Electronic Supplementary Information (ESI)

Two-dimensional Supramolecular Assemblies based on β-Cyclodextrin–grafted Graphene Oxide for Mitochondrial Dysfunction and Photothermal Therapy

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Experimental section

Instrumentation and methods

The morphologies of the synthesized graphene oxide (GO), GOCD and the TPM-Azo⊂GOCD supramolecular assemblies were observed by atom force microscopy (AFM, Dimension Icon, Bruker, USA). Intracellular distribution of the nanocomposites was monitored by a confocal microscope (FV1000, Olympus, Japan). The Fourier transformed infrared spectra GOCD and TPM-Azo were further assayed (FT-IR, Bio-rad, FTS6000, USA). Size distribution and Zeta potential of the nanocomposites were measured by a laser light scattering spectrometer (BI-200SM, Brookhaven, USA). A NIR laser beam was produced with a NIR laser system (808 nm, 1.5 W, 20 W/cm^2, Shuanghong, China). The photothermal properties of GOCD and TPM-Azo⊂GOCD were monitored by an infrared thermal imaging camera (Xintest, China). Cell death was examined by a flow cytometer (FACS Calibur, BD, USA). The luminescence density of the ATP-assaying samples was measured by a fluorescence microplate reader (Enspire, Perkin Elmer, USA). The FITC fluorescence of the mouse organs and tumors were monitored by an In-Vivo Imaging System (Xenogen, IVIS Lumina II, USA).

Preparation of GOCD, TPM-Azo and TPM-Azo⊂GOCD

To prepare TPM-Azo, 40 mg of transferrin (TF), 2 mg of polylysine (MW < 5, 000 D), 2 mg of polyethylene glycol (PEG-NH_2, MW < 5, 000 D), and 1 mg of MitP were simultaneously dissolved in PBS (50 mM, pH 7.2), and then 10 mg of EDC and 11 mg of NHSS were added into the solution. The mixture were stirred at 4 ºC for 12 h, dialyzed using dialysis bags (cut-off MW > 12, 000 D), and lyophilized in a freezing vacuum dryer, obtain TPM. To further modification of TPM with Azo, 5 mg of 4-(phenylazo) benzoic acid (Azo-COOH), 4 mg of EDC and 4.5 mg of NHSS were dissolved in PBS (50 mM, pH 7.2), and the solution were stirred at 20 ºC for 30 min. 20 mg of TPM were then added into the solution, and the mixture was further stirred at 4 ºC for 12 h. The solution was then dialyzed using dialysis bags (cut-off MW > 12, 000 D), obtain TPM-Azo.

To synthesize GOCD, GO was firstly prepared by modified Hummer's method.1 10 mg of the obtained GO was then suspended in 20 mL of dH_2O, and sonicated by a ultrasonic processor, followed by addition of 20 mL of PBS (100 mM, pH 7.2), and then 10 mg of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC), 11 mg of N-hydroxysulfosuccinimide (NHSS) and 10 mg of mono-6-deoxyl-6-ethylenediamino-β-CD were added. The mixture was shaken at 20ºC for 12 h and centrifuged, obtaining GOCD.

The TPM-Azo⊂GOCD supramolecular nanostructures were generated by mixing TPM-Azo and GOCD (each at a concentration of 2.5 mg/mL) and sonicating the mixture for 5 min.

Confocal microscopy

Human lung adenocarcinoma cells (A549, Cell Resource Center, China Academy of Medical Science, Beijing, China) were incubated with TPM-Azo (100 mg/L), GOCD (100 mg/L) or TPM-Azo⊂GOCD nanoassemblies (100 mg/L TPM-Azo plus 100 mg/L GOCD) for 24 h. The cells were then washed with PBS and stained with MitoTracker Red (100 nM, Sigma) and Hoechst 33342 (10 μg/mL, Sigma) at 37 °C for 40 min. The cells were then observed by confocal microscopy.

Cell viability assay

To evaluate the growth of the A549 cells, we treated them with TPM-Azo or TPM-Azo⊂GOCD nanoassemblies for 24 h as described in the preceding section. The extent of growth of the treated cells was then determined with a CCK-8 assay kit (DOJINDO, Japan). After 24 h of treatment, cell death rate was assessed by means of propidium iodide staining and subsequent flow cytometry analysis.

