at 15 minutes and analyzed under flow cytometry analyses.

Live/dead cell staining assay

The live/dead cell staining assay was performed on HeLa cells using Calcein AM/PI co-staining 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 **β-CD$^+$/1/siRNA** NPs (**β-CD$^+$** and **1**: 500 μM and **siRNA**: 1 μM) 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. The plates were further washed with 1X PBS and after that, imaged under Nikon Eclipse Ti with a 20 X objective.

3D Spheroid culture

MDA-MB231 cells were cultured to prepare the 3D spheroids, and the method adopted for the growth was the hanging drop method. The cells were grown on a T75 flask, and once it reached more than 90% confluency, cells were trypsinized and made cell stock solution with 5×10^3 cells and mounted over the lid of a petri dish and kept upside down with base filled with 15 mL of PBS to provide humidity for the spheroid. Followed by keeping a CO_2 incubator for 24 h, spheroid formation was confirmed using an optical microscope. After confirming the formation of spheroids, the spheroids were transferred to a 24-well plate mounted with 10 mm coverslip having 3:1 collagen to media, and it was incubated at 37 °C for 1 h. The spheroids were then incubated with **β-CD$^+$/1/siRNA** NPs (**β-CD$^+$** and **1**: 500 μM and **siRNA**: 1 μM) for 24 h at 37 °C. Once the incubation was over, the spheroids were fixed using 4% PFA for 15 min at 37 °C. Further the spheroids were permeabilized using 0.1% TritonX and incubated for 10 min at room temperature. Subsequently, 0.1 × TritonX + Phalloidin solution was added and incubated at 37 °C for 30 min. Spheroids were washed gently twice with 1 × PBS and mounted using Mowiol on a glass slide and kept overnight for drying and the spheroids were imaged using CLSM.

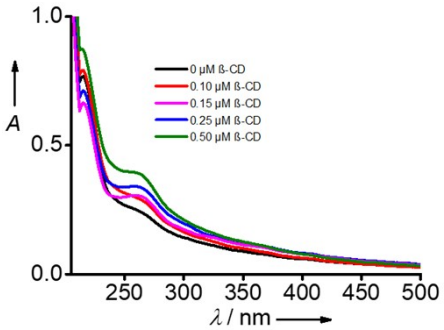


Figure S1. *Absorption spectral changes of **1** with the gradual increase of **β-CD**$^+$ concentration in water.*

