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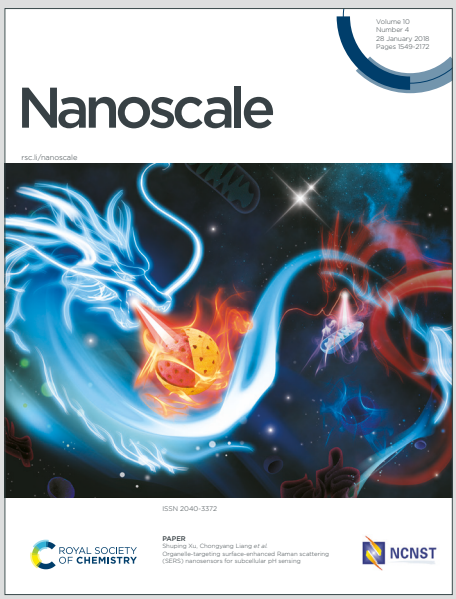

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ARTICLE

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

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Synergetic combination therapy is emerging as one of the most promising approaches for cancer treatment. Among the various therapeutic approaches, PDT has received particular attention due to its non-invasive nature. However, therapeutic performance of PDT is severally affected by tumour hypoxia. Herein, we report a supramolecular strategy for the fabrication of a PDT active 2D nanosheet loaded with a POD mimicking DNAzyme for the synergetic combination of PDT and CDT for targeted cancer therapy. Assembly of biotin functionalized BODIPY (**1**) and cationic β-cyclodextrin (**β-CD$^+$**) leads to the formation of **1/β-CD$^+$** nanosheet with positively charged **β-CD$^+$** on the surface of the sheet. The cationic face of **1/β-CD$^+$** sheet was then loaded with POD-mimicking **Hem**-loaded G-quadruplex aptamer (**Hem/DNA1)** via electrostatic interaction (**1/β-CD$^+$/Hem/DNA1)**. Cellular internalization of **1/β-CD$^+$/Hem/DNA1** nanosheet occurs via receptor-mediated endocytic pathway, which then undergoes lysosomal escape. Subsequently, **Hem/DNA1** on the surface of **1/β-CD$^+$/Hem/DNA1** reacts with endogenous H_2O_2 via the Fenton pathway produces $^{\bullet}OH$ and O_2. Moreover, under cellular conditions, **Hem** inside **1/β-CD$^+$/Hem/DNA1** nanosheet produces Fe^{2+}, which then undergoes another Fenton reaction to produce $^{\bullet}OH$ and O_2. The Fe^{3+} generated after Fenton reaction then in-situ get reduced to Fe^{2+} by glutathione for the next Fenton cycle. At the same time, photoirradiation of **1/β-CD$^+$** nanosheet using 635 nm laser produces $^1O_2$ via PDT pathway by using the endogenous O_2. The most remarkable feature of the present nanoformulation is the cooperativity in its therapeutic action, wherein O_2 produced during CDT pathway was used by **1/β-CD$^+$** sheet for improving its PDT efficacy in the hypoxic tumor microenvironment. This work represents a unique combination of CDT and PDT for targeted cancer therapy, wherein, CDT action of the nanoagent enhances the PDT efficacy and we strongly believe that this approach would encourage researchers to design similar combination therapy for the advancements in the treatment of cancer.

Introduction

Photodynamic therapy (PDT) is a promising treatment modality for cancer mainly due to its non-invasive nature and spatiotemporal controllability.[1,2] It involves the photoexcitation of a photosensitizer, preferably in the near-infrared (NIR) region, which then undergoes intersystem crossing from the singlet excited state to the triplet excited state. The triplet state of the sensitizer then acts as an energy donor to the ground state of molecular oxygen ($^3O_2$) and other molecules present in the tissue, producing highly cytotoxic reactive oxygen species (ROS) including singlet oxygen ($^1O_2$). These reactions lead to cell death at the photoirradiated region.[3–9] Although considerable advancement has been made in PDT-based cancer treatment, the condition of hypoxia (reduced oxygen concentration) in solid tumours significantly reduces the efficiency of PDT for cancer treatment.[10,11] Different strategies have been developed in recent years to overcome the tumour hypoxia condition such as by transporting additional O_2 to the tumor microenvironment[12,13] and by catalysing endogenous H_2O_2 to O_2.[14,15] Though these strategies showed enhanced supply of O_2 to the hypoxic tumours to some extent, the continuous consumption of O_2 by the mitochondria for the synthesis of ATP significantly hinders the efficacy of these approaches. Therefore, development of new strategies to boost O_2 concentration in hypoxic tumours is extremely important for PDT-based cancer therapy.[16–18]

Chemodynamic therapy (CDT) is another potential therapeutic approach for cancer treatment and has shown significant growth in recent years.[19,20] This involves the metal catalysed conversion of endogenous H_2O_2 into highly reactive hydroxyl radicals ($^{\bullet}OH$) via a Fenton or Fenton-like reaction.[21,22] It is important to note that one of the by-products of Fenton reaction is O_2. This clearly suggest that the integration of a CDT agent with the PDT system would be a promising approach for the improvement of PDT action by boosting the concentration of O_2. More importantly, CDT and PDT can work in a synergetic fashion wherein O_2 generated by Fenton rection can enhance the PDT action and at the same time $^{\bullet}OH$ generated via Fenton reaction is toxic to the cancer cells. Hence, this strategy offer the design of synergetic combination of PDT and CDT for the treatment of cancer.[23–28]