Western blotting

To detect cytosolic cytochrome C (Cyt $C_{(cyto)}$) and the control protein GAPDH, the treated A549 cells were homogenized in mitochondrial buffer with a Dounce homogenizer (20–50 strokes). The cell lysate was centrifuged at 1000 × g and 4 °C to remove nuclei and intact cells. The resulting suspension was further centrifuged at 12,000 × g to obtain the cytosol (supernatant) and the mitochondria (pellet). The levels of Cyt $C_{(cyto)}$ and GAPDH in the cytosol were detected using Cyt C monoclonal antibody and GAPDH monoclonal antibody, respectively (Abcam, USA). The Cyt $C_{(cyto)}$/GAPDH ratio was quantified by the Image J software (version 2, USA).

Extracellular mitochondrial aggregation

To assess the ability of the nanocomposites to induce extracellular mitochondrial aggregation, the isolated mitochondria were suspended in mitochondrial buffer with the protein content of 100 μg/mL, and stained 30 min with DAPI (5 μg/mL, Sigma). TPM-Azo (100 mg/L), GOCD (100 mg/L) or TPM-Azo⊂GOCD nanoassemblies (100 mg/L TPM-Azo plus 100 mg/L GOCD) were then added into the mitochondrial suspension. After mixed thoroughly and stood for 10 min, the mitochondria were observed by confocal microscopy.

Cell cycle analysis

To assess the effect of the nanocomposites on tumor cell cycle progression, the treated cells were washed twice with PBS, fixed 2 hours with 95% ethanol, and suspended in 50 mM citrate buffer (pH = 7.4). The fixed cells were further treated by RNase (200 μg/mL, Simga) at 37 °C for 60 min, stained by propidium iodide (20 mg/L) for 5 min, and examined by flow cytometry analysis.

ATP assay

To measure the intracellular ATP levels of the treated cells, the cells were broken using distilled water by violently votexing. The ATP levels in the cell lysates were then detected by the ATP Assay Kit (Beyotime, China). To reflect the ATP levels, the luminescence density was measured by a fluorescence microplate reader.

Viability assay

The A549 tumor cells or NIH3T3 non-cancerous cells were incubated with TPM-Azo (100 mg/L), GOCD (100 mg/L) or TPM-Azo⊂GOCD nanoassemblies (100 mg/L TPM-Azo plus 100 mg/L GOCD) for 24 h, and then irradiated by the NIR laser (808 nm, 1 W, 20 W/cm^2) for 10 min. The viability of the treated cells were then detected by the CCK-8 Assay Kit (DOJINDO, Japan). Cell death was assessed by propidium iodide (PI) staining after 24 h or 48 h.

Photothermal analysis

To measure photothermal conversion efficiency, GOCD (100 mg/L), TPM-Azo (100 mg/L) or TPM-Azo⊂GOCD (100 mg/L TPM-Azo plus 100 mg/L GOCD) with a NIR laser (808 nm, 1.5 W, 20 W/cm^2) for 5 min. Temperature was monitored in real time with an infrared thermal imaging camera.

***In vivo* tumor model**

To investigate the in vivo anti-tumor activity of the nanocomposites, the S180 tumor cells were suspended in distilled saline with the concentration of 10^7 cells mL^{-1}. The cell suspensions were subcutaneously inoculated into the 5 weeks-old female BALB/c mice. After inoculation for 4 days, the suspensions of GOCD (100 mg/kg), TPM-Azo (100 mg/kg) or TPM-Azo⊂GOCD (100 mg/kg TPM-Azo plus 100 mg/kg GOCD) were intravenously injected per day into the mice for 3 days, and then the tumor sites were irradiated by NIR laser (808 nm, 1.5 W) for 20 min. From the day that the tumors received NIR irradiation, the tumor volumes were monitored for 12 days. The mice were then anaesthetized, and the tumors were sampled, weighted, fixed by 4% formaldehyde solution, embedded with paraffin and sectioned into slices. The slices were then stained by the TdT-mediated dUTP nick-end labeling (TUNEL) assay kit (Beyotime, China).

To observe organ distribution of TPM-Azo⊂GOCD, the nanoassemblies (100 mg/kg TPM-Azo plus 100 mg/kg GOCD) were intravenously injected into the S180 tumor-burden mice. After 24 h of injection, the mice were anaesthetized, and the tumors and main organs (*i.e.*, lung, liver, heart, spleen, and kidney) were sampled. The FITC fluorescence of the organs and tumors were monitored by the In-Vivo Imaging System. The animal experiments were approved by the Animal Care and Use Committee at Nankai University.

