



Cite this: *Nanoscale*, 2024, **16**, 16195

## G<sub>4</sub>-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, the therapeutic performance of PDT is severely 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  $\beta$ -cyclodextrin ( $\beta$ -CD<sup>+</sup>) leads to the formation of a **1**/ $\beta$ -CD<sup>+</sup> nanosheet with positively charged  $\beta$ -CD<sup>+</sup> on the surface of the sheet. The cationic face of the **1**/ $\beta$ -CD<sup>+</sup> sheet was then loaded with a POD-mimicking Hemin-loaded G-quadruplex aptamer (**Hem/DNA1**) via electrostatic interactions (**1**/ $\beta$ -CD<sup>+</sup>/**Hem/DNA1**). Cellular internalization of the **1**/ $\beta$ -CD<sup>+</sup>/**Hem/DNA1** nanosheet occurs via a receptor-mediated endocytic pathway, which then undergoes lysosomal escape. Subsequently, **Hem/DNA1** on the surface of **1**/ $\beta$ -CD<sup>+</sup>/**Hem/DNA1** reacts with endogenous H<sub>2</sub>O<sub>2</sub> via the Fenton pathway to produce  $\cdot$ OH and O<sub>2</sub>. Moreover, under cellular conditions, **Hem** inside the **1**/ $\beta$ -CD<sup>+</sup>/**Hem/DNA1** nanosheet produces Fe<sup>2+</sup>, which then undergoes another Fenton reaction to produce  $\cdot$ OH and O<sub>2</sub>. The Fe<sup>3+</sup> generated after the Fenton reaction is then reduced *in situ* to Fe<sup>2+</sup> by glutathione for the next Fenton cycle. At the same time, photoirradiation of the **1**/ $\beta$ -CD<sup>+</sup> nanosheet using a 635 nm laser produces <sup>1</sup>O<sub>2</sub> via the PDT pathway by using endogenous O<sub>2</sub>. The most remarkable feature of the present nanoformulation is the cooperativity in its therapeutic action, wherein O<sub>2</sub> produced during the CDT pathway was used by the **1**/ $\beta$ -CD<sup>+</sup> sheet for improving its PDT efficacy in the hypoxic tumor micro-environment. This work represents a unique combination of CDT and PDT for targeted cancer therapy, wherein the 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 advancements in the treatment of cancer.

Received 5th April 2024,  
Accepted 28th July 2024  
DOI: 10.1039/d4nr01494d

rsc.li/nanoscale

## Introduction

Photodynamic therapy (PDT) is a promising treatment modality for cancer mainly due to its non-invasive nature and spatiotemporal controllability.<sup>1,2</sup> It involves the photo-excitation 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 (<sup>3</sup>O<sub>2</sub>) and other molecules present in the tissue, producing highly cytotoxic reactive oxygen species (ROS) including singlet oxygen (<sup>1</sup>O<sub>2</sub>). These reactions lead to cell death in the photoirradiated region.<sup>3–9</sup> 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.<sup>10,11</sup> Different strategies have been developed in recent years to overcome the condition of tumour hypoxia such as transporting additional O<sub>2</sub> to the tumor microenvironment<sup>12,13</sup> and catalysing endogenous H<sub>2</sub>O<sub>2</sub> to O<sub>2</sub>.<sup>14,15</sup> Though these strategies showed an enhanced supply of O<sub>2</sub> to the hypoxic tumours to some extent, the continuous consumption of O<sub>2</sub> by the mitochondria for the synthesis of ATP significantly hinders the efficacy of these

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4nr01494d>







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

sharp absorption bands were observed for Hem in water in the case of Hem/DNA1 (1/1  $\mu\text{M}$ , Fig. 1b). This must be due to the end stacking of Hem on the DNA1 G-quadruplex, which prevents its aggregation in water.

