Supporting Information

for

G_4-hemin loaded 2D nanosheets for combined and targeted chemo-photodynamic cancer therapy

Gowtham Raj,[a] Tamraparni Ghosh,[a] Vasudev D.S,[a] Harsha P.,[a] Devu B. Kumar,[b] Justin Prasad,[a] Athul V.B,[b] Abhimanyu S.M.[b] and Reji Varghese[a]*

[a] School of Chemistry
Indian Institute of Science Education and Research (IISER) Thiruvananthapuram
Thiruvananthapuram-695 551, India; Email: reji@iisertvm.ac.in

[b] School of Biology
Indian Institute of Science Education and Research (IISER) Thiruvananthapuram
Thiruvananthapuram-695 551, India

TABLE OF CONTENTS

Experimental methods

Chemicals

Analytical grade β-Cyclodextrin, Hemin, Phalloidin, Fluorescein Isothiocyanate and **DNA1** were obtained from Sigma Aldrich. Hoechst, 2′,7′- dichlorofluorescin diacetate (DCFH-DA), acridine orange (AO), propidium iodide (PI), Annexin V-FITC, Tetramethylrhodamine, Methyl Ester Perchlorate (TMRM), Collagen I, rat tail were purchased from Thermo Fischer Scientific. All of these chemicals were used without further purification. Deionized (DI) water was used for all the studies.

Instruments

AFM imaging was carried out on Multimode SPM (Veeco Nanoscope V). The probe used for imaging was an antimony-doped silicon cantilever with a resonance frequency of 300 kHz and a spring constant of 40 Nm^{-1}. TEM analyses were carried out on an FEI Tecnai 30 G2 (120 kV). Absorption spectra were recorded on a Peltier-attached Shimadzu UV-3600 Vis-NIR spectrophotometer in a quartz cuvette of 10 mm path length. DLS analyses were done on a Malvern Zetasizer NanoZs equipped with a 655 nm laser. Experiments were performed at 25°C at a backscattering angle of 173°. Confocal images have been carried out using Nikon Eclipse Ti with a 100 X oil immersion objective. Flow cytometry analysis was carried out using the FACS LSR Fortessa Flow Cytometer (BD, USA). MTT assay has been carried on Tecan Infinite 200 PRO microplate reader.

Synthesis of Hem/DNA1

The preparation of **Hem/DNA1** was achieved through a one-step thermal annealing process. Briefly, **DNA1** (5'-GGTGGTGGTGGTTGGGTGGTGGTGGAAAAAAAAA-3') (1 μM) was diluted in K^+-EDTA buffer followed by annealing to 90 °C. The reaction mixture was kept at 90 °C for 10 minutes and allowed to cool to room temperature. When the temperature reaches 60 °C **Hem** (1 μM) was added to form **Hem/DNA1**. Subsequently, cut-off filtration was performed to remove any unbound **Hem** from the solution.

Sample preparation for TEM and AFM analysis

For the TEM analyses, an annealed solution of **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA**) was drop cast onto a 400-mesh carbon-coated copper grid (Ted Pella, Inc.) and the excess sample was wiped off using tissue paper. The process was repeated 2-3 times, and samples were kept for drying. The samples were then analyzed using TEM

FEI Tecnai 30 G2 (120 kV). For the AFM analyses, **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA**) was drop cast onto a freshly cleaved mica surface and allowed to dry overnight, and the images were taken using Multimode SPM (Veeco Nanoscope V) AFM.

Measurement of peroxidase-like activity of 1/β-CD$^+$/Hem/DNA1

The peroxidase-like activity of **Hem/DNA1** (1 μM) was studied using 3,5,3',5'-tetramethylbenzidine (TMB) as the substrate. The oxidation of TMB (200 μM) using H_2O_2 (200 μM) catalyzed by **Hem/DNA1** (1 μM) in NaAc (pH 6.5) buffer was studied. The absorbance of oxidized TMB at 650 nm was monitored to assess the catalytic performance of the CDT agents.