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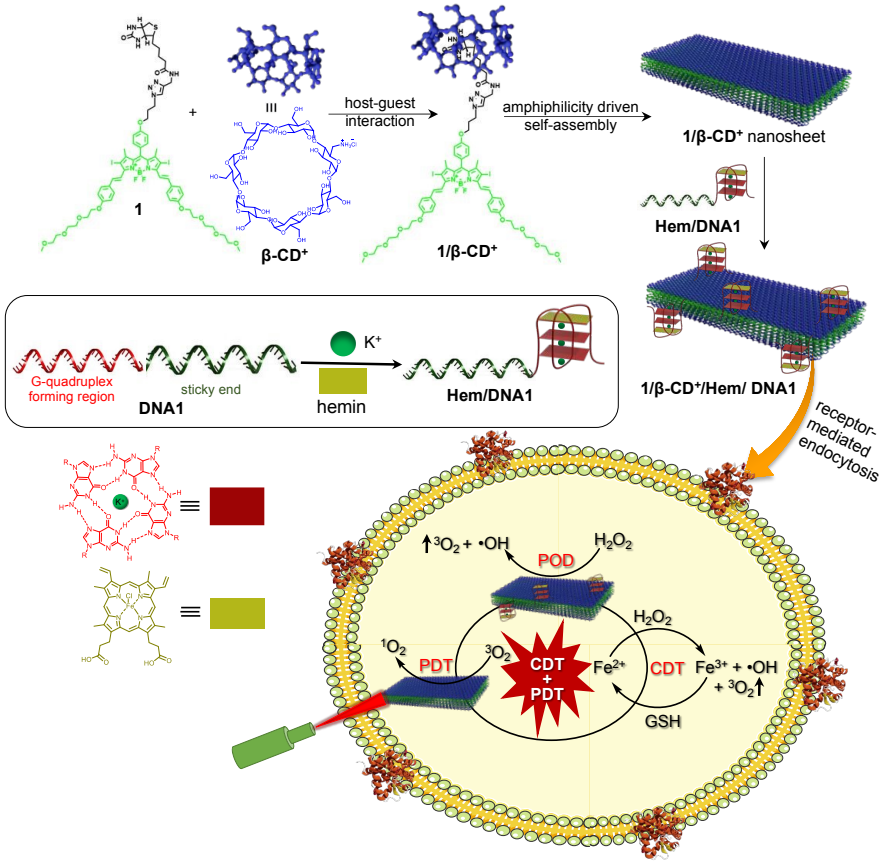

Scheme 1. Scheme showing the non-covalent synthesis of **1/β-CD⁺** complex and its spontaneous self-assembly to the formation of **1/β-CD⁺** nanosheet. Integration of **Hem/DNA1** onto the surface of **1/β-CD⁺** nanosheet via electrostatic interactions is also shown. Cellular internalization of **1/β-CD⁺/ Hem/DNA1** into MDA-MB-231 cells through receptor mediated endocytosis and the mechanism of combined PDT and CDT are also demonstrated.

We have recently reported a fluorinated boron-dipyrromethene (BODIPY)-based nanosheet as a nanoformulation for combined antisense DNA delivery and PDT.[29] The nanosheet exhibited high extinction coefficient in the NIR region, excellent $^1O_2$ generation upon photoirradiation and efficient delivery of antisense DNA.[30,31] Because the surface of the nanosheet is cationic in nature, it permits the integration of any functional DNA onto its surface via electrostatic interaction. In view of this, we envisioned that the incorporation of a peroxidase (POD)-mimicking DNAzyme[32-35] onto PDT active nanosheet would be a promising nanoformulation to achieve the design of synergetic combination of CDT and PDT.[36–38] For example, G-rich DNA is known to fold G-quadruplexes that show high binding affinity to hemin (**Hem**) to form **Hem**-bound G-quadruplex, which is known to exhibit POD activity to produce •OH and O_2.[39] In addition, G-quadruplex can function as an aptamer for specific cancer cell and hence offer targeted delivery of therapeutic agents into cancerous cells.[40,41]

Herein, we report a supramolecular strategy for the fabrication of a PDT active 2D nanosheet loaded with a POD-mimicking DNAzyme for the synergetic combination of PDT and CDT-based cancer therapy. As reported previously, the host-guest interaction between biotin functionalized BODIPY (**1**) and cationic β-cyclodextrin (**β-CD⁺**) in aqueous medium resulted in the formation of **1/β-CD⁺** nanosheet with positively charged **β-CD⁺** exposed onto the surface of the sheet.[29] The cationic faces of **1/β-CD⁺** were then loaded with POD-mimicking **Hem**-loaded G-quadruplex aptamer (**Hem/DNA1)** via electrostatic interaction to form **1/β-CD⁺/Hem/DNA1** nanosheet. Importantly, **DNA1** (DNAzyme) on the surface of the nanosheet can act as a targeting ligand for the overexpressed nucleolin protein on the surface of cancer cells (eg.: MDA-MB-231) for the selective delivery of PDT and CDT agents.[42–44] After selective cellular internalization of **1/β-CD⁺/Hem/DNA1** nanosheet via receptor-mediated endocytic pathway, it undergoes lysosomal escape due to the cationic charge on the surface of the nanosheet, which potentially prevents its degradation at the lysosome. Subsequently, **Hem/DNA1** on the surface of **1/β-CD⁺/Hem/DNA1** reacts with endogenous H_2O_2 via the Fenton pathway produces •OH and O_2. Moreover, under cellular conditions, over time, **Hem** inside **1/β-CD⁺/Hem/DNA1** nanosheet produces Fe^{2+} by the enzymatic action of intracellular heme oxygenase.[45] The in-situ generated Fe^{2+} then undergoes another Fenton reaction to produce •OH and O_2. The Fe^{3+} generated after Fenton reaction then in-situ get reduced to Fe^{2+} by glutathione (GSH) for the next Fenton cycle. At the same time, photoirradiation of **1/β-CD⁺** nanosheet using 635 nm laser produces $^1O_2$ via PDT by using endogenous O_2. The most remarkable feature of the nanoformulation is the cooperativity in its therapeutic action, wherein O_2 produced during CDT pathway was used by **1/β-CD⁺** sheet for improving its PDT efficacy in the hypoxic tumor microenvironment (Scheme 1).