Ethical statement

All of the biological experiments, including the animal experiments and the cell culturing experiments, were performed in compliance with the guidelines of the Animal Care and Use Committee at Nankai University and the experiment guidelines of the College of Life Science at Nankai University. The Committee approved all of the experiments.

Statistical analysis

Three replicates of each experiment were performed, and values reported herein are means ± standard deviations. Differences between groups were compared by a one-way analysis of variance test ($P < 0.05$). All statistical tests were performed using the SPSS software package (version 20, IBM, USA).

Supplementary Figures

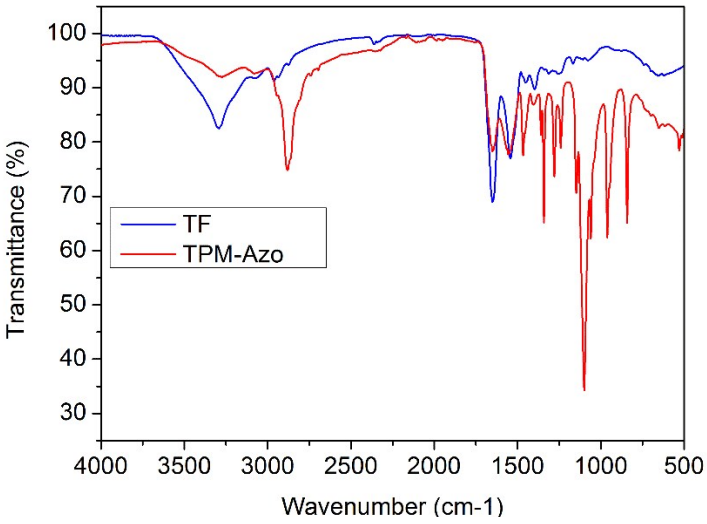

Fig. S1 FT-IR spectrum of TF and TPM-Azo.

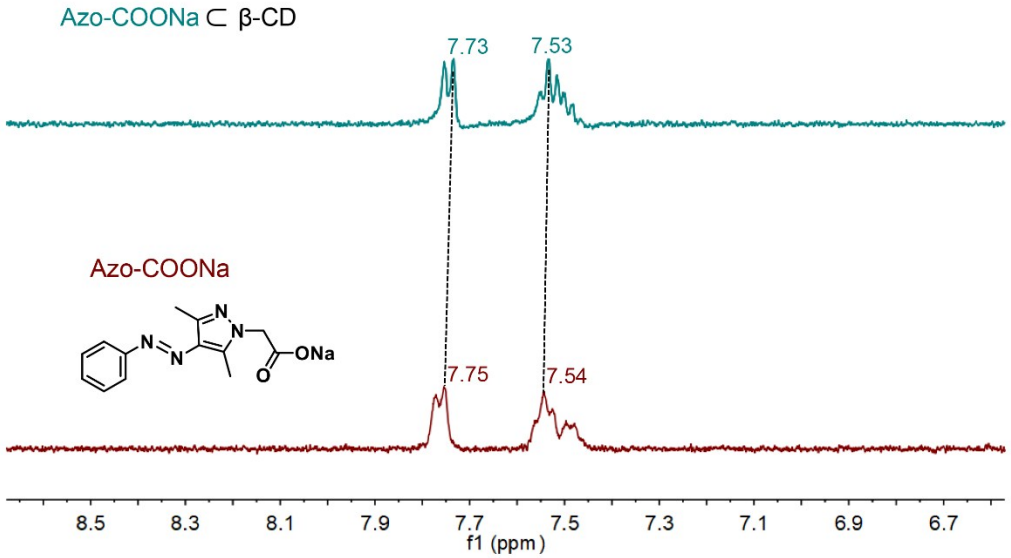

Fig. S2. Partial ^{1}H NMR spectra (400 MHz, D_2O, 25°C) of trans-Azo-COONa and trans-Azo- COONa+β-cyclodextrin (1 : 1).

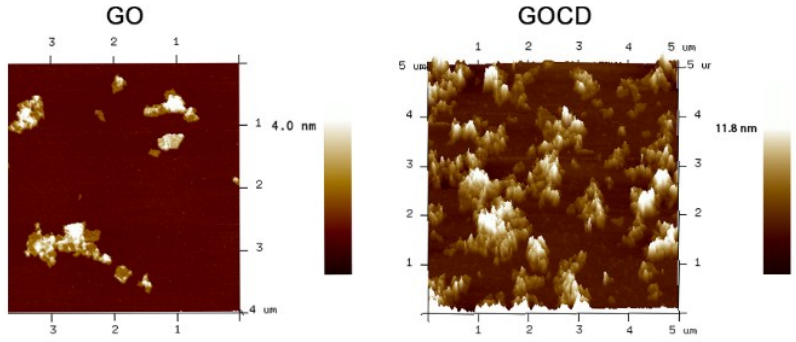

Fig. S3 Atom force microscopy (AFM) images of the synthesized GO and GOCD nanosheets.

