Electronic Supplementary Information (ESI)

A tumour mRNA-triggered nanoassembly for enhanced fluorescence imaging-guided photodynamic therapy

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Experiment section

Chemicals and Materials

The DNA Sequences listed in Table S1 were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). Melamine, magnesium chloride ($MgCl_2$), Tris(hydroxymethyl)aminomethane were purchased from Sigma-Aldrich (Shanghai, China). CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit were purchased from Promega (Wisconsin, USA). HeLa cells (human cervical carcinoma cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). RPMI-1640 medium, penicillin, streptomycin, heat-inactivated fetal bovine serum (Invitrogen) were purchased from Thermo Fisher Scientific (MA, USA). Lyso-Tracker Red (LysoTracker® Red), Singlet Oxygen Sensor Green (SOSG) and 2,7-dichlorofluorescin diacetate (DCFH-DA) were obtained from Thermo Fisher Scientific (MA, USA). All the chemicals used in this work were of analytical grade and directly used without further purification. Ultrapure water was obtained through a Millipore Milli-Q water purification system (Billerica, MA) and had an electric resistance >18.25 MΩ.

Instruments

UV-vis absorption spectrum was measured on UV2450 (Shimadzu, Japan). The fluorescence spectra were recorded at room temperature in a quartz cuvette on F-7000 fluorescence spectrophotometer (Hitachi, Japan). All fluorescence images were acquired on Nikon TI-E+A1 SI confocal laser scanning microscope (Japan). The transmission electron microscopy (TEM) images were obtained on a field-emission high-resolution 2100F TEM (JEOL, Japan) at an acceleration voltage of 200 kV. X-ray diffraction (XRD) patterns of g-C_3N_4 samples were collected via a D8 Advance X-ray diffractometer (Bruker, USA) with Cu-Kα radiation (λ=1.5418 Å). The infrared absorption spectroscopic measurements were taken with g-C_3N_4 powders in KBr pieces on a Nexus 870 FT-IR spectrophotometer (Thermo Electron, USA) under continuous N_2 purge. Zeta potential measurements of samples was were conducted on a Malvern Zetasizer 3000 HS particle size analyzer (Malvern Instruments, UK) in air at room

temperature.

Preparation of g-C_3N_4 nanosheets

The bulk g-C_3N_4 light yellow powder was prepared from melamine according to the previously reported method.[1] The g-C_3N_4 nanosheets were prepared as follows: 100 mg of bulk g-C_3N_4 powder was dispersed in 10 mL of 5 M HNO_3 and refluxed for 24 h. The refluxed product was centrifuged at 14000 rpm for 15 min, washed with pure water to neutral pH, and redispersed in 10 mL of water. The obtained mixture was sonicated for 16 h and then centrifuged at 8000 rpm for 15 min to remove the residual unexfoliated g-C_3N_4 nanoparticles before use. Finally, the highly water-dispersible g-C_3N_4 nanosheets was obtained and stored in darkness at room temperature. The concentration of the g-C_3N_4 nanosheet solution was ~0.7 mg/mL.

Quenching effect of g-C_3N_4 nanosheets

Firstly, the hairpin probes (1μM HP1* and 1μM HP2) were denatured in TAE buffer (25 mM Tris-HCl buffer containing 5 mM $MgCl_2$, pH 8.3) at 95 °C for 5 min and quickly cooled to 0 °C for 1 h to make the probe fold into a hairpin structure. Then, 100 μL of reaction solution containing HP1* (100 nM), HP2 (100 nM), and 20 mM HEPES buffer (500 mM KCl, pH 7.9) were mixed with g-C_3N_4 nanosheets at different concentrations, followed by incubation at 37 °C for 30 min. Finally, the fluorescence intensity of the reaction mixture at 670 nm was measured on F-7000 Fluorescence Spectrophotometer under the excitation at 565 nm.

Calculation of DNA probes loading on g-C_3N_4 nanosheets

The detailed procedure for calculating the concentration of HP1/HP2 on the surface of g-C_3N_4 nanosheets was performed as following: after incubation of HP1/HP2 (150 nM) with g-C_3N_4 nanosheets (140 μg/mL) at 37 °C for 30 min, the mixture was centrifuged at 20000 rpm for 30 min and the supernatants were obtained. Then, the absorbance of DNA in the supernatants was measured and the DNA concentration (~100 nM) on the surface of g-C_3N_4 nanosheets was calculated by subtracting the amount of HP1/HP2 in the supernatant mixture from the total amount of HP1/HP2 added into g-C_3N_4 solution.