Results and discussion

Initially, PDT (**1/β-CD⁺**) and CDT (**Hem/DNA1**) agents were synthesized independently. Syntheses of **β-CD⁺** and **1** was achieved as per previous reported procedures.[46–48] Non-covalent synthesis of **1/β-CD⁺** supramolecular amphiphile was achieved by annealing **β-CD⁺** (20 μM in water) and **1** (20 μM in DMSO) at 90 °C for 5 minutes, followed by slow cooling to room temperature, as reported earlier.[29] Unassembled **β-CD⁺** and **1** present in the solution was removed by repeated centrifugation (3 times at 3000 rpm for 5 minutes), and the supernatant obtained was used for further experiments. It was earlier reported by us that **1/β-CD⁺** amphiphile spontaneously self-assemble into 2D nanosheet with lateral dimensions in the range of several hundred of nm to μm with thickness of the sheet is in the range of 6–10 nm (Figure S1).[29] Similarly, **Hem/DNA1** DNAzyme was synthesized by annealing **DNA1** (5′-GGTGGTGGTGGTTGGGTGGTGGTGGAAAAAAAAA-3′) (1 μM) and **Hem** (1 μM) in Tris EDTA KCl buffer (pH 7) at 60 °C, followed by slow cooling to room temperature. The solution was then subjected to centrifugal cut-off filtration to remove any unbound **Hem**. In the presence of K^+, G-rich oligonucleotide undergoes intramolecular folding to form G-quadruplex, which facilitates the end stacking of **Hem** to form **Hem/DNA1** DNAzyme.[39] Circular dichroism (CD) spectrum of **Hem/DNA1** revealed the characteristic spectral features of antiparallel G-quadruplex (Figure 1a).[49] Furthermore, no change in the CD spectrum was observed even the end stacking of **Hem** on the G-quadruplex, implying its structural stability. The loading of **Hem** onto **DNA1** was confirmed by absorption spectral studies. Broad absorption spectrum was observed for **Hem** (1 μM) in water due to its very poor water solubility and hence **Hem** exists as aggregated species in water. Whereas, intense and sharp absorption bands were observed for **Hem** in DMSO, implying that **Hem** exists as monomeric species in DMSO. Interestingly, monomer-like absorption spectral features with intense and sharp absorption bands were observed for **Hem** in water in the case of **Hem/DNA1** (1/1 μM, Figure 1b). This must be due to the end stacking of **Hem** on **DNA1** G-quadruplex, which prevents its aggregation in water.

We then evaluated POD-mimicking activity of **Hem/DNA1** by using tetramethylbenzidine (TMB) oxidation assay (Figure 1c). For this, **Hem/DNA1** (1/1 μM) was treated with TMB (200 μM) in the presence of H_2O_2 (200 μM) and the emergence of the peak corresponding to oxidized TMB at 650 nm was monitored. As expected, a strong peak at 650 nm was observed for **Hem/DNA1**-treated samples demonstrating its excellent catalytic activity. On the other hand, only negligible activity was observed for free **Hem**. Subsequently, **Hem/DNA1** was loaded onto the surface of **1/β-CD⁺** sheet by annealing **Hem/DNA1** (1/1 μM) and **1/β-CD⁺** (20/20 μM) at 40 °C for 10 minute, followed by slow cooling to room temperature to yield **1/β-CD⁺/Hem/DNA1**. The loading efficiency of **Hem/DNA1** onto the surface of **1/β-CD⁺** nanosheet was found to be ~100 %. Details of the calculation of loading efficiency is provided in the supporting information (Figure S2). Zeta potential analyses of **Hem/DNA1** and **1/β-CD⁺** showed values of −24.2 and +15.27 mV, respectively (Figure 1d). Whereas, **1/β-CD⁺/Hem/DNA1** showed a zeta potential value of +7.9 mV. This significant reduction in the zeta potential value for **1/β-CD⁺/Hem/DNA1** (+7.9 mV) compared to **1/β-CD⁺** sheet (+15.27 mV) can be attributed to the successful loading of negatively charged **Hem/DNA1** onto the cationic surface of **1/β-CD⁺** sheet via electrostatic interactions. Interestingly, the sheet morphology of **1/β-CD⁺** was found to remain intact even after the loading of

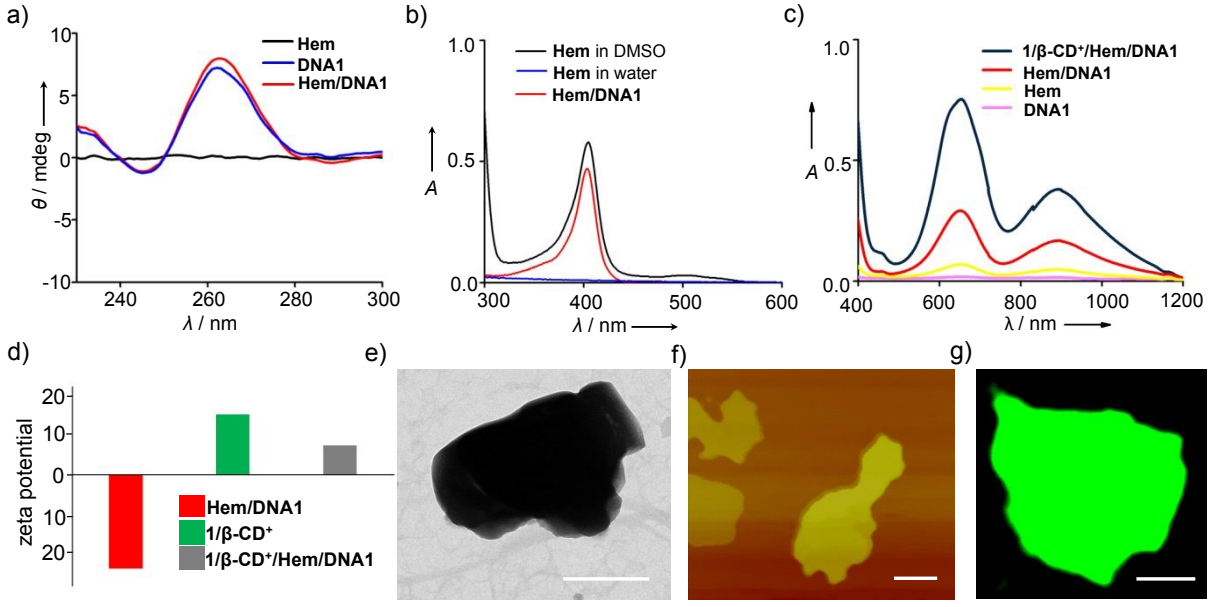

Figure 1. (a) Comparison of CD spectra of **DNA1**, **Hem**, and **Hem/DNA1**. (b) Comparison of absorption spectra of **Hem** (in H_2O and DMSO) and **Hem/DNA1**. (c) Comparison of absorption spectra of TMB-treated solutions of **DNA1**, **Hem**, **Hem/DNA1** and **1/β-CD⁺/Hem/DNA1**. (d) Comparison of zeta potentials of **Hem/DNA1**, **1/β-CD⁺** and **1/β-CD⁺/Hem/DNA1**. (e) TEM and (f) AFM images of **1/β-CD⁺/Hem/DNA1** (scale bars represent 500 nm). (g) CLSM image of **1/β-CD⁺/Hem/DNA2** (**DNA2** is FAM labelled **DNA1**) (scale bar represents 5 μm).