We then evaluated the POD-mimicking activity of Hem/DNA1 by using the tetramethylbenzidine (TMB) oxidation assay (Fig. 1c). For this, Hem/DNA1 (1/1  $\mu\text{M}$ ) was treated with TMB (200  $\mu\text{M}$ ) in the presence of  $\text{H}_2\text{O}_2$  (200  $\mu\text{M}$ ) 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 the Hem/DNA1-treated sample 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 the  $1/\beta\text{-CD}^+$  sheet by annealing Hem/DNA1 (1/1  $\mu\text{M}$ ) and  $1/\beta\text{-CD}^+$  (20/20  $\mu\text{M}$ ) at 40  $^\circ\text{C}$  for 10 minutes, followed by slow cooling to room temperature to yield  $1/\beta\text{-CD}^+/\text{Hem/DNA1}$ . The loading efficiency of Hem/DNA1 onto the surface of the  $1/\beta\text{-CD}^+$  nanosheet was found to be  $\sim 100\%$ . Details of the calculation of the loading efficiency are provided in the ESI (Fig. S2<sup>†</sup>). Zeta potential analyses of Hem/DNA1 and  $1/\beta\text{-CD}^+$  showed values of  $-24.2$  and  $+15.27$  mV, respectively (Fig. 1d), whereas  $1/\beta\text{-CD}^+/\text{Hem/DNA1}$  showed a zeta potential value of  $+7.9$  mV. This significant reduction in

the zeta potential value for  $1/\beta\text{-CD}^+/\text{Hem/DNA1}$  ( $+7.9$  mV) compared to that of the  $1/\beta\text{-CD}^+$  sheet ( $+15.27$  mV) can be attributed to the successful loading of negatively charged Hem/DNA1 onto the cationic surface of the  $1/\beta\text{-CD}^+$  sheet via electrostatic interactions. Interestingly, the sheet morphology of  $1/\beta\text{-CD}^+$  was found to remain intact even after the loading of Hem/DNA1 as evident from the transmission electron microscopy (TEM) (Fig. 1e) and atomic force microscopy (AFM) (Fig. 1f) analyses. The nanosheet morphology of  $1/\beta\text{-CD}^+/\text{Hem/DNA1}$  was further confirmed by confocal laser scanning microscopy (CLSM) analyses using DNA1 labelled with FAM (DNA2), which showed the formation of green fluorescent 2D nanosheets (Fig. 1g). Furthermore, the  $1/\beta\text{-CD}^+/\text{Hem/DNA1}$  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 the  $1/\beta\text{-CD}^+/\text{Hem/DNA1}$  nanosheet, which revealed no change in the characteristic absorption of the nanosheet with respect to time, at least for up to 6 h (Fig. S3<sup>†</sup>). We also evaluated whether the POD-mimicking activity of Hem/DNA1 is maintained after loading onto the nanosheet. As expected, a strong absorption peak at 650 nm was observed for  $1/\beta\text{-CD}^+/\text{Hem/DNA1}$ -treated samples, validating its catalytic activity even after loading onto the nanosheet (Fig. 1c).



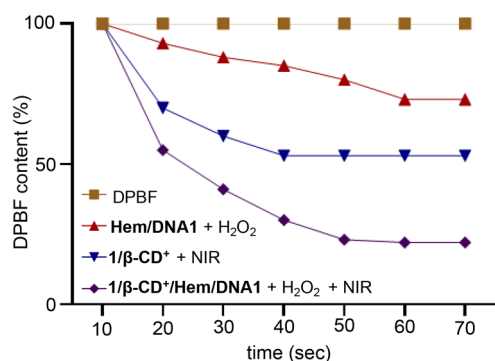


**Fig. 1** (a) Comparison of the CD spectra of DNA1, Hem, and Hem/DNA1. (b) Comparison of the absorption spectra of Hem (in H<sub>2</sub>O and DMSO) and Hem/DNA1. (c) Comparison of the absorption spectra of TMB-treated solutions of DNA1, Hem, Hem/DNA1 and 1/β-CD<sup>+</sup>/Hem/DNA1. (d) Comparison of the zeta potentials of Hem/DNA1, 1/β-CD<sup>+</sup> and 1/β-CD<sup>+</sup>/Hem/DNA1. (e) TEM and (f) AFM images of 1/β-CD<sup>+</sup>/Hem/DNA1 (scale bars represent 500 nm). (g) CLSM image of 1/β-CD<sup>+</sup>/Hem/DNA2 (DNA2 is FAM-labelled DNA1) (scale bar represents 5 μm).