Gel electrophoresis analysis

Gel electrophoresis was conducted on 3% (w/v) agarose gel containing gold view (0.5 μg/mL) and ethidium bromide (0.5 μg/mL) in 0.5 × TBE (44 mmol/L Tris-Boric Acid; 1 mmol/L EDTA) at room temperature. 10 μL DNA sample (Final concentration of HP1 and HP2 were both 100 nM) was mixed with 1 μL 10 × loading buffer and then added in the well of agarose gel. The gel samples were run at 100 V for 70 min. Then, the gel images were obtained on Tanon 4200SF gel imaging system.

***In vitro* detection of the target RNA**

Firstly, the hairpin probes (1μM HP1 and 1μM HP2) were denatured in TAE buffer (25 mM Tris-HCl buffer containing 5 mM $MgCl_2$, pH 8.3) at 95 °C for 5 min and then quickly cooled to 0 °C for 1 h to make the probe perfectly fold into a hairpin structure. 100 μL of reaction solution containing g-C_3N_4 nanosheets (140 μg/mL), HP1 (100 nM), HP2 (100 nM), and 20 mM HEPES buffer (500 mM KCl, pH 7.9) were mixed with the target RNA at different concentrations (0-100 nM), followed by incubation at 37 °C for 4 h. Finally, the fluorescence spectra from 570 to 650 nm of the reaction mixture were recorded on F-7000 Fluorescence Spectrophotometer using an excitation wavelength of 535 nm.

For selectivity assay, 100 μL of mixture solution containing g-C_3N_4 nanosheets (140 μg/mL), HP1 (100 nM), HP2 (100 nM), and 20 mM HEPES buffer (500 mM KCl, pH 7.9) were incubated with different target RNA including 50 nM target RNA, Mis-1, Mis-2 , Mis-3, and Mis-4 at 37 °C for 4 h, respectively. Then the fluorescence intensity of the reaction mixture at 585 nm were measured on F-7000 Fluorescence Spectrophotometer under the excitation at 535 nm.

***In vitro* monitoring $^1O_2$ generation of g-C_3N_4 nanosheets**

g-C_3N_4 nanosheets (140 μg/mL) was mixed with SOSG (1μM) in 20 mM HEPES buffer (500 mM KCl, pH 7.9) and exposed to LED light (450 nm, 10 mW cm^{-2}) for 15 min. Then the fluorescence spectra were recorded from 505 to 600 nm using an excitation wavelength of 488 nm.

Cell culture

HeLa cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere incubator containing 5 wt %/vol CO_2.

***In vitro* cytotoxicity of g-C_3N_4 nanosheets**

The cytotoxicity of the g-C_3N_4 nanosheet was evaluated by a CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit. HeLa cells were seeded at 5×10^3 cells per well. All the cells were first cultured at 37 °C for 24 h. Then different concentrations (0-350 μg/mL) of g-C_3N_4 nanosheets was added and incubated for another 24 h. Afterwards, the culture medium was removed and cells were washed twice with 1×PBS (200 μL). Then, 20 μL CellTiter Reagent diluted with 100 μL of growth medium was added and incubated at 37 °C for 2 h. The absorbance measurements at 490 nm was obtained on a Thermo Scientific Multiskan Microplate Reader (Thermo Fisher, USA).

Fluorescence imaging of g-C_3N_4 nanosheets in living cells.

Fluorescence imaging of living cells was performed as follows: HeLa cells were plated on a 35 mm Petri dish with a 10 mm bottom well for 24 h. For colocalization assay, HeLa cells were first incubated with 140 μg/mL g-C_3N_4 nanosheets in 1 mL of the culture medium for 6 h at 37 °C. Then Lyso-Tracker Red (100 nM) were added and incubated for another 15 min. Fluorescence emission from 425 to 505 nm of g-C_3N_4 nanosheets was collected with an excitation wavelength of 405 nm. Fluorescence emission of Lyso-Tracker Red were collected at 593-620 nm under excitation at 560 nm.