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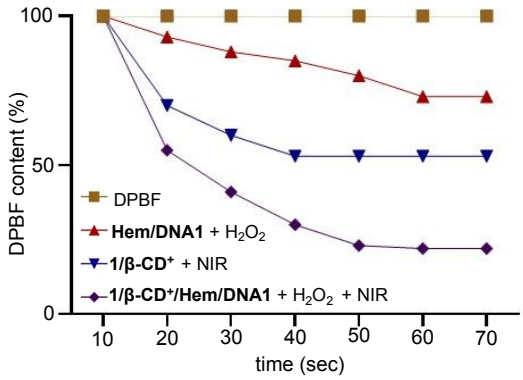

Figure 2. Plot of absorbance changes of DPBF at 420 nm of DPBF-treated solutions of **Hem/DNA1**, **1/β-CD⁺** and **1/β-CD⁺/Hem/DNA1** under photoirradiation with 635 nm laser for 70 sec (0.75 W/cm²).

Hem/DNA1 as evident from the transmission electron (TEM) (Figure 1e) and atomic force microscopic (AFM) (Figure 1f) analyses. Nanosheet morphology of **1/β-CD⁺/Hem/DNA1** was further confirmed by confocal laser scanning microscopic (CLSM) analyses using **DNA1** labelled with FAM (**DNA2**), which showed the formation of green fluorescent 2D nanosheets (Figure 1g). Furthermore, **1/β-CD⁺/Hem/DNA1** nanosheet was found to be structurally stable in PBS buffer and 10 % serum at pH 7.0. This was evident from the time-dependent absorption spectral changes of **1/β-CD⁺/Hem/DNA1** nanosheet, which revealed no change in the characteristic absorption of the nanosheet with respect to time, at least up to 6 h (Figure S3). We also evaluated whether the POD-mimicking activity of **Hem/DNA1** retains after loading onto the nanosheet. As expected, a strong absorption peak at 650 nm was observed for **1/β-CD⁺/Hem/DNA1**-treated samples, validating its catalytic activity even after loading onto the nanosheet (Figure 1c).

One of the by-products of CDT action of **Hem/DNA1** is O_2 and hence the in-situ generated O_2 can be utilized by the photosensitizer (**1/β-CD⁺**) for the enhanced generation of $^1O_2$ under the hypoxic conditions of tumour microenvironment. The CDT-assisted enhanced PDT of **1/β-CD⁺/Hem/DNA1** was studied using 1,3-diphenylisobenzofuran (DPBF) degradation assay. For this, **Hem/DNA1** (1/1 μM) and **1/β-CD⁺/Hem/DNA1** (50/50/1/1 μM) nanosheets solutions in water (pH 6.5) containing H_2O_2 (200 μM) and DPBF (200 μM) was irradiated with a 635 nm laser (0.75 W/cm²) for 70 sec and the decrease in the absorption band of DPBF at 420 nm was monitored with respect to time (Figure 2). Absorption spectrum of DPBF was considered as the control (100 %) and no degradation was observed in this case. A decrease of 27 % in the absorption of DPBF was observed for **Hem/DNA1**-treated sample compared to DPBF alone, indicating the degradation of DPBF by the in-situ generated •OH by the CDT action of **Hem/DNA1**. Interestingly, a drastic decrease of 73 % in DPBF absorption was observed for **1/β-CD⁺/Hem/DNA1**-treated sample. This enhanced degradation of DPBF in the case of **1/β-CD⁺/Hem/DNA1** compared to **Hem/DNA1** must be attributed to the synergetic action of CDT and PDT, wherein O_2 generated during the CDT action of **Hem/DNA1** enhances the PDT action of **1/β-CD⁺**.

Having demonstrated the synergetic action of CDT and PDT, the therapeutic performance of **1/β-CD⁺/Hem/DNA1** nanosheet for combined CDT and PDT-based cancer therapy