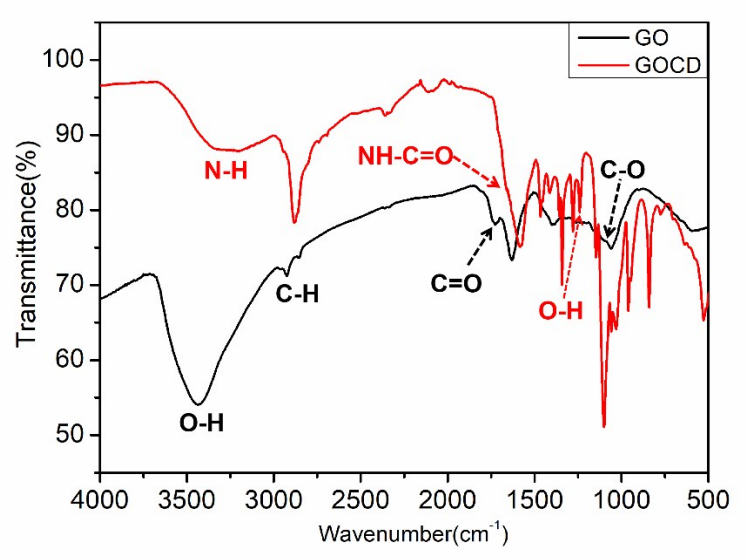

Fig. S4 FT-IR spectrum of GO and GOCD.

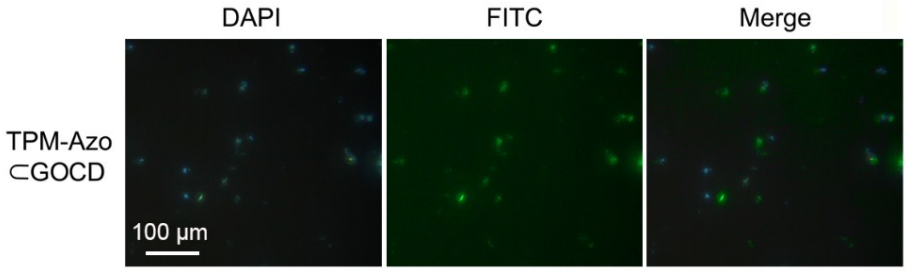

Fig. S5 UV-irradiation-induced disassembly of the mitochondrial aggregates by transformation of TPM-*trans*-Azo to TPM-*cis*-Azo in TPM-Azo⊂GOCD.

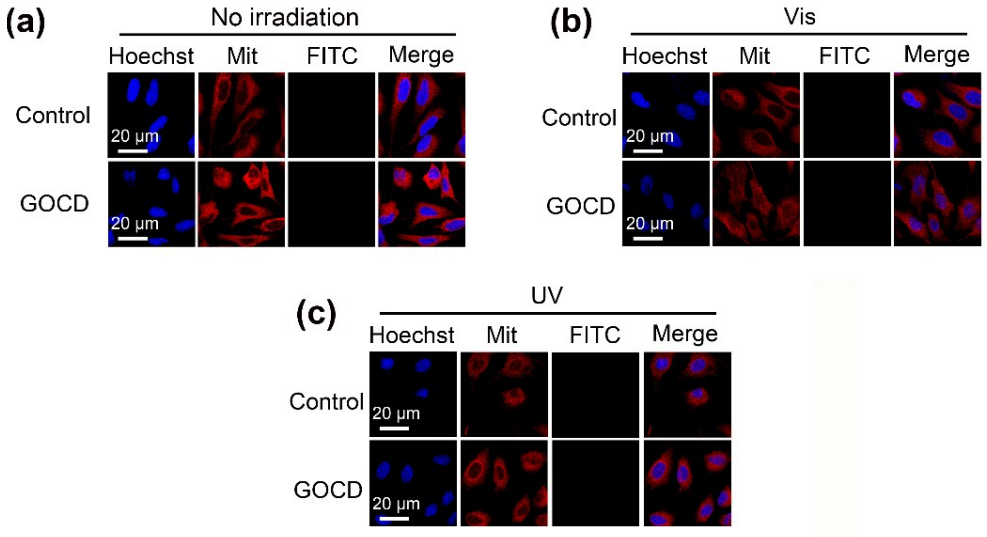

Fig. S6 Confocal microscopy images of the A549 cells without nanocomposite treatment (Control) or treated by GOCD under no irradiation (a), Vis irradiation (b) or UV irradiation (c).