Measurement of DPBF degradation

DPBF was used to measure extracellular $^1O_2$ produced by **1/β-CD$^+$/Hem/DNA1**. For this purpose, DPBF alone, **1/β-CD$^+$** nanosheet (50 μM) in water containing DPBF (200 μM) was irradiated with a 635 nm laser at 0.75 W/cm^2 for 70 sec and the decrease in the absorption of DPBF was monitored with respect to time. Similarly, DPBF degradation in the presence of Hem/DNA1 (1/1 μM) + H_2O_2 (200 μM) (pH 6.5) and 1/β-CD+/Hem/DNA1 (50 μM 1/β-CD+ and Hem/DNA1 (1/1 μM) + H_2O (200 μM) + NIR light pH 6.5 was also monitored by a UV−vis spectrometer.

Cellular uptake studies

MDA-MB-231 cells were seeded in μ-Slide 8 well slides at a seeding density of 1000 cells per well and cultured with DMEM containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 for 24 h. After confluency has reached up to 70 %, cell culture media was replaced with new cell culture media containing **1/β-CD$^+$/Hem/DNA2** (1 μM with respect to **DNA2**) and the cells were further incubated for 12 h. After 12 h of incubation, the medium was replaced with fresh DMEM containing 10 μL Hoechst 33342 (0.35 mg/mL), 10 μL Lysotracker deep red (0.35 mg/mL), 10 μL Mitotracker deep red (0.35 mg/mL). After 15 minutes of incubation, the medium was removed and the cells were washed three times with PBS and imaged using CLSM (Nikon Eclipse Ti).

Detection of ROS inside the cells

MDA-MB-231 cells were seeded in μ-Slide 8 well slides at a seeding density of 1000 cells per well and cultured with DMEM containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 for 24 h. After confluency had reached up to 70 %, cell culture media was replaced with the medium **Hem/DNA1** (1/1 μM), **1/β-CD$^+$** (20 μM **1/β-CD$^+$**), **1/β-CD$^+$/Hem/DNA1** and the cells were further incubated for 2 h. After the incubation, illuminated with NIR light (635 nm laser at 0.75

W/cm^2) for 10 minutes cells after stained with ROS-sensitive dye DCFHDA (10 μM) in FBS-free medium and kept for another 4 h of incubation, washed off three times with PBS and imaged using CLSM.

Evaluation of lysosomal integrity

The acridine orange (AO) staining assay was used to evaluate the lysosomal integrity during cellular apoptosis. Briefly, MDA-MB-231 were seeded in μ-Slide 8 well slides at a seeding density of 1000 cells per well and cultured with DMEM containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 for 24 h. After confluency had reached up to 70 %, cell culture media was replaced with the medium containing **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA1**). The cells were further incubated for 2 h followed by NIR light illumination (635 nm laser at 0.75 W/cm^2) for 10 minutes and kept for another 4 h of incubation. After the incubation period was over, cells were then stained with AO (20 μM) in PBS for 20 min and washed off three times with PBS and imaged using CLSM.

Evaluation of the mitochondrial membrane potential

To investigate the mitochondrial membrane potential, MDA-MB-231 cells were seeded in μ-Slide 8 well slides at a seeding density of 1000 cells per well and cultured with DMEM containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 for 24 h. After confluency has reached up to 70 %, cell culture media was replaced with the with the medium containing **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA1**). The cells were further incubated for 2 h followed by NIR light illumination (635 nm laser at 0.75 W/cm^2) for 10 minutes and kept for another 4 h of incubation. After the incubation period was over, cells were then stained with AO (20 μM) in PBS for 20 min and washed off three times with PBS and imaged using CLSM. After the incubation period was over, cells were then stained with TMRM (10 μM) in PBS for 20 min and washed off three times with PBS band imaged using CLSM.

MTT assay

Cell-viability was studied by methyl thiazolyl tetrazolium (MTT) assay. For this, MDA-MB-231 cells was seeded in 96-well plates at 100 cells per well and cultured for 24 h until it reached confluency. We studied the toxicity of **β-CD$^+$** and **1/β-CD$^+$** nanosheets in the dark in MDA-MB-231 cells. For this purpose, **β-CD$^+$** and **1/β-CD$^+$** nanosheets under dark of varying concentrations (1-20 μM) were treated with MDA-MB-231 cells for 24 h. We then studied the combined chemo/photodynamic therapy based on enhanced toxicity in MDA-MB-231 cells. For this purpose,

MDA-MB-231 cells were treated with **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA**) for 2 h followed by NIR light illumination (635 nm laser at 0.75 W/cm^2) for 10 minutes and then cultured for 24 h. After incubating for 24 h, the medium was replaced with fresh culture medium and 20 μL of 5 mg/mL MTT solution was added. The cells were incubated for another 4 h and 100 μL DMSO was added to solubilize the formazan crystals and the absorbance at 565 nm was measured using a microplate reader to evaluate the cytotoxicity.