One of the by-products of the CDT action of Hem/DNA1 is O<sub>2</sub> and hence the *in situ* generated O<sub>2</sub> can be utilized by the photosensitizer (1/β-CD<sup>+</sup>) for the enhanced generation of <sup>1</sup>O<sub>2</sub> under hypoxic conditions of the tumour microenvironment. The CDT-assisted enhanced PDT of 1/β-CD<sup>+</sup>/Hem/DNA1 was studied using the 1,3-diphenylisobenzofuran (DPBF) degradation assay. For this, Hem/DNA1 (1/1 μM) and 1/β-CD<sup>+</sup>/Hem/DNA1 (50/50/1/1 μM) nanosheet solutions in water (pH 6.5) containing H<sub>2</sub>O<sub>2</sub> (200 μM) and DPBF (200 μM) were irradiated with a 635 nm laser (0.75 W cm<sup>-2</sup>) for 70 s and the decrease in the absorption band of DPBF at 420 nm was monitored with respect to time (Fig. 2). The absorption spectrum of DPBF was considered as the control (100%) and no degradation was

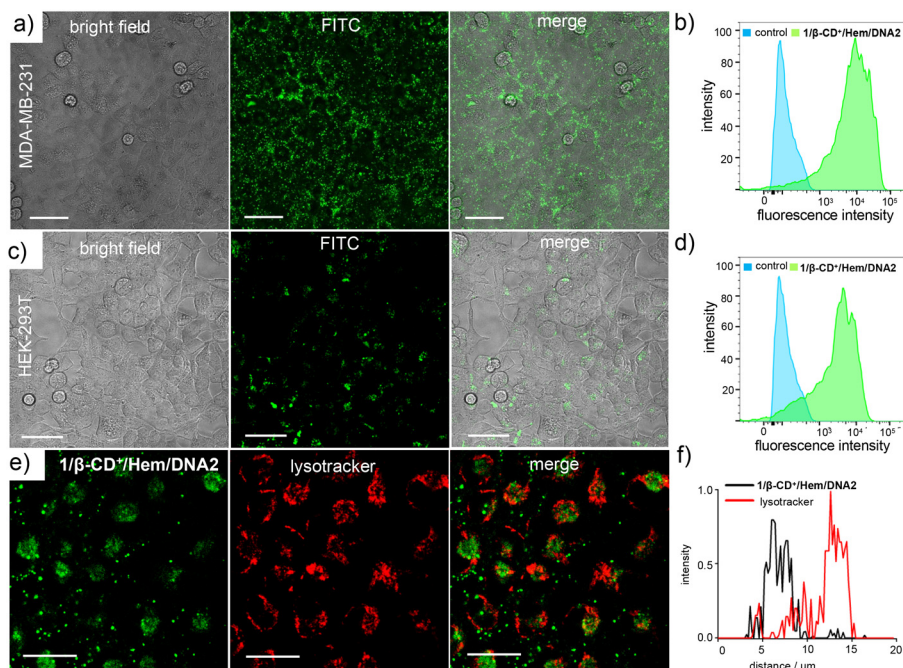
observed in this case. A decrease in DPBF absorption of 27% was observed for the Hem/DNA1-treated sample compared to DPBF alone, indicating the degradation of DPBF by the <sup>•</sup>OH *in situ* generated by the CDT action of Hem/DNA1. Interestingly, a drastic decrease in DPBF absorption of 73% was observed for the 1/β-CD<sup>+</sup>/Hem/DNA1-treated sample. This enhanced degradation of DPBF in the case of 1/β-CD<sup>+</sup>/Hem/DNA1 compared to Hem/DNA1 must be attributed to the synergistic action of CDT and PDT, wherein O<sub>2</sub> generated during the CDT action of Hem/DNA1 enhances the PDT action of 1/β-CD<sup>+</sup>.

Having demonstrated the synergistic action of CDT and PDT, the therapeutic performance of the 1/β-CD<sup>+</sup>/Hem/DNA1 nanosheet for combined CDT and PDT-based cancer therapy 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<sup>+</sup>/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 fluorescence for the cells that was mainly distributed through the cytosolic regions of the cells (Fig. 3a and b). This indicates the efficient internalization of 1/β-CD<sup>+</sup>/Hem/DNA2 into MDA-MB-231 cells. It is important to note that Hem/DNA1 acts not only as a CDT agent, but also as a targeting ligand for MDA-MB-231 cells. Targeting cancer cells is due to the molecular recognition of nucleolin protein overexpressed on the surface of MDA-MB-231 cells by Hem/DNA2. Therefore, Hem/DNA2 preferably permits the selective internalization of the nanoformulation into the cancer cells compared to the normal healthy cell lines. The selective internalization of 1/β-CD<sup>+</sup>/Hem/DNA2 was evaluated by com-



**Fig. 2** Plot of absorbance changes of DPBF at 420 nm of DPBF-treated solutions of Hem/DNA1, 1/β-CD<sup>+</sup> and 1/β-CD<sup>+</sup>/Hem/DNA1 under photoirradiation with a 635 nm laser for 70 s (0.75 W cm<sup>-2</sup>).