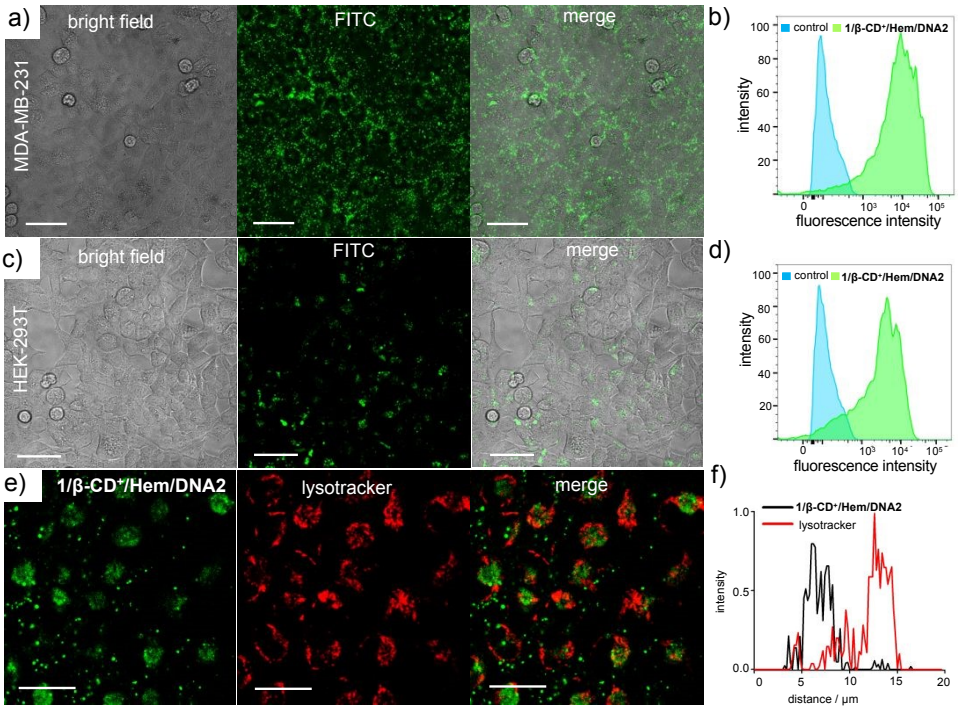

Figure 3. (a) CLSM images of **1/β-CD⁺/Hem/DNA2**-treated MDA-MB-231 cells (scale bar represents 50 μm) and (b) the corresponding FACS analyses. (c) CLSM images of **1/β-CD⁺/Hem/DNA2**-treated HEK-293T cells (scale bar represents 50 μm) and (d) the corresponding FACS analyses. (e) CLSM images of **1/β-CD⁺/Hem/DNA2**-treated MDA-MB-231 cells stained with lysotracker red (scale bar represents 25 μm) and (f) the corresponding line analyses.

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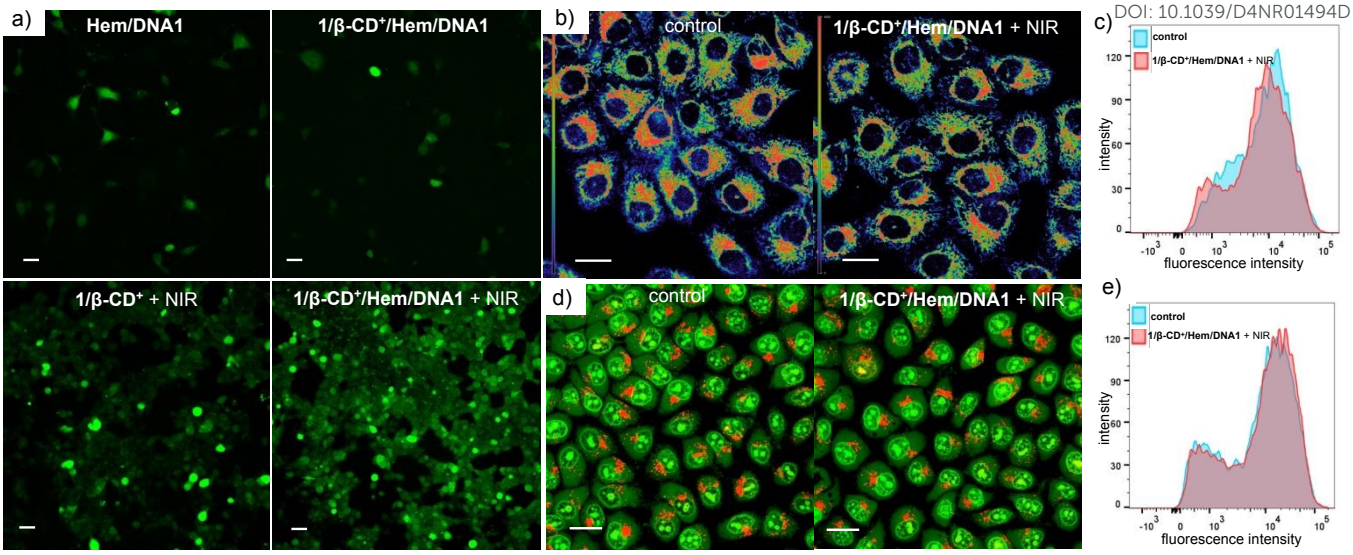

Figure 4. (a) DCFH-DA assay: CLSM images of **Hem/DNA1**, **1/β-CD⁺/Hem/DNA1, 1/β-CD⁺** + NIR and **1/β-CD⁺/Hem/DNA1** + NIR-treated MDA-MB-231 cells (635 nm laser with power of 0.75 W/cm²) (scale bar represents 25 μm). (b) TMRM assay: CLSM images of **1/β-CD⁺/Hem/DNA1**-treated MDA-MB-231 cells stained with TMRM under photoirradiation at 635 nm laser (0.75 W/cm²) (scale bar represents 25 μm) and (c) the corresponding FACS analyses. (d) 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/cm²) (scale bar represents 25 μm) and (e) the corresponding FACS analyses.

was studied using triple-negative human breast cancer cells, MDA-MB-231, as a representative cell line. Initially, the cellular internalization of **1/β-CD⁺/Hem/DNA2** (20/20/1/1 μM) was investigated using CLSM analyses by monitoring the green fluorescence of **DNA2**. After 12 h of incubation, CLSM analyses revealed strong green florescence for the cells and was mainly distributed through the cytosolic regions of the cells (Figure 3a and b). This indicates the efficient internalization of **1/β-CD⁺/Hem/DNA2** into MDA-MB-231 cells. It is important to note that **Hem/DNA1** not only acts as a CDT agent, but also acts as a targeting ligand for MDA-MB-231 cells. Targeting of the cancer cells was due to the molecular recognition of nucleolin protein overexpressed on the surface of MDA-MB-231 cells by **Hem/DNA2**. Thereby, **Hem/DNA2** permits the selective internalization of the nanoformulation into the cancer cells preferably compared to the normal healthy cell lines. The selective internalization of **1/β-CD⁺/Hem/DNA2** was evaluated by comparing its cellular internalization between HEK-293T (normal cell line), which has negligible expression of nucleolin on its surface, and MDA-MB-231 cancer cells (Figure 3a–d). In accordance with our design, CLSM analyses clearly showed strong green fluorescence for **1/β-CD⁺/Hem/DNA2**-treated MDA-MB-231 cells (Figure 3a and b) when compared to the corresponding HEK-293T cells (Figure 3c and d). In support of this, fluorescence-activated cell sorting (FACS) analyses revealed high mean fluorescence intensity (MFI) shift for MDA-MB-231 (11490) compared to HEK-293T cells (4440).