Live/Dead cell assay

The cytotoxicity of **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA**) was analyzed using live/dead cell assay. For this, MDA-MB-231 cells were seeded in μ-Slide 8 well slides at a seeding density of 1000 cells per well and cultured with DMEM containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 for 24 h. After confluency had reached up to 70 %, cell culture media was replaced with the medium containing **1/β-CD$^+$/Hem/DNA1** and for 2 h followed by NIR light illumination (635 nm laser at 0.75 W/cm^2) for 10 minutes and then cultured for 24 h. After the incubation period was over, cells were washed with PBS and calcein AM (1 μM) and propidium iodide (10 μL from 10 μg/mL) in fresh medium were added and incubated for 30 min. The plates were further washed with 1X PBS and then imaged using CLSM.

Annexin V-FITC/PI assay

Cell apoptosis was studied using Annexin V-FITC/PI assay. For this, HeLa cells were seeded in μ-Slide 8 well slides at a seeding density of 1000 cells per well and cultured with DMEM containing 10 % fetal bovine serum (FBS) at 37 °C in 5% CO_2 for 24 h. After confluency had reached up to 70 %, cell culture media was replaced with the medium containing **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA**) for 2 h followed by NIR light illumination (635 nm laser at 0.75 W/cm^2) for 10 minutes and then cultured for 24 h . After the incubation period was over, cells were washed with PBS. The Annexin V-FITC (5 μL) in Annexin V-FITC binding buffer (200 μL), and PI solution (10 μL from 10 μg/mL) were added and incubated for 20 min. Washed with 1 X PBS and then imaged using CLSM. 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, **1/β-CD$^+$/Hem/DNA1** (20 μM with respect to **1/β-CD$^+$** and 1 μM with respect to **Hem/DNA**) for 2 h followed by NIR light illumination (635 nm laser at 0.75 W/cm^2) for 10 minutes and then cultured

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 1 X 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 PI solution (10 µL from 10 µg/mL) and centrifuged the cell suspension at 150 RCF for 3 minutes and supernatant were removed. Cells were then washed with Annexin binding buffer and resuspended in Annexin binding buffer to 300 µL volume. The cell suspension was then analyzed using a flow cytometer.

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 , **1/β-CD$^+$/Hem/DNA1** (20 µM with respect to **1/β-CD$^+$** and 1 µM with respect to **Hem/DNA**) for 2 h followed by NIR light illumination (635 nm laser at 0.75 W/cm^2) for 10 minutes and then cultured for 24 h. 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 tthe spheroids were imaged using CLSM. The rate of invasion of the cells from the spheroid was observed with respect to control, and the invasion index was calculated using the following formula.

$$Invasion\ index = \frac{(area\ covered\ by\ the\ cells\ after\ migration - area\ of\ the\ speroid)}{area\ of\ the\ speroid} \quad \ldots\ldots\ldots \text{(equation 1)}$$

Fluorescence-activated cell sorting (FACS) analyses

For all the microscopic imaging, corresponding FACS analyses were carried out for quantification. For this purpose, HeLa cells were seeded at a cell density of 1×10^3 cells in 24 well plate and

grown in a 5 % CO_2 incubator at 37 °C in for 24 h. Once the cells had reached 90 % confluency, treatments were done according to the experiments. Once the incubation time was over, cells were stained according to the experiments as mentioned in the previous protocols and the media was changed with fresh media. The cells were collected by trypsinization and washed three times with 1× PBS. Cells were then collected in 300 µL PBS in a FACS tube and analysed using flow cytometry.