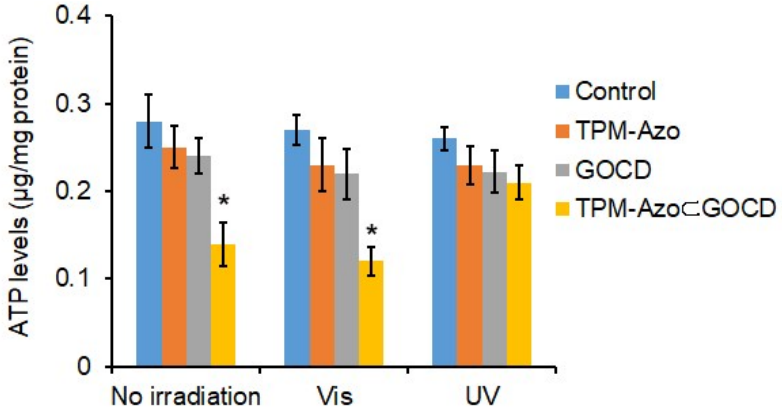

Fig. S7 ATP levels in the cells treated by GOCD, TPM-Azo or TPM-Azo⊂GOCD upon visible-light (Vis) and UV irradiation. * Indicates a significant difference between the TPM-Azo⊂GOCD group and other groups ($P < 0.05$).

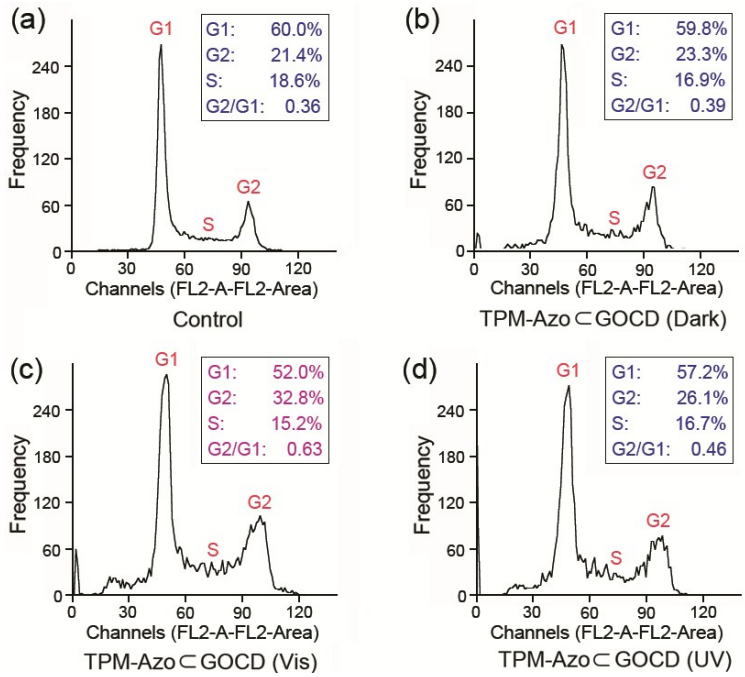

Fig. S8 Cell cycle arrest induced by the TPM-Azo⊂GOCD nanoassemblies in A549 tumor cells.

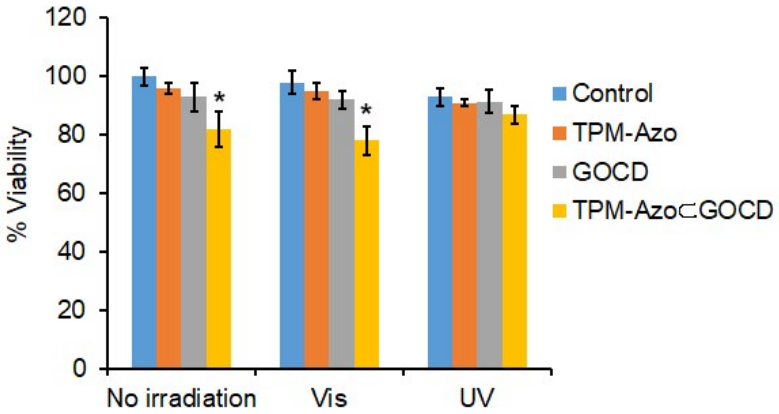

Fig. S9 Cell viability of the A549 tumor cells treated by GOCD, TPM-Azo or TPM-Azo⊂GOCD upon visible-light (Vis) and UV irradiation. * indicates a significant difference between the TPM-Azo⊂GOCD group and other groups ($P < 0.05$).