**Fig. 3** (a) CLSM images of  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$ -treated MDA-MB-231 cells (scale bar represents  $50\ \mu\text{m}$ ) and (b) the corresponding FACS analyses. (c) CLSM images of  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$ -treated HEK-293T cells (scale bar represents  $50\ \mu\text{m}$ ) and (d) the corresponding FACS analyses. (e) CLSM images of  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$ -treated MDA-MB-231 cells stained with LysoTracker Red (scale bar represents  $25\ \mu\text{m}$ ) and (f) the corresponding line analyses.

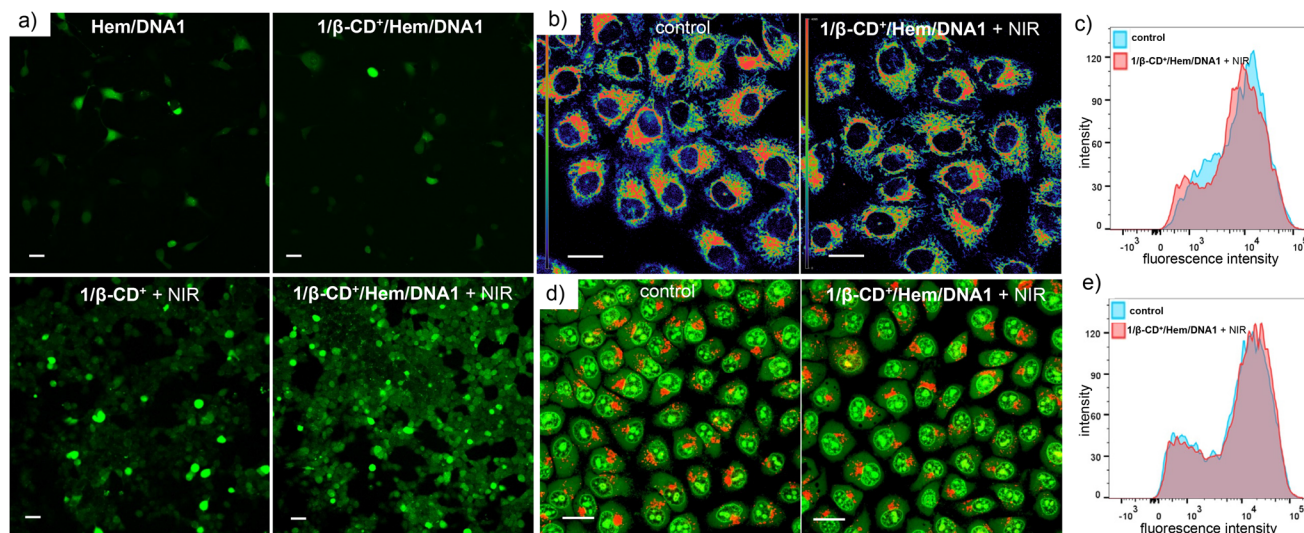
paring its cellular internalization between HEK-293T (normal cell line), which has negligible expression of nucleolin on its surface, and MDA-MB-231 cancer cells (Fig. 3a–d). In accordance with our design, CLSM analyses clearly showed strong green fluorescence for  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$ -treated MDA-MB-231 cells (Fig. 3a and b) when compared to the corresponding HEK-293T cells (Fig. 3c and d). In support of this, fluorescence-activated cell sorting (FACS) analyses revealed a high mean fluorescence intensity (MFI) shift for MDA-MB-231 (11 490) compared to HEK-293T cells (4440).

Typically any foreign materials entering into a cell *via* the endocytic pathway ultimately reach the lysosome and becomes degraded in the lysosome due to its acidic pH. In order to check whether the nanosheet reaches the lysosome after its cellular internalization, colocalization studies were performed after staining the lysosome of the cells using LysoTracker Red (Fig. 3e). As is evident from the CLSM analyses, only very poor colocalization was observed for the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$  nanosheet with LysoTracker Deep Red. This suggests that the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$  nanosheet undergoes lysosomal escape immediately after receptor-mediated endocytosis and escapes degradation in the lysosome. This was further supported by the corresponding line analyses, which showed a relatively low Pearson coefficient value of 0.048 (Fig. 3f). We believe that the lysosomal escape of the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$  nanosheet is due to the net positive charges present on the surface of the nanosheet, which favours lysosomal membrane destabilization, as reported previously by our own group and others.<sup>50</sup>

The lysosomal escape of  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$  prevents the degradation of the nanosheet at the acidic lysosomes, thereby improving the efficacy of the nanoformulation.