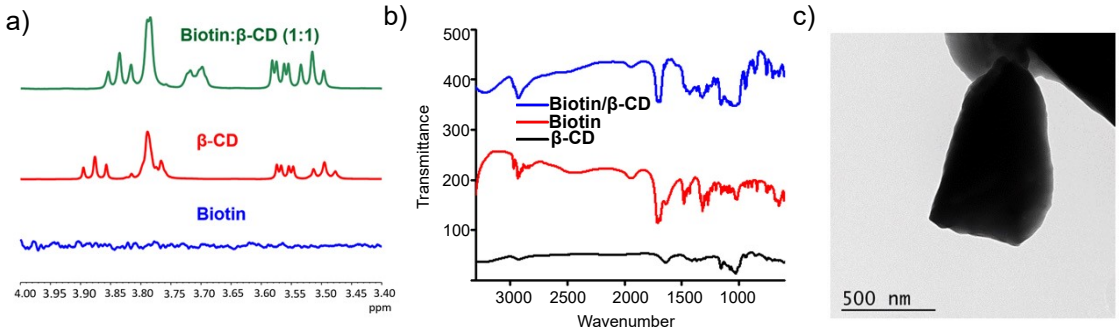

Figure S1. Comparison of (a) ^{1}H-NMR and (b) IR spectra of **biotin**, **β-CD** and **biotin-β-CD** complex. (c) TEM image of **1/β-CD$^+$**.

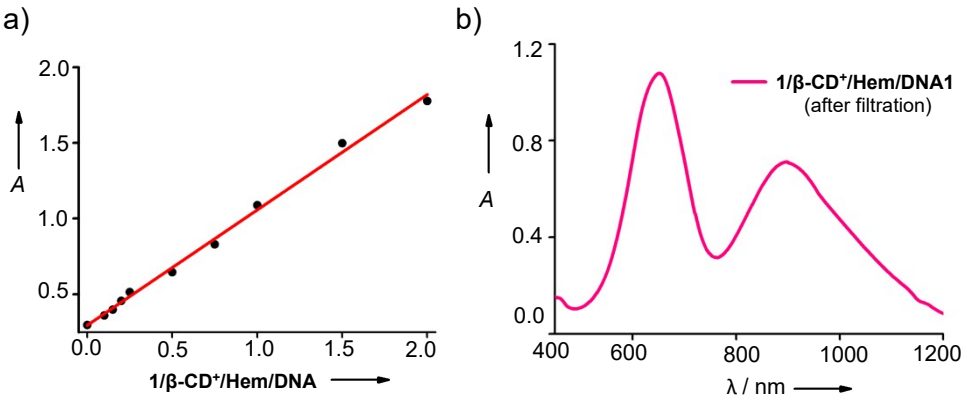

Figure S2. (a) Calibration curve for calculating the loading of **Hem/DNA1** onto **1/β-CD$^+$** nanosheet. Calibration plot was obtained by monitoring the oxidation of TMB by **1/β-CD$^+$/Hem/DNA1** nanosheet with varying concentration of **Hem/DNA1** (0 to 2 µM) keeping **1/β-CD$^+$** concentration constant at 20 µM. (b) Absorption spectrum of oxidized TMB using a known concentration of **1/β-CD$^+$/Hem/DNA1** nanosheet.