Fluorescence imaging of mRNA in living cells

HeLa cells were plated on a 35 mm Petri dish with a 10 mm bottom well for 24 h. Then HeLa cells were incubated with the HP1/HP2@g-C_3N_4 nanoassembly at 37 °C for 6 h. Before the incubation, the HP1/HP2@g-C_3N_4 nanoassembly were obtained as follows: The hairpin probes (1μM HP1 and 1μM HP2) were denatured in TAE buffer (25 mM Tris-HCl buffer containing 5 mM $MgCl_2$, pH 8.3) at 95 °C for 5 min and then quickly cooled to 0 °C for 1 h to make the probe perfectly fold into a hairpin structure. Then, g-C_3N_4 nanosheets (140 μg/mL) were incubated with the mixture containing 100 nM

HP1 and 100 nM HP2 in TAE buffer for 30 min at 37 °C. The HP1/HP2@g-C_3N_4 nanoassembly were achieved. Finally, fluorescence images of HeLa cells were captured by using a Nikon confocal laser scanning microscope. Fluorescence emission from 425 to 505 nm of g-C_3N_4 nanosheets was collected under excitation at 405 nm. Fluorescence emission from 593 to 620 nm of TAMRA was collected under excitation at 560 nm.

The research process to regulate the expression level of survivin mRNA in HeLa cells by the inhibitor is as follows: HeLa cells were first treated with different concentration (0, 5, 20 nM) of YM155 for 24 h to inhibit the expression of survivin mRNA, and then incubated with the HP1/HP2@g-C_3N_4 nanoassembly for 6 h. Finally, fluorescence images of HeLa cells were captured by using a Nikon confocal laser scanning microscope.

Monitoring intracellular ROS generation of g-C_3N_4 nanosheets

Intracellular ROS generation was evaluated by using 2,7-dichlorofluorescin diacetate (DCFH-DA). HeLa cells were plated on a 35 mm Petri dish with a 10 mm bottom well for 24 h. HeLa cells were incubated with 140 μg/mL g-C_3N_4 nanosheets at 37 °C for 6 h and subsequently stained with DCFH-DA for 30 min followed by LED light (λ=450 nm; Power density: 20 mW cm^{-2}) irradiation for 10 min. Afterwards, the cells were washed twice with 1×PBS and then supplemented with fresh medium. Finally, fluorescence images of HeLa cells were captured using the Nikon confocal laser scanning microscope. Fluorescence emission of g-C_3N_4 nanosheets from 505 nm to 550 nm was collected under excitation at 405 nm. Fluorescence emission of DCFH-DA from 505 nm to 550 nm was collected under excitation at 488 nm.

***In vitro* photodynamic therapy**

To evaluate the PDT efficacy of the g-C_3N_4 nanosheets, HeLa cells were seeded at 5×10^3 cells per well in 96-well plates at 37 °C for 24 h. Then different concentrations of g-C_3N_4 nanosheets was added and incubated for another 6 h. Afterwards, the cells were washed twice with 1×PBS and supplemented with fresh medium. A LED light source (~450 nm) was applied as the light source to irradiate the 96-well plates. After exposure to LED (20 mW cm^{-2}) for 20 min, the cells were allowed to incubate for an

additional 12 h. Cell viability was measured with a Cell Proliferation Assay kit. For dependent cell viability of HeLa cells incubated with g-C_3N_4 nanosheets after different irradiation time, 5×10^3 cells per well in 96-well plates were incubated with 140 μg/mL g-C_3N_4 nanosheets at 37 °C for 24 h. After exposure to LED (20 mW cm^{-2}) for different time periods, the cells were allowed to incubate for an additional 12 h. Cell viability was measured with a Cell Proliferation Assay kit.

Live/Dead Cell Staining

HeLa cells were plated on a 35 mm Petri dish with a 10 mm bottom well for 24 h. HeLa cells were incubated with 140 μg/mL g-C_3N_4 nanosheets at 37 °C for 6 h and then substituted with fresh RMPI-1640. The irradiated group was exposed to LED light (20 mW cm^{-2}) for 20 min, and the other group was kept in the dark. After another 12 h of incubation, the cells were treated with 500 μL of calcein AM (5 μM) /PI (10 μM) solution for 40 min at 37 °C without light and substituted with fresh RMPI-1640. Finally, fluorescence images of HeLa cells were captured by using a Nikon confocal laser scanning microscope. Fluorescence emission at 515 nm of calcein AM was collected under excitation at 488 nm. Fluorescence emission at 617 nm of PI was collected under excitation at 560 nm.