After establishing the escape of the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$  nanosheet from the lysosomes, the ROS generation efficiency of the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1$  nanosheet inside MDA-MB-231 cells was investigated using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a fluorescent probe for ROSs. For this, MDA-MB-231 cells were treated with **Hem/DNA1**,  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1$ ,  $1/\beta\text{-CD}^+ + \text{NIR}$  or  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1 + \text{NIR}$  ( $635\ \text{nm}$  laser with a power of  $0.75\ \text{W cm}^{-2}$  for 10 minutes) and *in situ* ROS generation inside the cell was monitored using the DCFH-DA assay, which produces fluorescent green 2,7-dichlorofluorescein (DCF) upon ROS detection (Fig. 4a). CLSM analyses clearly revealed that the maximum green fluorescence intensity of DCF was associated with  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1 + \text{NIR}$ -treated cells compared to **Hem/DNA1**,  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1$ , and  $1/\beta\text{-CD}^+ + \text{NIR}$ -treated cells. These results unequivocally confirm that  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1 + \text{NIR}$ -treated cells produce maximum ROSs inside the cells upon laser irradiation and this must be attributed to the synergetic action of CDT and PDT. In accordance with the CLSM analysis, FACS analyses disclosed MFI shift values of 6052, 1240, 463 and 127 for  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1 + \text{NIR}$ ,  $1/\beta\text{-CD}^+$ , **Hem/DNA1** and untreated MDA-MB-231 cells, respectively (Fig. S5†). A maximum MFI shift was observed for  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}1 + \text{NIR}$ -treated cells. As expected, no significant ROS generation was observed for  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA}2$ -treated cells in the absence of photoirradiation.





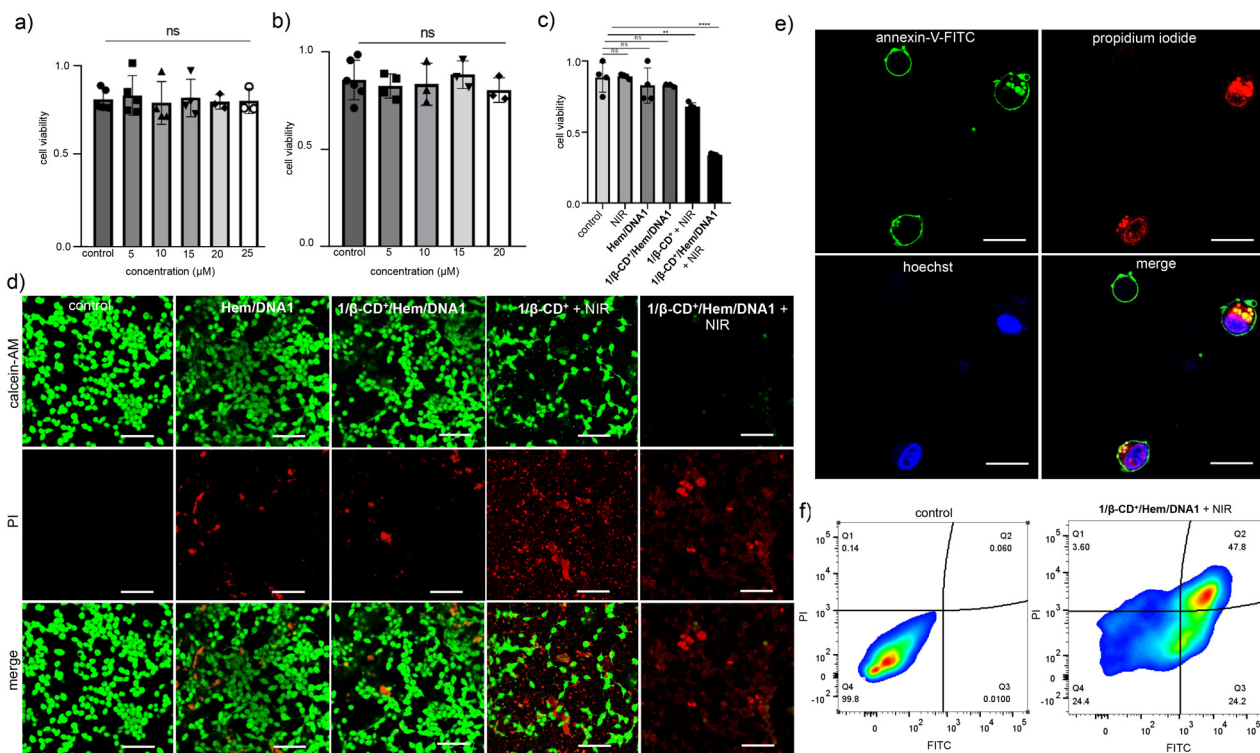
**Fig. 4** (a) DCFH-DA assay: CLSM images of Hem/DNA1,  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1,  $1/\beta$ -CD<sup>+</sup> + NIR and  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 + NIR-treated MDA-MB-231 cells (635 nm laser with a power of  $0.75 \text{ W cm}^{-2}$ ) (scale bar represents  $25 \mu\text{m}$ ). (b) TMRM assay: CLSM images of  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1-treated MDA-MB-231 cells stained with TMRM under photoirradiation with a 635 nm laser ( $0.75 \text{ W cm}^{-2}$ ) (scale bar represents  $25 \mu\text{m}$ ) and (c) the corresponding FACS analyses. (d) AO assay: CLSM images of  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1-treated MDA-MB-231 cells stained with AO under photoirradiation with a 635 nm laser ( $0.75 \text{ W cm}^{-2}$ ) (scale bar represents  $25 \mu\text{m}$ ) and (e) the corresponding FACS analyses.