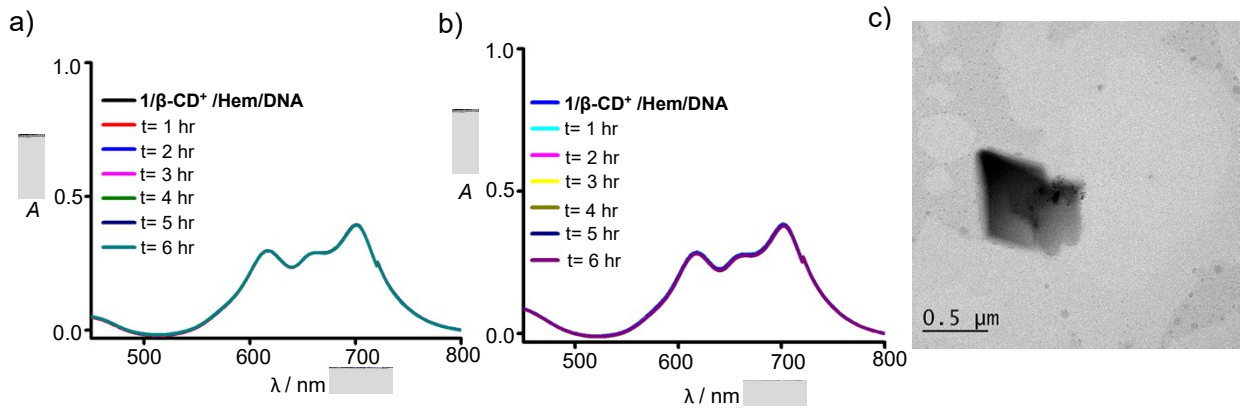

Figure S3. Time dependent absorption changes of **1/β-CD⁺/Hem/DNA1** nanosheet (a) in PBS buffer at pH 7.0 and (b) in 10 % serum. (c) A representative TEM image **1/β-CD⁺/Hem/DNA1** nanosheet after incubating in 10 % serum for 6h.

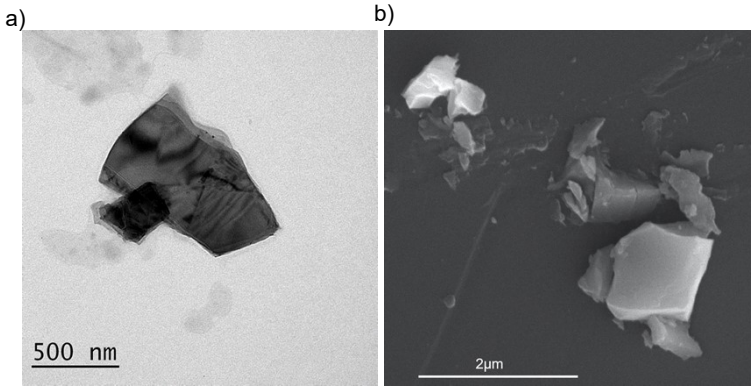

Figure S4. (a) TEM and (b) SEM images of **1/β-CD⁺** nanosheet.

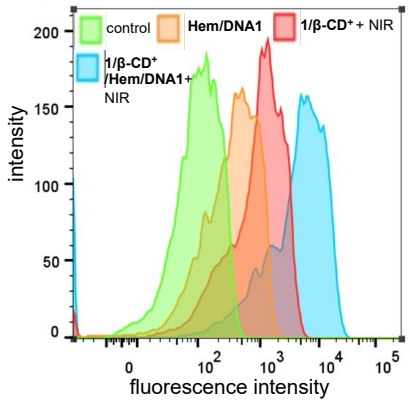

Figure S5. FACS analyses of **Hem/DNA1**, **1/β-CD⁺** + NIR and **1/β-CD⁺/Hem/DNA1** + NIR-treated MDA-MB-231 cells (635 nm laser with power of 0.75 W/cm^2 for 10 minutes).

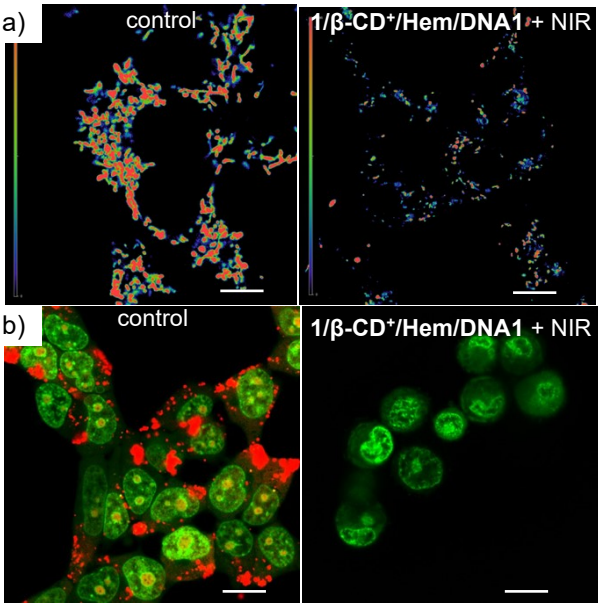

Figure S6. (a) TMRM assay: CLSM images of **1/β-CD$^+$/Hem/DNA1**-treated MDA-MB-231 cells stained with TMRM under photoirradiation at 635 nm laser (0.75 W/cm^2) (scale bar represents 25 μm) after 24 h of incubation. (b) AO assay: CLSM images of **1/β-CD$^+$/Hem/DNA1**-treated MDA-MB-231 cells stained with AO under photoirradiation at 635 nm laser (0.75 W/cm^2) (scale bar represents 25 μm) after 24 h of incubation.