Typically any foreign materials entering into a cell via endocytic pathway ultimately reaches at the lysosome and get degraded at the lysosome due to its acidic pH. In order to check whether the nanosheet reaches at the lysosome after its cellular internalization, colocalization studies were performed after staining the lysosome of the cells using lysotracker red (Figure 3e). As evident from the CLSM analyses that only very poor colocalization was observed for **1/β-CD⁺/Hem/DNA2** nanosheet with lysotracker deep red. This suggest that **1/β-CD⁺/Hem/DNA2** nanosheet undergoes lysosomal escape immediately after the receptor-mediated endocytosis and escape the degradation at the lysosome. This was further supported by the corresponding line analyses, which showed a relatively low Pearson Coefficient value of 0.048 (Figure 3f). We believe that the lysosomal escape of **1/β-CD⁺/Hem/DNA2** nanosheet is due to the net positive charges present on the surface of the nanosheet, which favours the lysosomal membrane destabilization, as reported previously by our own group and others.[50] The lysosomal escape of **1/β-CD⁺/Hem/DNA2** prevents the degradation of the nanosheet at the acidic lysosomes and thereby improving the efficacy of the nanoformulation.

After establishing the escape of **1/β-CD⁺/Hem/DNA2** nanosheet from the lysosomes, the ROS generation efficiency of **1/β-CD⁺/Hem/DNA1** nanosheet inside MDA-MB-231 cell was investigated using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as a fluorescent probe for ROS. For this, MDA-MB-231 cells were treated with **Hem/DNA1**, **1/β-CD⁺/Hem/DNA1**, **1/β-CD⁺** + NIR or **1/β-CD⁺/Hem/DNA1** + NIR (635 nm laser with a power of 0.75 W/cm² for 10 minutes) and the in-situ ROS generation inside the cell was monitored by DCFH-DA assay, which produces green fluorescent 2,7- dichlorofluorescein (DCF) upon ROS detection (Figure 4a). CLSM analyses clearly revealed that maximum green fluorescence intensity of DCF was associated with **1/β-CD⁺/Hem/DNA1** + NIR-treated cells compared to **Hem/DNA1**, **1/β-CD⁺/Hem/DNA1**, **1/β-CD⁺** + NIR-treated cells. These results unequivocally confirm that **1/β-CD⁺/Hem/DNA1** + NIR-treated cells produces maximum ROS inside the cells upon laser irradiation and this must be attributed to the synergetic action of CDT and PDT. In accordance with the CLSM, FACS analyses disclosed MFI shift values of 6052, 1240, 463 and 127

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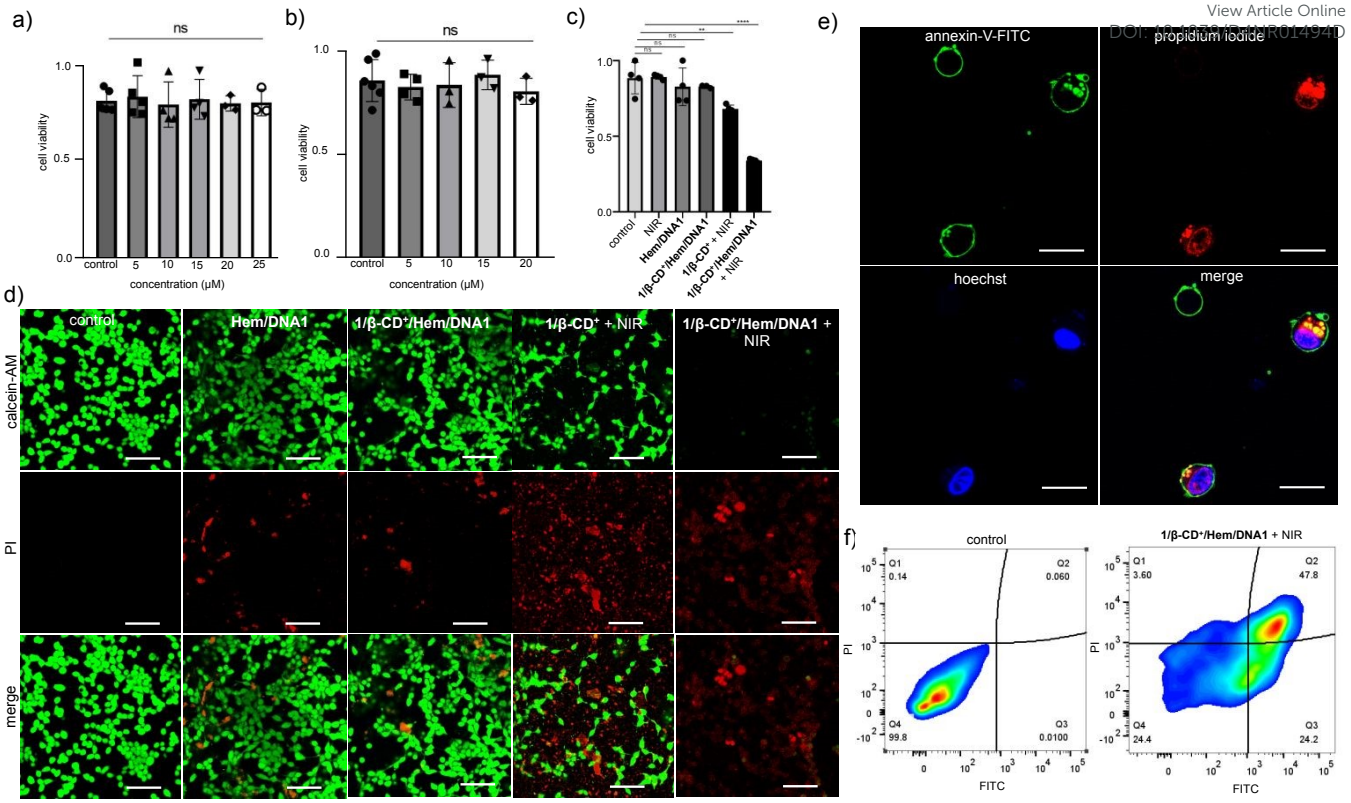