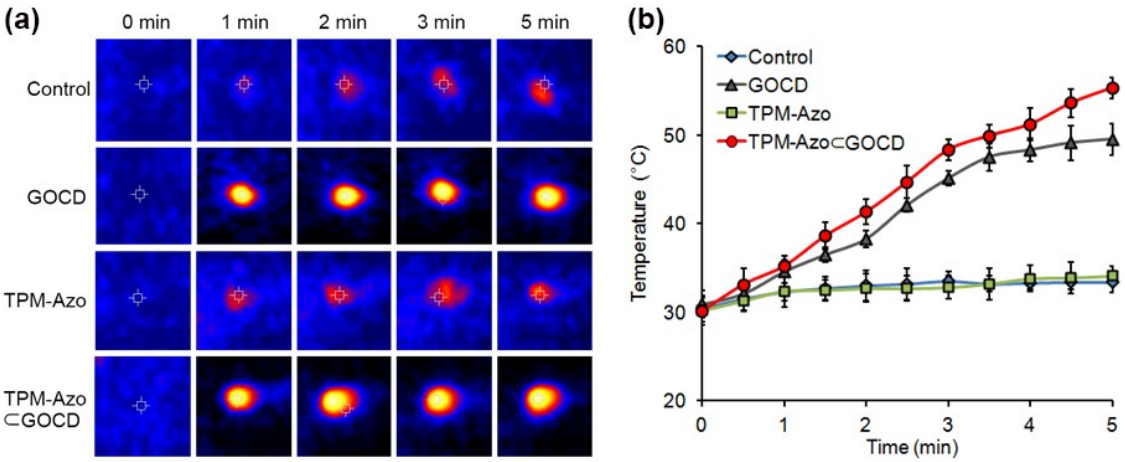

Fig. S10 Temperature increase of GOCD, TPM-Azo or TPM-Azo⊂GOCD upon exposure to NIR light (808 nm, 1.5 W).

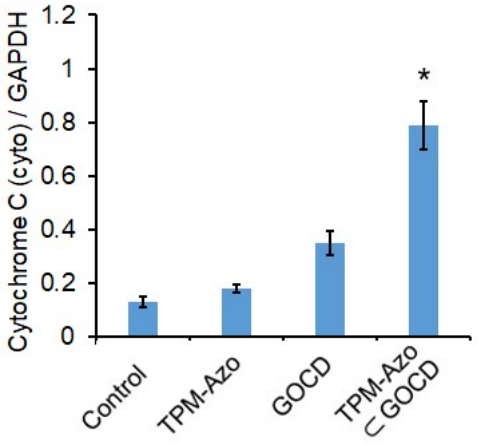

Fig. S11 Cytochrome C release from the mitochondria to the cytosol (Cytochrome c $_{(cyto)}$) in the A549 tumor cells under exposure to the NIR light. GAPDH was detected as the control. * indicates a significant difference between the TPM-Azo⊂GOCD group and other groups ($P < 0.05$).

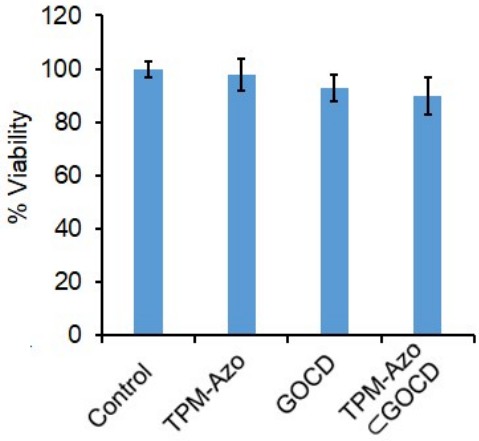

Fig. S12 Viability of the non-cancerous NIH3T3 cells treated by GOCD, TPM-Azo or TPM-Azo⊂GOCD nanoassemblies with NIR treatment. There is no significant difference between the groups ($P < 0.05$).