Because ROSs such as  $^1\text{O}_2$  and  $\cdot\text{OH}$  are highly oxidizing in nature, they can induce damage to organelles like lysosomes and mitochondria, which can potentially lead to apoptosis by various signalling pathways. In view of this, we examined the structural integrity of these organelles in the presence of ROSs generated by the combined CDT and PDT. The mitochondrial damage caused by the ROSs was evaluated using the tetramethylrhodamine methyl ester perchlorate (TMRM) assay (Fig. 4b and c).<sup>51</sup> Since mitochondria have typical membrane potentials in the range of  $-180 \text{ mV}$ , they can accumulate high concentrations of cationic dyes like TMRM. If mitochondria are damaged or depolarized, dye accumulation gradually decreases, which leads to a diminishing of its fluorescence intensity. For this, MDA-MB-231 cells were treated with  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 ( $20/20/1/1 \mu\text{M}$ ) for 2 h, then kept under NIR light illumination ( $635 \text{ nm}$  laser at  $0.75 \text{ W cm}^{-2}$ ) for 10 minutes followed by 3 h of incubation. The cells were then stained with TMRM, and changes in the green fluorescence intensity of TMRM were probed using CLSM analyses. In this case as well, CLSM analyses of  $1/\beta$ -CD<sup>+</sup>/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 (Fig. 4b). This was further quantified by FACS analyses (Fig. 4c), which disclosed similar MFI shift values for the  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1-treated cells (12 674) and the corresponding untreated control cells (12 660). The lysosomal integrity was further evaluated using an acridine orange (AO) assay (Fig. 4d and e).<sup>52,53</sup> Acridine orange emits green fluorescence when bound to dsDNA in the nucleus and red fluorescence inside lysosomes. 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  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 ( $20/20/1/1 \mu\text{M}$ ) for 2 h followed by NIR light illumination for 10 minutes followed by 3 h of incubation. Subsequently, the cells were co-stained with AO and evaluated using CLSM analyses. The CLSM images of  $1/\beta$ -CD<sup>+</sup>/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 the ROSs generated by the  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 nanosheet (Fig. 4d). This was further confirmed by FACS analyses, which showed MFI shift values of 16 476 and 17 527 for the control and  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 nanosheet-treated MDA-MB-231 cells, respectively (Fig. 4e). However, it must be noted that TMRM and AO staining assays revealed significant damage to lysosomes and mitochondria after 24 h of incubation (Fig. S6†). These results indicate that ROSs cause damage to the cell organelles over longer time-scales of incubation, but remain intact during shorter incubation times.