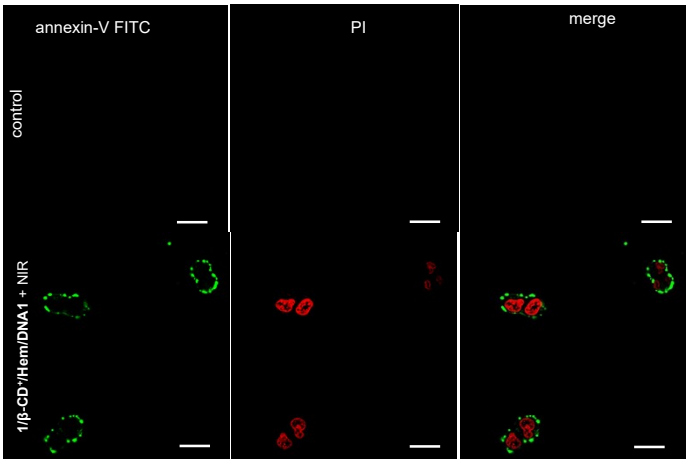

Figure S7. Annexin V-FITC/PI assay: CLSM images of **1/β-CD$^+$/Hem/DNA1**-treated MDA-MB-231 cells under photoirradiation using 635 nm laser (0.75 W/cm^2) and the corresponding untreated control cells (scale bar represents 25 μm).

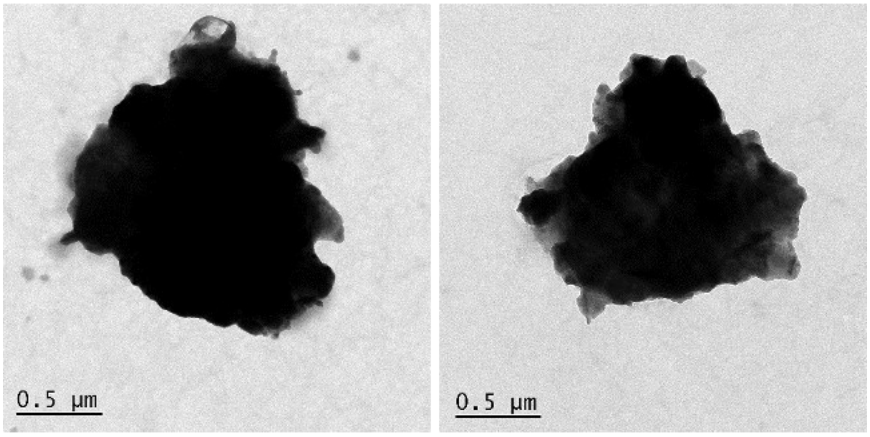

Figure S8. Additional TEM images of **1/β-CD$^+$/Hem/DNA1**.

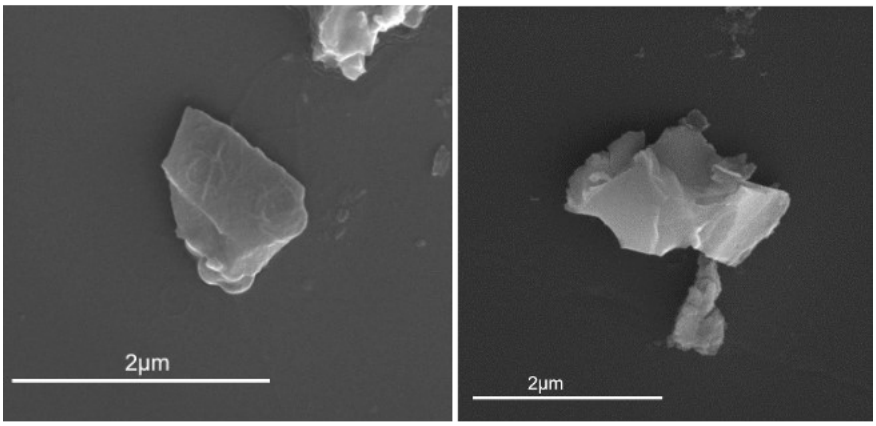

Figure S9. SEM images of **1/β-CD$^+$/Hem/DNA1**.