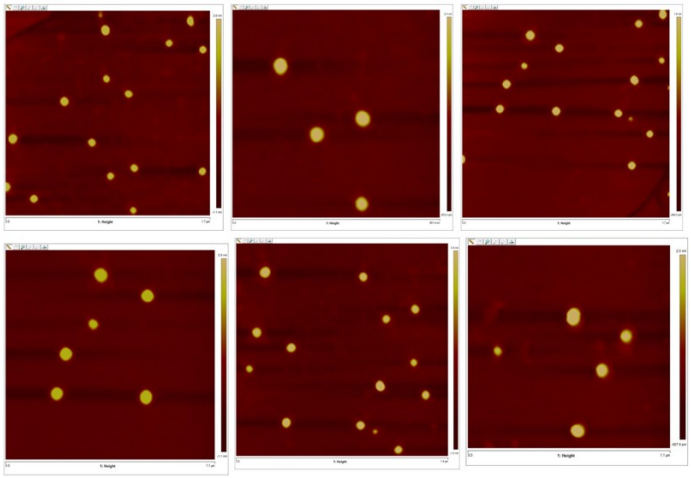


Figure S2. *AFM images of **β-CD**$^+$**/1** NPs.*

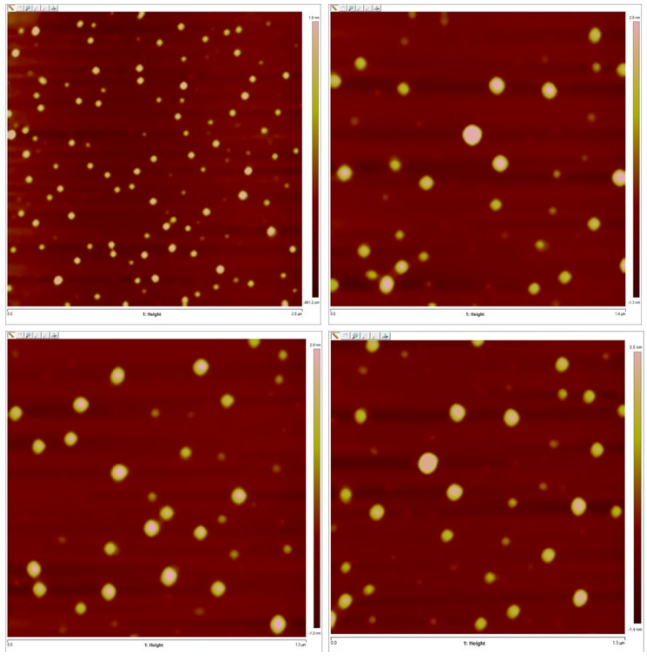


Figure S3. *Additional AFM images of **β-CD**$^+$**/1/siRNA** NPs.*

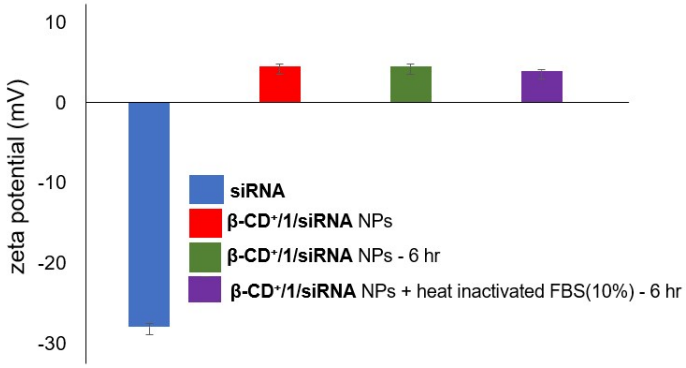


Figure S4. *Zeta potential analyses of* ***β-CD*$^{+}$*/1/siRNA*** *NPs under various experimental conditions.*

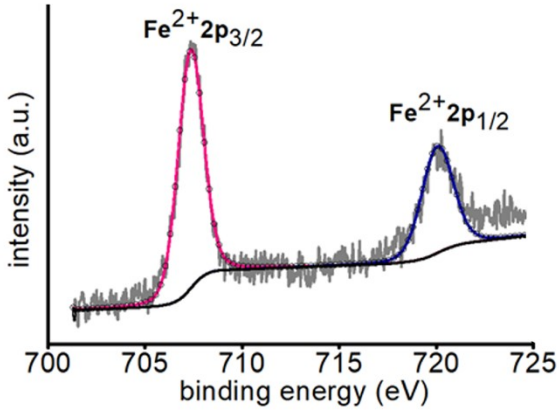


Figure S5. *XPS spectrum of* ***1***.

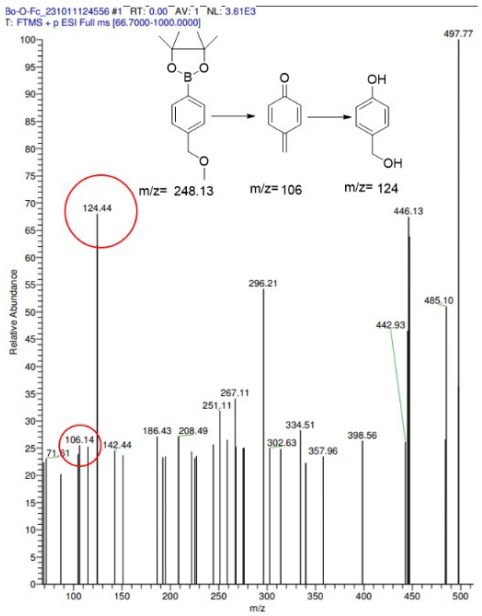


Figure S6. *LRMS spectrum of a reaction mixtures consisting of* H_2O_2 *(9 mM), triethylamine (0.9 mM), and* ***1*** *(0.9 mM) in acetonitrile/water (10/1.1, v/v).*