After demonstrating selective internalization and ROS generation inside the cancer cells, the cytotoxicity of  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 towards MDA-MB-231 cells was studied using the MTT assay.<sup>54</sup> Initially, toxicities of  $\beta$ -CD<sup>+</sup> and  $1/\beta$ -CD<sup>+</sup> nanosheets towards MDA-MB-231 cells in the dark were studied (Fig. 5a and b). For this,  $\beta$ -CD<sup>+</sup> (Fig. 5a) and  $1/\beta$ -CD<sup>+</sup> (Fig. 5b) nanosheets of varying concentrations ( $1$ – $20 \mu\text{M}$ ) in the dark were treated with MDA-MB-231 cells for 24 h. As expected, no cytotoxicity was observed even at the higher concentration of  $20 \mu\text{M}$ . Subsequently, the cytotoxicities of MDA-MB-231 cells treated with Hem/DNA1 ( $1/1 \mu\text{M}$ ),  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 ( $20/20/1/1 \mu\text{M}$ ),  $1/\beta$ -CD<sup>+</sup> ( $20/20 \mu\text{M}$ ) + NIR, and  $1/\beta$ -CD<sup>+</sup>/Hem/DNA1 + NIR ( $20/20/1/1 \mu\text{M}$ ) for 2 h ( $635 \text{ nm}$  laser with a power of  $0.75 \text{ W cm}^{-2}$  for 10 minutes) were studied (Fig. 5c). As expected no significant cytotoxicity was observed for Hem/DNA1 and





**Fig. 5** (a)–(c) MTT assay: cell viability at varying concentrations of (a)  $1/\beta\text{-CD}^+$  and (b)  $1/\beta\text{-CD}^+$ -treated MDA-MB-231 cells in the dark. (c) MTT assay of Hem/DNA1,  $1/\beta\text{-CD}^+$ /Hem/DNA1,  $1/\beta\text{-CD}^+$  + NIR and  $1/\beta\text{-CD}^+$ /Hem/DNA1 + NIR-treated MDA-MB-231 cells (635 nm laser with a power of  $0.75 \text{ W cm}^{-2}$ ). (d) Calcein-AM/PI live–dead assay: CLSM images of Hem/DNA1,  $1/\beta\text{-CD}^+$ /Hem/DNA1,  $1/\beta\text{-CD}^+$  + NIR and  $1/\beta\text{-CD}^+$ /Hem/DNA1 + NIR-treated MDA-MB-231 cells (scale bar represents  $100 \mu\text{m}$ ). (e) Annexin V-FITC/PI assay: CLSM images of  $1/\beta\text{-CD}^+$ /Hem/DNA1-treated MDA-MB-231 cells under photoirradiation using a 635 nm laser ( $0.75 \text{ W cm}^{-2}$ ) (scale bar represents  $25 \mu\text{m}$ ) and (f) the corresponding FACS analyses.

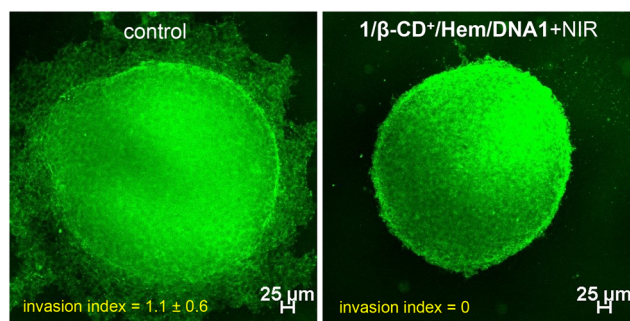
$1/\beta\text{-CD}^+$ /Hem/DNA1 in the absence of NIR light, indicating the poor therapeutic efficacy. On the other hand, significant cytotoxicity was observed for  $1/\beta\text{-CD}^+$  and  $1/\beta\text{-CD}^+$ /Hem/DNA1-treated cells under NIR light irradiation. Interestingly, enhanced cytotoxicity was observed for  $1/\beta\text{-CD}^+$ /Hem/DNA1-treated cells compared to  $1/\beta\text{-CD}^+$ -treated cells. A cell death extent of 65% was observed for  $1/\beta\text{-CD}^+$ /Hem/DNA1-treated cells, whereas only 32% cell death was observed for  $1/\beta\text{-CD}^+$ -treated cells. These results are in full agreement with the enhanced ROS generation for  $1/\beta\text{-CD}^+$ /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 the  $1/\beta\text{-CD}^+$ /Hem/DNA1 nanosheet towards MDA-MB-231 cells, the calcein-AM/PI co-staining assay was performed (live/dead assay) (Fig. 5d). Calcein-AM interacts with the esterase present inside live cancer cells and produces 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 cells 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.<sup>43</sup> To demonstrate the