Table S1. Sequences of the synthetic oligonucleotides used in the experiments (5′-3′).[a]

Name	Sequence (5'-3')
Target	CACCGCAUCUCUACAUUCAAGA
Mis-1	CACGGCAUCUCUACAUUCAAGA
Mis-2	CACCGCAUCUGUACAUUCAAGA
Mis-3	CACCGCAUCUCUACAUUGAAGA
Mis-4	CACGCCAUCUCUACAUUCAAGA
HP1	AT(BHQ2)CTCTACATTCAAGTACACCGCATCTTGAATGTAGAGAT(TAMRA)GCGGTG
HP2	AAGTCACCGCATCTCTACATTCAAGATGCGGTGACTTGAATGTA
HP1*	ATCTCTACATTCAAGTACACCGCATCTTGAATGTAGAGAT(Cy5)GCGGTG

[a] The target RNA sequence marked with green is initiator, which is complementary to the purple sequence of HP1 and HP1*. The bases marked with blue are the mismatched bases of target RNA. Underline sequences indicate complementary regions of the probes to form hairpin DNA structure.

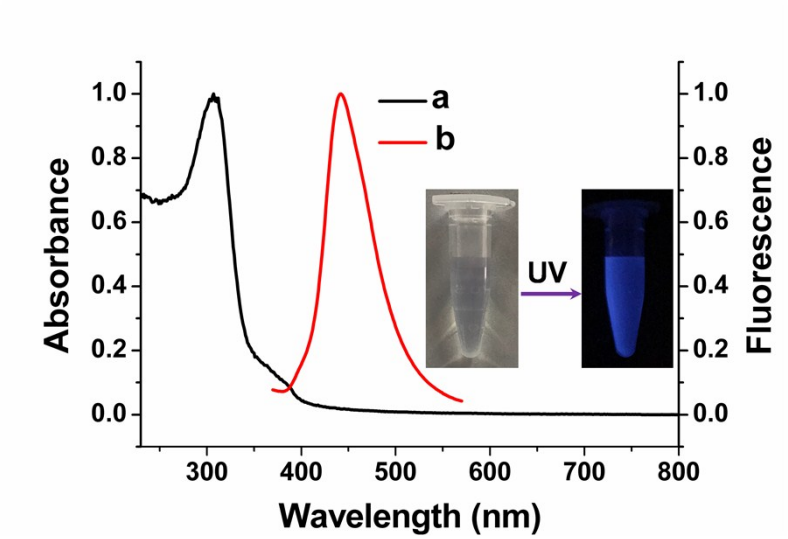

Fig. S1. UV-vis absorption spectrum of g-C_3N_4 nanosheets (a, black), fluorescence emission spectrum of g-C_3N_4 nanosheets (b, red). Inset shows the color change of g-C_3N_4 nanosheets solution under daylight and UV light.

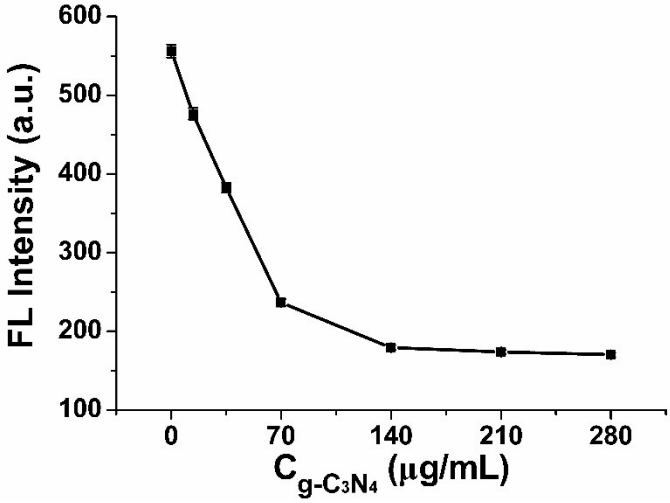

Fig. S2. Quenching effect of g-C_3N_4 nanosheets at various concentrations on the fluorescence of HP1*/HP2. The concentration of HP1*/HP2 is 100 nM. The concentrations of g-C_3N_4 nanosheets ranged from 0 to 280 mg/mL.