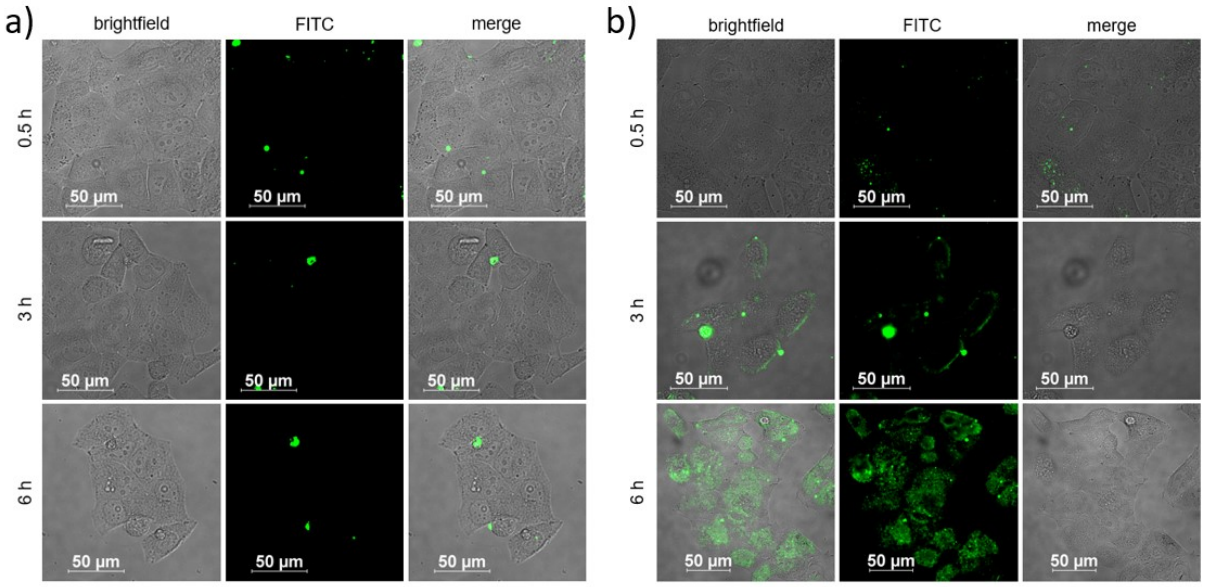


***Figure S7**. (a) Time-dependent CLSM images for the cellular internalization of **siRNA-FAM** and (b) **β-CD⁺/1/siRNA-FAM** NPs into HeLa cells.*

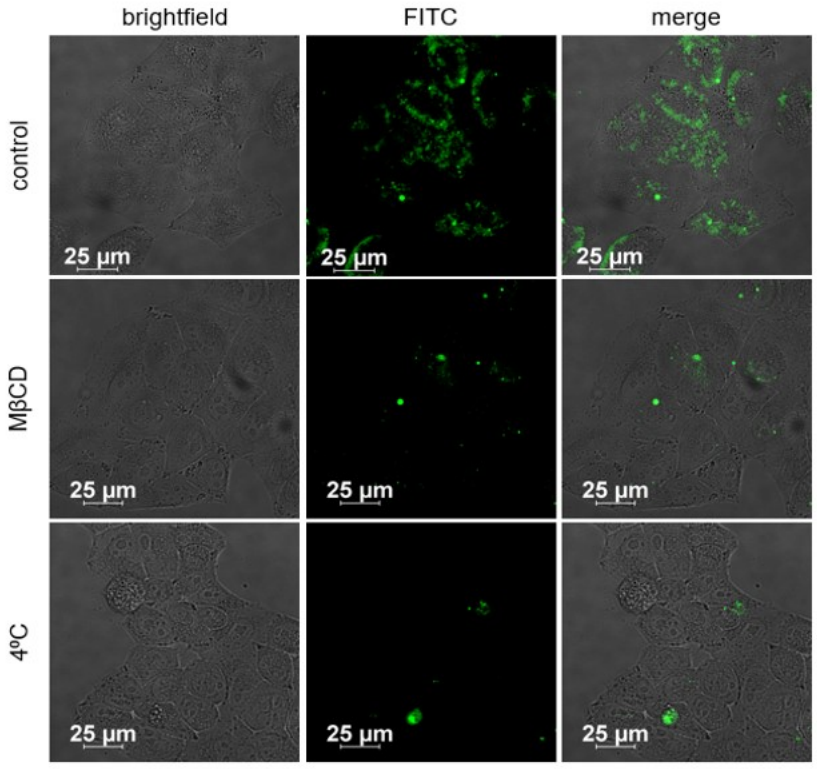


***Figure S8**. CLSM images of MβCD (50 µM) pre-treated HeLa cells treated with **β-CD⁺/1/siRNA-FAM** NPs (middle row) and at 4 ℃ (middle row).*