Figure 5. (a)–(c) MTT assay: cell viability at varying concentrations of (a) **β-CD⁺** and (b) **1/β-CD⁺**-treated MDA-MB-231 cells in the dark. (c) MTT assay for **Hem/DNA1**, **1/β-CD⁺/Hem/DNA1, 1/β-CD⁺** + NIR and **1/β-CD⁺/Hem/DNA1** + NIR-treated MDA-MB-231 cells (635 nm laser with a power of 0.75 W/cm²). (d) Calcein-AM/PI live-dead assay: CLSM images of **Hem/DNA1**, **1/β-CD⁺/Hem/DNA1, 1/β-CD⁺** + NIR and **1/β-CD⁺/Hem/DNA1** + NIR-treated MDA-MB-231 cells (scale bar represents 100 μm). (e) 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/cm²) (scale bar represents 25 μm) and (f) the corresponding FACS analyses.

for **1/β-CD⁺/Hem/DNA1** + NIR, **1/β-CD⁺**, **Hem/DNA1** and untreated MDA-MB-231 cells, respectively (Figure S5). Maximum MFI shift was observed for **1/β-CD⁺/Hem/DNA** + NIR-treated cells. As expected, no significant ROS generation was observed for **1/β-CD⁺/Hem/DNA**-treated cells in the absence of photoirradiation.

Because ROS species such as $^1O_2$ and $^{\bullet}OH$ are highly oxidizing in nature and induce damage to organelles like lysosomes and mitochondria, which can potentially leads to apoptosis by various signalling pathways. In view of this, we examined the structural integrity of these organelles in the presence of ROS species generated by the combined CDT and PDT. The mitochondrial damage caused by the ROS was evaluated using tetramethylrhodamine methyl ester perchlorate (TMRM) assay (Figure 4b and c).[51] Since mitochondria have typical membrane potentials in the range of –180 mV, they can accumulate high concentrations of cationic dyes like TMRM. If mitochondria are damaged or depolarized, dye accumulation gradually decreases, which leads to the diminishing of its fluorescence intensity. For this, MDA-MB-231 cells were treated with **1/β-CD⁺/Hem/DNA1** (20/20/1/1 μM) for 2 h, then kept under NIR light illumination (635 nm laser at 0.75 W/cm²) for 10 minutes followed by 3 h of incubation. Cells were then stained with TMRM, and changes in green fluorescence intensity of TMRM were probed using CLSM analyses. In this case as well, CLSM analyses of **1/β-CD⁺/Hem/DNA1**-treated MDA-MB-231 cells showed no decrease in the fluorescence intensity when compared to the corresponding control cells, indicating the healthy nature of the mitochondria (Figure 4b). This was further quantified by FACS analyses (Figure 4c), which disclosed similar MFI shift values for **1/β-CD⁺/Hem/DNA1** treated (12674) and the corresponding untreated control cells (12660). The lysosomal integrity was further evaluated using an acridine orange (AO) assay (Figure 4d and e).[52,53] Acridine orange emits green fluorescence when bound to dsDNA in the nucleus and red fluorescence inside lysosome. If the lysosome is damaged, it leads to a decrease in the red fluorescence of AO. For this purpose, MDA-MB-231 cells were treated with **1/β-CD⁺/Hem/DNA1** (20/20/1/1 μM) for 2 h followed by NIR light illumination for 10 minutes followed by 3 h incubation. Subsequently, the cells were co-stained with AO and evaluated using CLSM analyses. CLSM images **1/β-CD⁺/Hem/DNA1**-treated cells showed no noticeable decrease in the red fluorescence of AO with respect to the control cells, indicating that the lysosomes were not damaged by ROS generated by **1/β-CD⁺/Hem/DNA1** nanosheet (Figure 4d). This was further confirmed by FACS analyses, which showed MFI shift values of 16476 and 17527 for the control and **1/β-CD⁺/Hem/DNA1** nanosheet-treated MDA-MB-231 cells, respectively (Figure 4e). However it must be noted that TMRM and AO staining assays revealed significant damage to lysosome and mitochondria after 24 h of incubation (Figure S6). These results conclude that

ROS cause damage to the cell organelles at a larger time scale of incubation, but are intact at smaller incubation time.

After demonstrating the selective internalization and ROS generation inside the cancer cells, the cytotoxicity of **1/β-CD⁺/Hem/DNA1** towards MDA-MB-231 cells was studied using MTT assay.[54] Initially, toxicities of **β-CD⁺** and **1/β-CD⁺** nanosheets towards MDA-MB-231 cells in the dark was studied (Figure 5a and b). For this, **β-CD⁺** (Figure 5a) and **1/β-CD⁺** (Figure 5b) nanosheets under dark of varying concentrations (1-20 μM) were treated with MDA-MB-231 cells for 24 h. As expected, no cytotoxicity was observed even at a higher concentration of 20 μM. Subsequently, cytotoxicity of MDA-MB-231 cells treated with **Hem/DNA1** (1/1 μM), **1/β-CD⁺/Hem/DNA1** (20/20/1/1 μM), **1/β-CD⁺** (20/20 μM) + NIR, and **1/β-CD⁺/Hem/DNA1** + NIR (20/20/1/1 μM) for 2 h (635 nm laser with a power of 0.75 W/cm² for 10 minutes) were studied (Figure 5c). As expected no significant cytotoxicity was observed for **Hem/DNA1** and **1/β-CD⁺/Hem/DNA1** in the absence of NIR light, indicating the poor therapeutic efficacy. On the other hand, significant cytotoxicity was observed for **1/β-CD⁺** and **1/β-CD⁺/Hem/DNA1**-treated cell under NIR light irradiation. Interestingly, enhanced cytotoxicity was observed for **1/β-CD⁺/Hem/DNA1**-treated cells compared to **1/β-CD⁺**-treated cells. A cell death of 65 % was observed for **1/β-CD⁺/Hem/DNA1**-treated cells, whereas, only 32 % cell death was observed for **1/β-CD⁺**-treated cells. These results are in full agreement with the enhanced ROS generation for **1/β-CD⁺/Hem/DNA1**-treated cells and fully support our hypothesis of synergetic CDT and PDT actions.