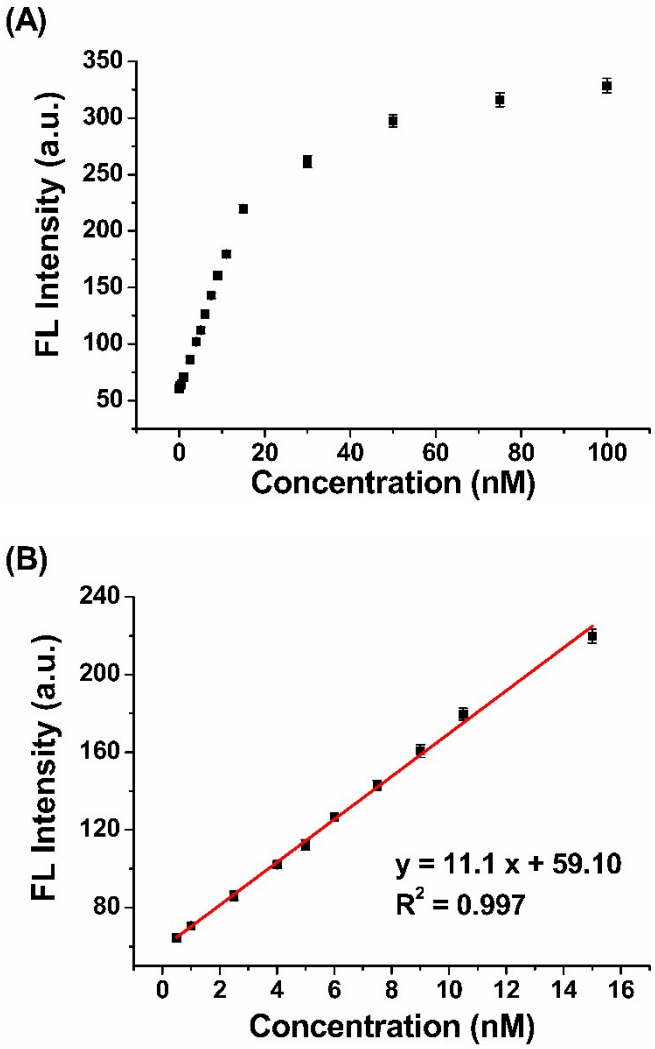

Fig. S3. (A) Fluorescence calibration curve of HP1/HP2@g-C_3N_4 nanoassembly in response to different concentrations (0-100 nM) of target RNA in 20 mM HEPES buffer (500 mM KCl, pH 7.9) for 4 h at 37 ℃. (B) Linear fitting of fluorescence intensity toward the concentration of the target from 0.5 nM to 15 nM. λex/em = 535/580 nm. The concentration of HP1/HP2 is 100 nM.

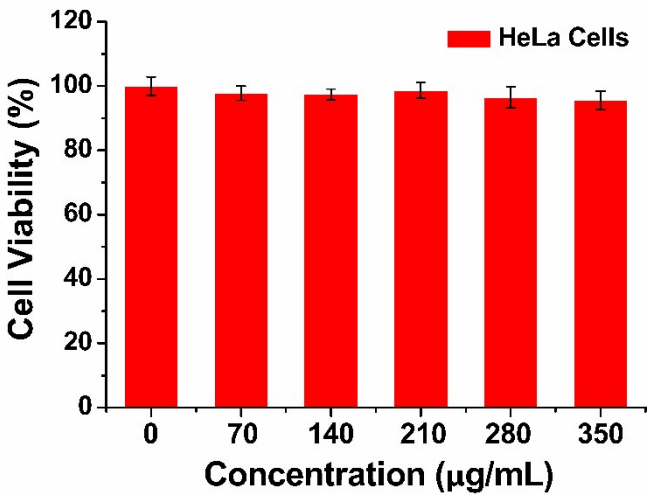

Fig. S4. The effect of HP1/HP2@g-C_3N_4 nanoassembly (0-350 μg/mL) on the viability of HeLa cells. The viability of the cells without g-C_3N_4 nanosheets is defined as 100%. The results are the means ± SD of three experiments.