live/dead assay, the cells were initially treated with Hem/DNA1,  $1/\beta\text{-CD}^+$ /Hem/DNA1,  $1/\beta\text{-CD}^+$  + NIR, and  $1/\beta\text{-CD}^+$ /Hem/DNA1 + NIR for 2 h (635 nm laser with a power of  $0.75 \text{ W cm}^{-2}$  for 10 minutes). As shown in Fig. 5d,  $1/\beta\text{-CD}^+$ /Hem/DNA1 + NIR-treated cells exhibited mainly red fluorescence of PI and negligible green fluorescence of calcein-AM compared to Hem/DNA1,  $1/\beta\text{-CD}^+$ /Hem/DNA1, and  $1/\beta\text{-CD}^+$  + NIR-treated cells. These results conclude the enhanced cytotoxicity of  $1/\beta\text{-CD}^+$ /Hem/DNA1 + NIR compared to  $1/\beta\text{-CD}^+$  + NIR, Hem/DNA1 and  $1/\beta\text{-CD}^+$ /Hem/DNA1. The live/dead cell assay also supports our hypothesis of enhanced cytotoxicity for  $1/\beta\text{-CD}^+$ /Hem/DNA1 + NIR due to the synergetic combination of CDT and PDT.

In order to understand the cell death mechanism of the present nanoformulation, the Annexin V-FITC (AV)/PI staining assay was carried out on  $1/\beta\text{-CD}^+$ /Hem/DNA1-treated MDA-MB-231 cells under NIR light illumination. As shown in Fig. 5e and Fig. S7,<sup>†</sup>  $1/\beta\text{-CD}^+$ /Hem/DNA1 (20/20/1/1  $\mu\text{M}$ )-treated cells exhibited the intense green fluorescence of Annexin V-FITC around the cell membrane and the strong red fluorescence of PI from the nuclei, revealing that most of the cell population is in the late stage of the apoptotic pathway. This is further confirmed through the FACS analyses (Fig. 5f). For this purpose, MDA-MB-231 cells were treated with the  $1/\beta\text{-CD}^+$ /Hem/DNA1 nanosheet under NIR light illumination and analysed by flow cytometry after staining with Annexin V-FITC





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

(AV)/PI. In accordance with the CLSM, the cell population mainly shifted to the quadrant corresponding to the apoptotic pathway. These results collectively reveal that the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA1}$  nanosheet under NIR light illumination induces cell death *via* the oxidative stress-induced apoptotic pathway.

After demonstrating the *in vitro* performance of the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA1}$  nanosheet, we studied the efficiency of the nanoformulation in the multicellular tumor spheroid model using a 3D cell culture (Fig. 6). The 3D-multicellular tumor spheroid mimics the *in vivo* tumor and undergoes proliferation in all directions similar to malignant tumors. In a 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.<sup>55</sup> To this end, 3D tumour spheroids of MDA-MB-231 cells were prepared using the standard hanging drop method.<sup>56,57</sup> The invasion potential of the 3D spheroids was studied in the presence of the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA1}$  nanosheet after NIR light irradiation to understand its effect on spheroid invasion. For this, 3D spheroids were incubated with  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA1}$  ( $20/20/1/1 \mu\text{M}$ ) nanosheets for 4 h, kept under irradiation at 635 nm ( $0.75 \text{ W cm}^{-2}$ ) for 10 minutes followed by 24 h of incubation. Untreated spheroids were considered as controls that showed the maximum invasion potential (invasion index =  $1.1 \pm 0.6$ ) as they showed migration in all directions. On the other hand, the  $1/\beta\text{-CD}^+/\text{Hem}/\text{DNA1}$ -treated sample showed a 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 nano-

formulation results in the formation of highly toxic ROSS along with the generation of  $\text{O}_2$  as one of the bioproducts, which can boost the concentration of  $\text{O}_2$  in the tumour micro-environment. This in turn enhances 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 the CDT action of the nanoagent enhances the PDT efficacy and we strongly believe that this approach will encourage researchers to design similar combination therapies for 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.

## Data availability

The data supporting this article have been included as part of the ESI.†

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

Financial support from the SERB (CRG/2022/002612) is gratefully acknowledged. Sarika Mohan. S is acknowledged for help with the FACS analyses.

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