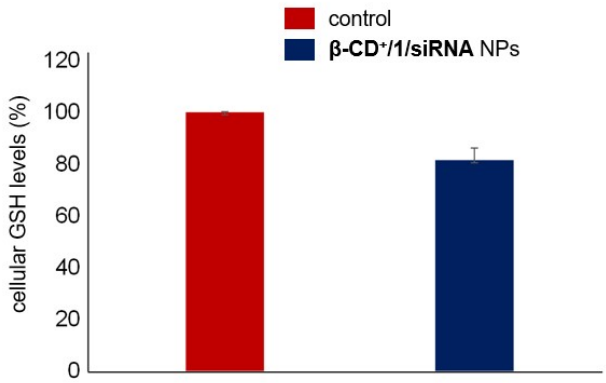


Figure S9. *Cellular GSH-level analysis monitored after incubation with* ***β-CD⁺/1/siRNA*** *NPs for 24 h through the GSH/GSSG detection kit.*

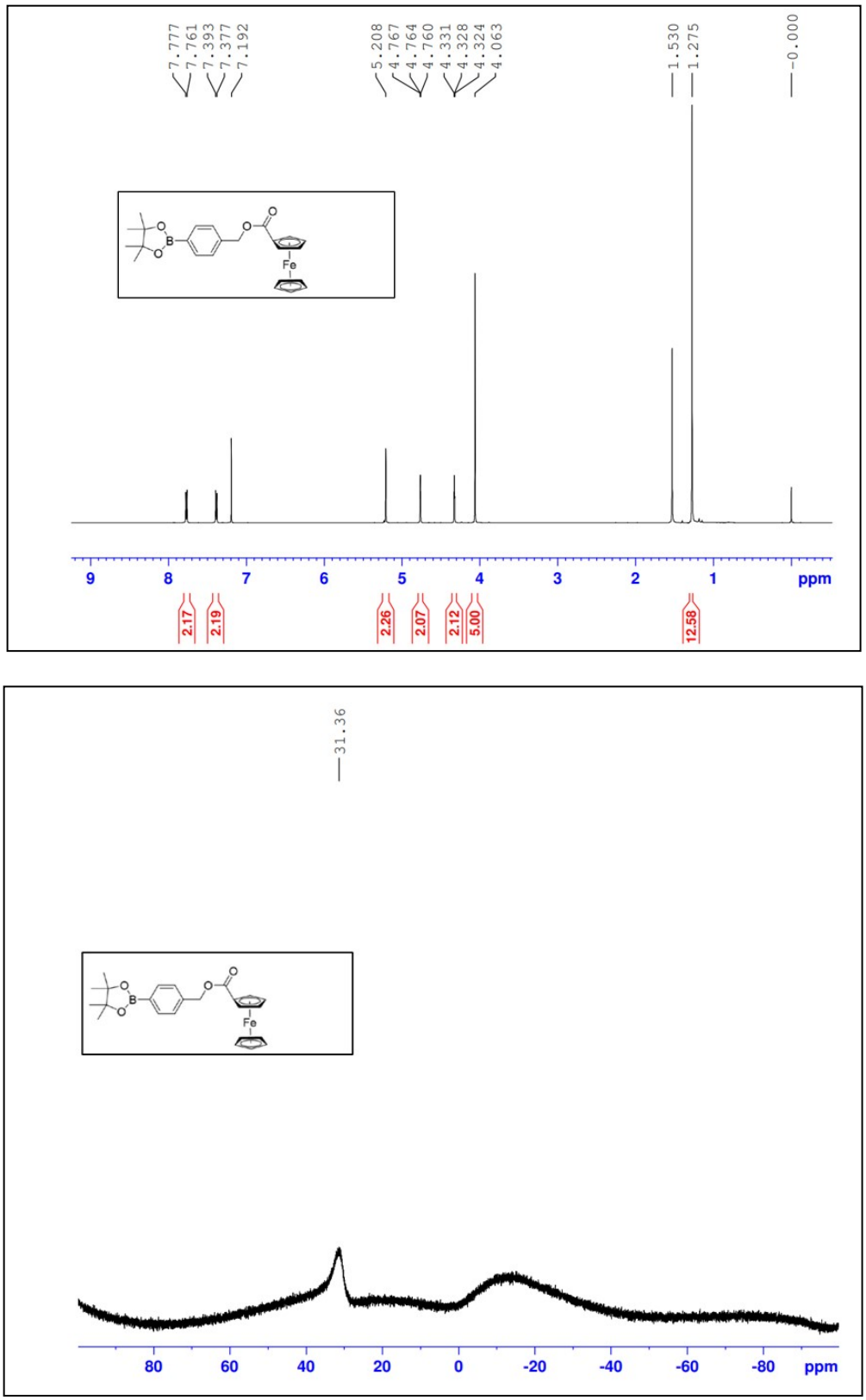
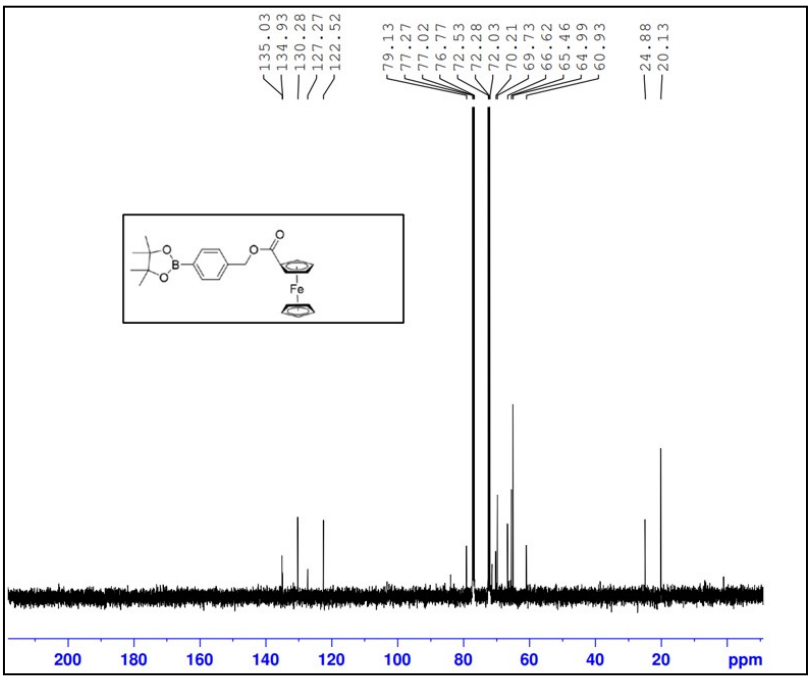