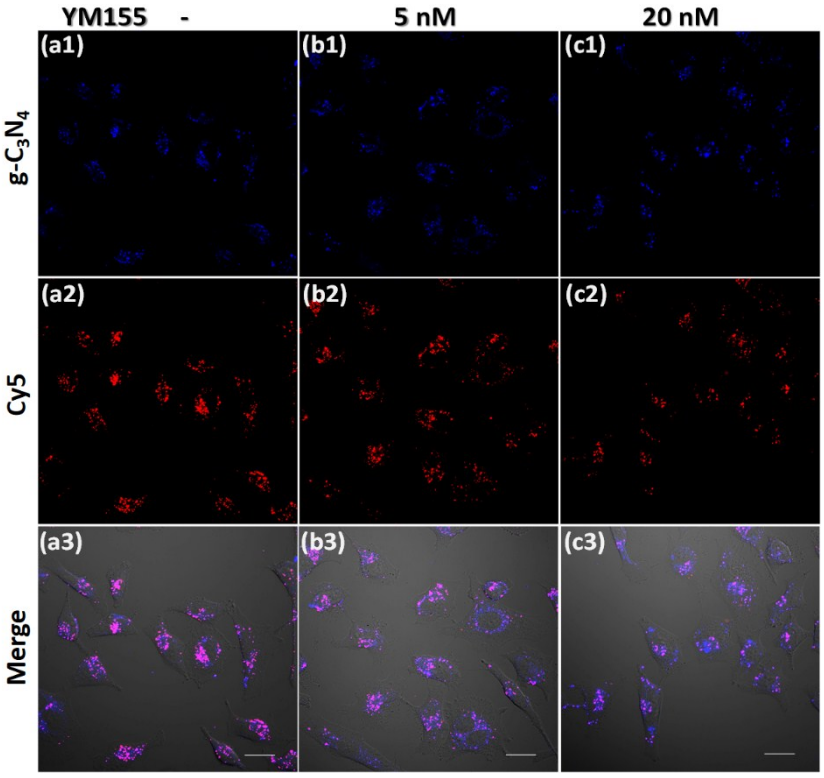

Fig. S5. Fluorescence and DIC overlay images of HeLa cells after being treated with the survivin suppressant (YM155) and HP1/HP2@g-C_3N_4 nanoassembly. (a1, a2, a3) Cells treated with HP1/HP2@g-C_3N_4 nanoassembly for 6 h. (b1, b2, b3) Cells treated with YM155 (5 nM) for 24 h and subsequently incubated with HP1/HP2@g-C_3N_4 nanoassembly for 6 h. (c1, c2, c3) Cells treated with YM155 (20 nM) for 24 h and subsequently incubated with HP1/HP2@g-C_3N_4 nanoassembly for 6 h. Scale bars are 25 μm.

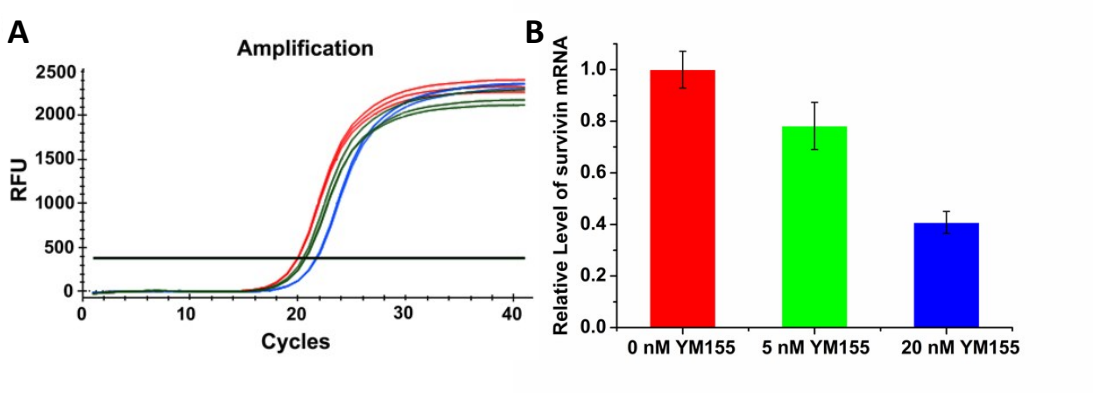

Fig. S6. (A) RT-PCR analysis for HeLa cells treated with the survivin suppressant (YM155). 0 nM YM155 (red line), 5 nM YM155 (green line), 20 nM YM155 (blue line) (B) Calculated relative expression levels of survivin mRNA in HeLa cells treated with the inhibitor (YM155) for 24 h.