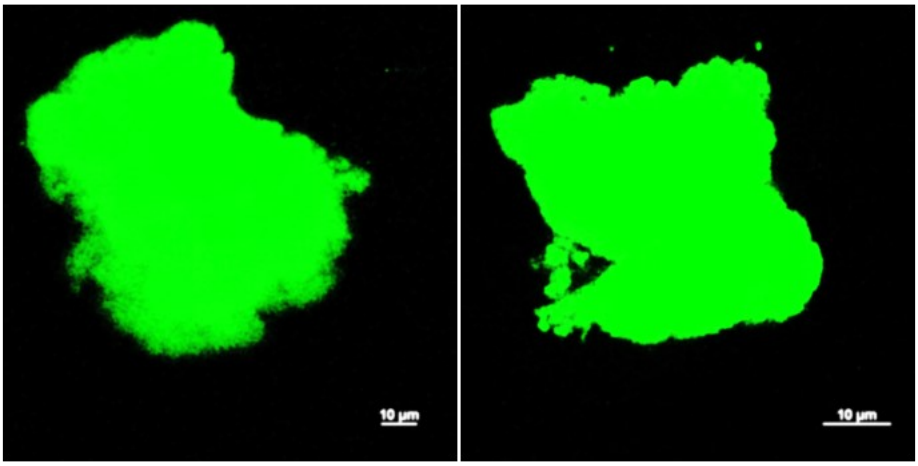

Figure S10. Additional CLSM images of **1/β-CD$^+$/Hem/DNA2**.

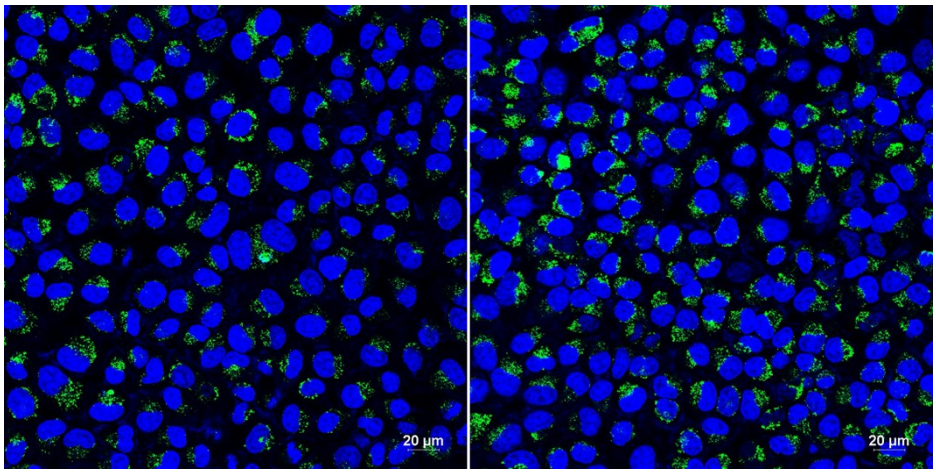

Figure S11. Additional CLSM images of cellular internalization of **1/β-CD$^+$/Hem/DNA2**.