To further support the cytotoxicity induced by the combined actions of CDT and PDT by **1/β-CD⁺/Hem/DNA1** nanosheet towards MDA-MB-231 cells, calcein-AM/PI co-staining assay was performed (live/dead assay) (Figure 5d). Calcein-AM interacts with the esterase present inside the live cancer cells and produce green fluorescence from the cells due to the cleavage of the acetoxymethyl (AM) ester-protecting group of calcein-AM. On the other hand, no ester cleavage is possible inside the dead cell as the concentration of esterase is very minimal and hence no green fluorescence is expected for the dead cells. Similarly, propidium iodide (PI) cannot pass through the cell membrane of live cells, whereas it can stain the dead cell to give red fluorescence.[43] To demonstrate the live/dead assay, cells were initially treated with **Hem/DNA1**, **1/β-CD⁺/Hem/DNA1**, **1/β-CD⁺** + NIR, **1/β-CD⁺/Hem/DNA1** + NIR for 2 h (635 nm laser with a power of 0.75 W/cm² for 10 minutes). As shown in Figure 5d, **1/β-CD⁺/Hem/DNA1** + NIR -treated cells exhibited mainly red fluorescence of PI and negligible green fluorescence of calcein-AM compared to **Hem/DNA1**, **1/β-CD⁺/Hem/DNA1**, **1/β-CD⁺** + NIR-treated cells. These results conclude the enhanced cytotoxicity of **1/β-CD⁺/Hem/DNA1** + NIR compared to **1/β-CD⁺** + NIR, **Hem/DNA1** and **1/β-CD⁺/Hem/DNA1**. Live/dead assay also support our hypothesis of enhanced cytotoxicity for **1/β-CD⁺/Hem/DNA1** + NIR due to the synergetic combination of CDT and PDT.

In order to understand the cell death mechanism of the present nanoformulation, annexin V-FITC(AV)/PI staining assay was carried out on **1/β-CD⁺/Hem/DNA1**-treated MDA-MB-231 cells under NIR light illumination. As shown in Figure 5e and Figure S7, **1/β-CD⁺/Hem/DNA1** (20/20/1/1 μM)-treated cells exhibited intense green fluorescence of annexin V-FITC around the cell membrane and strong red fluorescence of PI from the nuclei, revealing that the most of the cell population is in the late stage of apoptotic pathway. This is further confirmed through the FACS analyses (Figure 5f). For this purpose, MDA-MB-231 cells were treated with **1/β-CD⁺/Hem/DNA1** nanosheet under NIR light illumination and analysed by flow cytometry after staining with annexin V-FITC (AV)/PI. In accordance with the CLSM, the cell population mainly shifted to the quadrant corresponds to the apoptotic pathway. These results collectively conclude that **1/β-CD⁺/Hem/DNA1** nanosheet under NIR light illumination induces cell death via oxidative stress-induced apoptotic pathway.

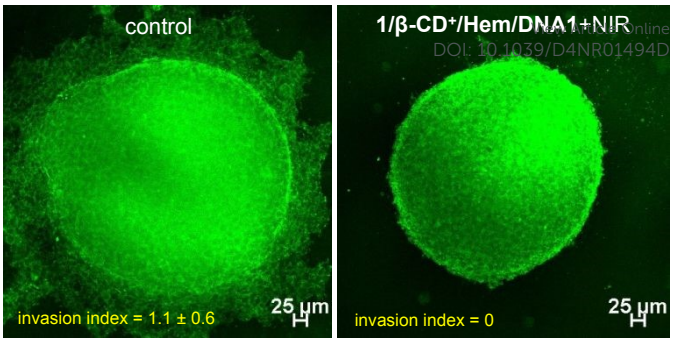

Figure 6. CLSM images of **1/β-CD⁺/Hem/DNA1**-treated MDA-MB-231 3D spheroids after NIR light illumination (0.75 W cm^{-2} for 10 minutes) (right) and the corresponding untreated control spheroids (left).

After demonstrating the in vitro performance of **1/β-CD⁺/Hem/DNA1** nanosheet, we studied the efficiency of the nanoformulation in multicellular tumor spheroid model using 3D cell culture (Figure 6). The 3D-multicellular tumor spheroid mimics the in vivo tumor and they undergoes proliferation in all directions similar to malignant tumors. In 3D cell culture, cells clump together to form a stable 3D spheroid-like structure, wherein the communication and signalling between the cells are maximized, leading to efficient proliferation and invasion over time.[55] To this end, 3D tumour spheroids of MDA-MB-231 cells were prepared using the standard hanging drop method.[56,57] The invasion potential of the 3D spheroids was studied in the presence of **1/β-CD⁺/Hem/DNA1** nanosheet after NIR light irradiation to understand its effect on the spheroid invasion. For this, 3D spheroids were incubated with **1/β-CD⁺/Hem/DNA1** (20/20/1/1 μM) nanosheet for 4 h, kept under irradiation at 635 nm (0.75 W/cm²) for 10 minutes followed by 24 h of incubation. Untreated spheroids were considered as controls that showed maximum invasion potential (invasion index = 1.1 ± 0.6) as they showed migration in all the directions. On the other hand, **1/β-CD⁺/Hem/DNA1**-treated sample showed drastic reduction in the invasion potential (invasion index = 0) of the spheroids due to the combined therapeutic actions of CDT and PDT. These observations are in good agreement with the in vitro studies.

Conclusions

In summary, we have reported a supramolecular strategy for the fabrication of a PDT active 2D nanosheet loaded with a POD-mimicking DNAzyme for the synergetic combination of PDT and CDT for targeted cancer therapy. The most important feature of the present nanoformulation is the synergetic combination of CDT and PDT actions. The CDT action of the nanoformulation results in the formation of highly toxic ROS along with the generation of O_2 as one of the bioproducts, which can boost the concentration of O_2 in the tumour microenvironment. This in turn enhance the PDT action of the nanoformulation, which is otherwise found to be less effective due to tissue hypoxia. Excellent therapeutic activity was demonstrated both in vitro and in vivo for targeted cancer therapy. This work represents a unique combination of CDT and PDT for targeted cancer therapy, wherein, CDT action of the nanoagent enhances the PDT efficacy and we strongly believe that this approach would encourage researchers to design similar combination therapy for the advancements in the treatment of cancer.

Author Contributions

The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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Data availability statement

The data supporting this article have been included as part of the Supplementary Information