Figure S10. *^{1}H (above) and ^{11}B (below) NMR spectra of* ***1***.



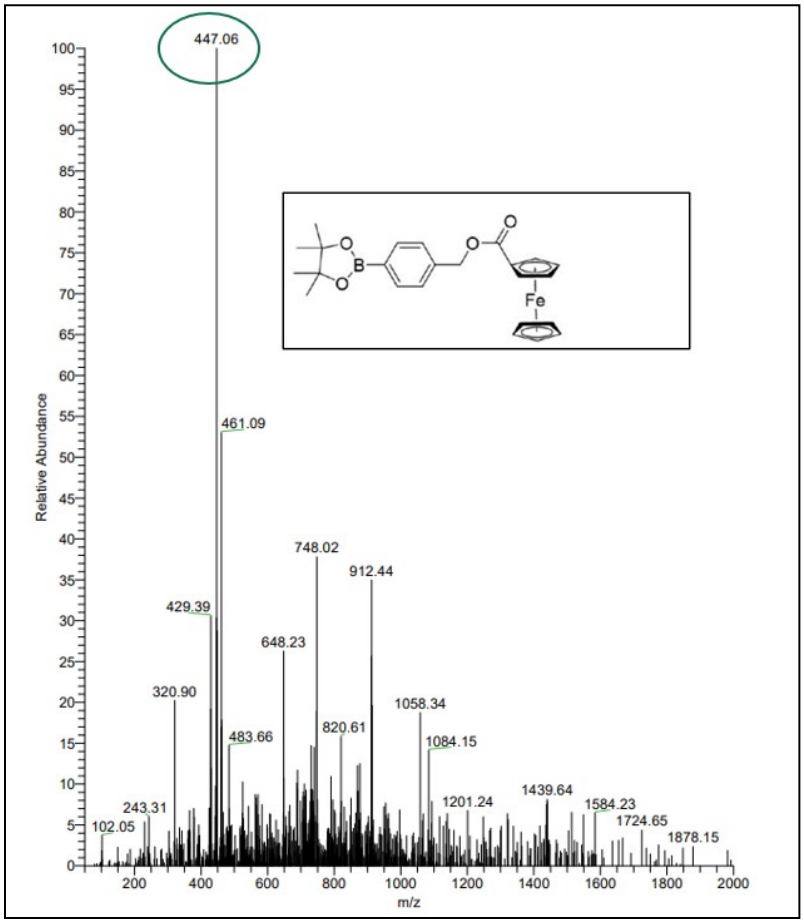


Figure S11. *^{13}C (above) and LRMS (below) spectra of **1**.*

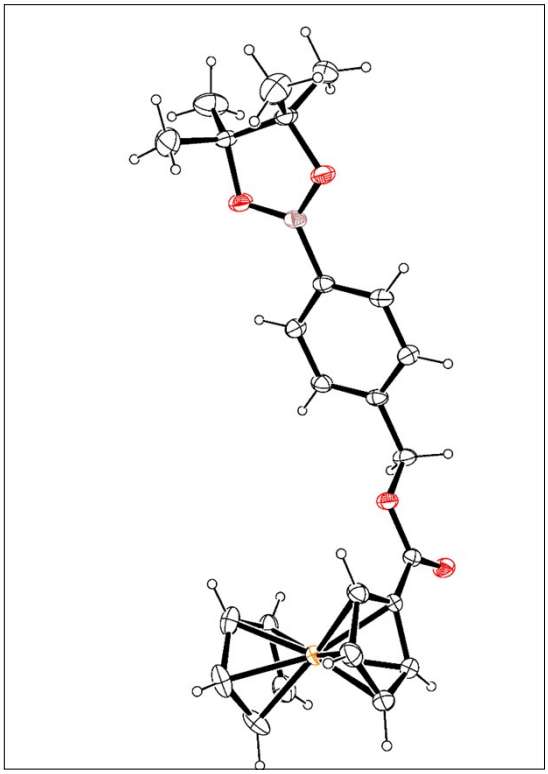


Figure S12. *ORTEP diagram of **1**.*

Crystal data of 1.

Identification code	shelx	
Empirical formula	C24 H27 B Fe O4	
Formula weight	446.11	
Temperature	296(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P 21/n	
Unit cell dimensions	a = 6.8843(6) Å	α= 90°.
	b = 29.823(3) Å	β= 102.271(3)°.
	c = 10.8712(10) Å	γ = 90°.
Volume	2181.0(3) $Å^3$	
Z	4	
Density (calculated)	1.359 Mg/m^3	
Absorption coefficient	0.719 mm^{-1}	
F(000)	936	
Crystal size	0.095 x 0.085 x 0.038 mm^3	
Theta range for data collection	2.354 to 25.000°.	

Index ranges	-8<=h<=8, -35<=k<=35, -12<=l<=12
Reflections collected	28755
Independent reflections	3838 [R(int) = 0.0670]
Completeness to theta = 25.000°	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.973 and 0.935
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3838 / 0 / 275
Goodness-of-fit on F^2	1.075
Final R indices [I>2sigma(I)]	R1 = 0.0549, wR2 = 0.1355
R indices (all data)	R1 = 0.0803, wR2 = 0.1498
Extinction coefficient	n/a
Largest diff. peak and hole	0.577 and -0.311 e.$Å^{-3}$
CCDC number	2259673

References:

1) S. K. Albert, H. V. P. Thelu, M. Golla, N. Krishnan and R. Varghese, *Nanoscale*, 2017, **9**, 5425–5432.
2) W. Tang and S.-C. Ng, *Nat. Protoc*., 2008, **3**, 691–697.