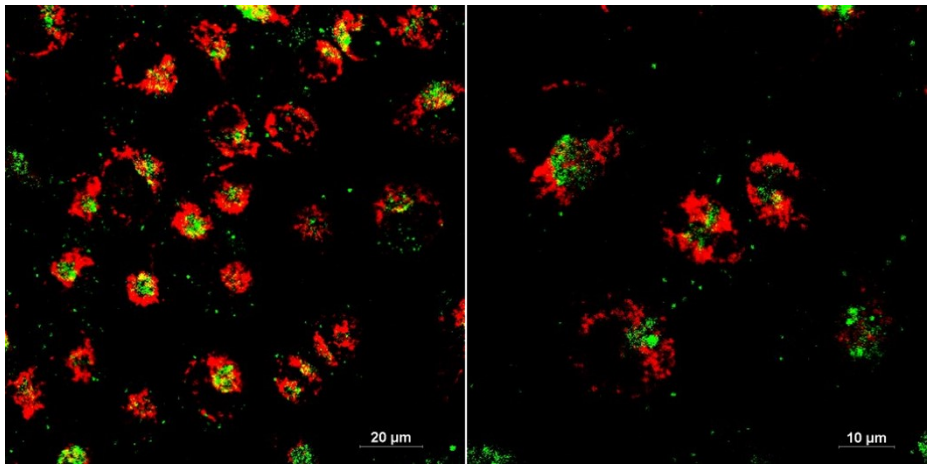

Figure S12. Additional CLSM images of lysosomal escape of **1/β-CD$^+$/Hem/DNA2**.

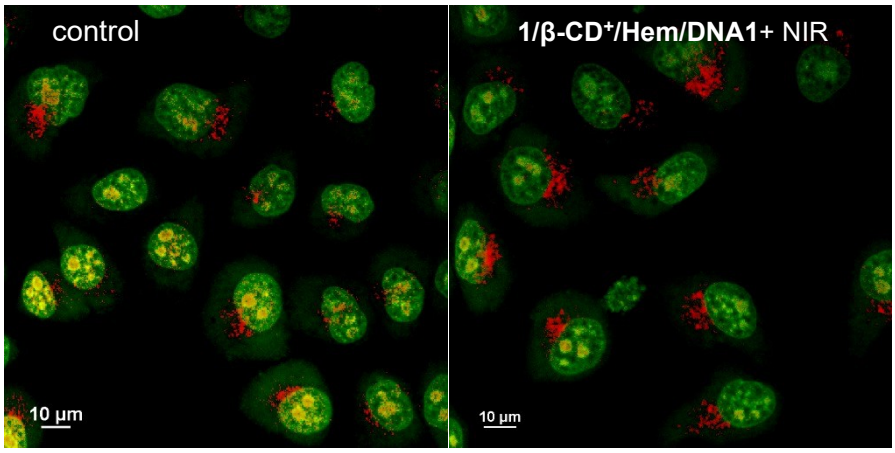

Figure S13. Additional CLSM images of acridine orange assay **1/β-CD$^+$/Hem/DNA1** after NIR illumination.

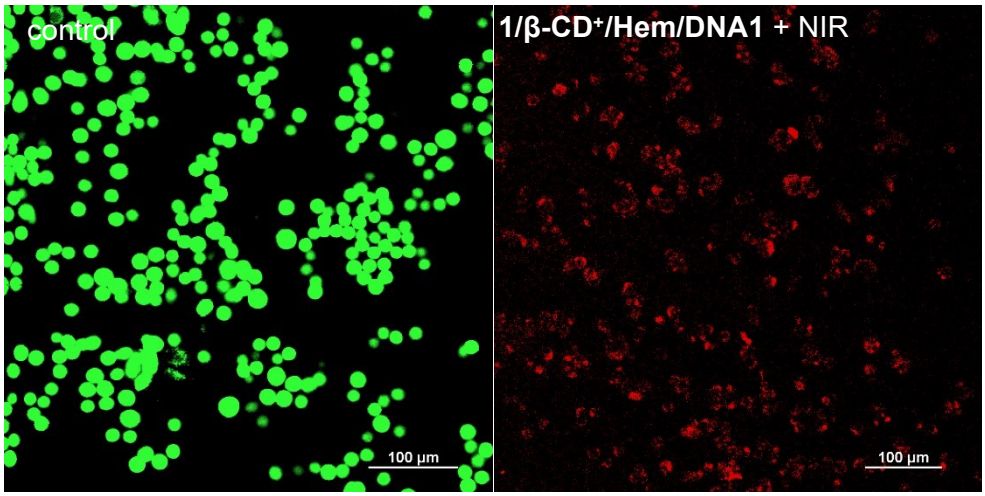

Figure S14. Additional CSLM images of live/dead cell assay of **1/β-CD$^+$/Hem/DNA1** after NIR illumination.