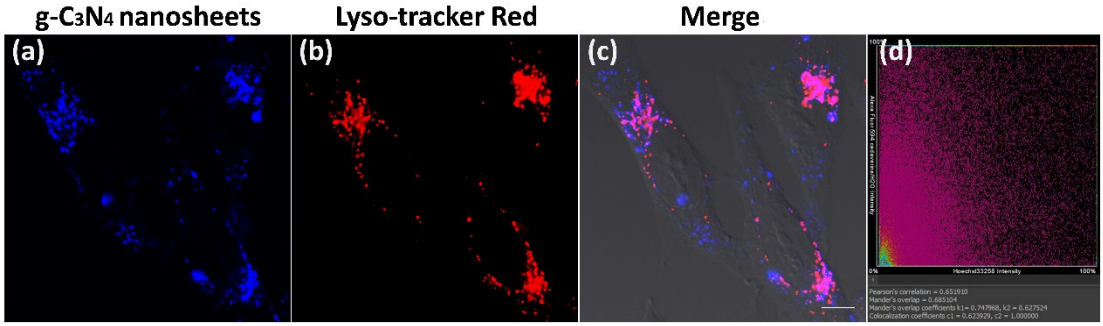

Fig. S7. Colocalization study for transfected HeLa cells with LysoTracker. HeLa cells were first incubated with HP1/HP2@g-C_3N_4 nanoassembly (140 μg/mL) for 6 h (a), and then incubated with 100 nM Lyso-Tracker Red (b) for 15 min. Scale bar =10 μm. The third panel represents fluorescence overlaid with the bright-field image. (d) The scatter plot analysis of co-localization in fluorophore between Lyso-Tracker Red and g-C_3N_4 nanosheets.

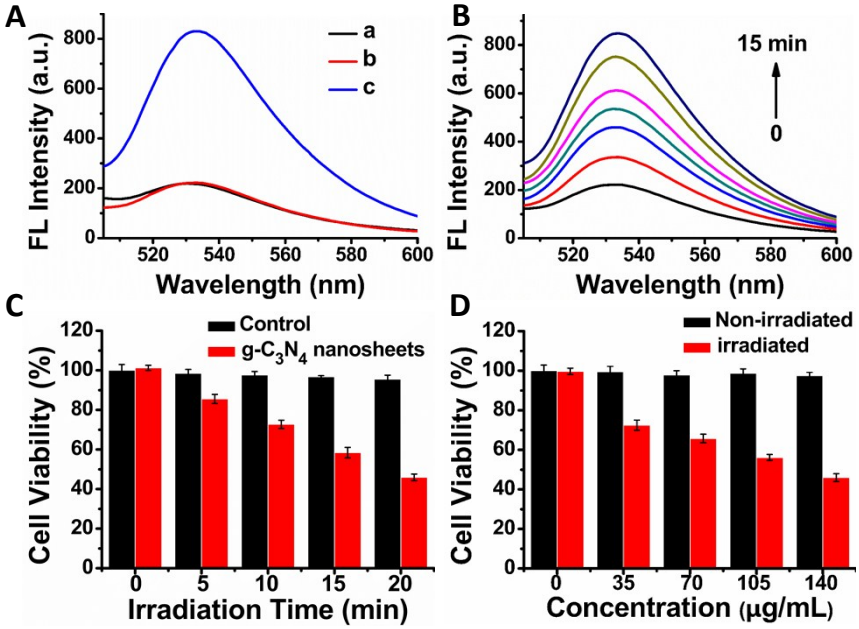

Fig. S8. (A) The fluorescence signal of singlet oxygen sensor green (SOSG) for $^1O_2$ monitoring. (a) 1μM SOSG with LED light for 15min, (b) 1μM SOSG + g-C_3N_4 nanosheets without light, (c) 1μM SOSG + g-C_3N_4 nanosheets with LED light (10 mW cm^{-2}) for 15 min (B) The fluorescence signal of singlet oxygen sensor green (SOSG) for $^1O_2$ monitoring when g-C_3N_4 nanosheets irradiated by LED light (10 mW cm^{-2}) for different time. (C) Viability of HeLa cells incubated with 140 μg/mL g-C_3N_4 nanosheets for different irradiation time intervals (LED light power density: 20 mW cm^{-2}). (D) Concentration dependent cell viability of HeLa cells incubated with g-C_3N_4 nanosheets (LED light power density: 20 mW cm^{-2}; irradiation time: 20 min).

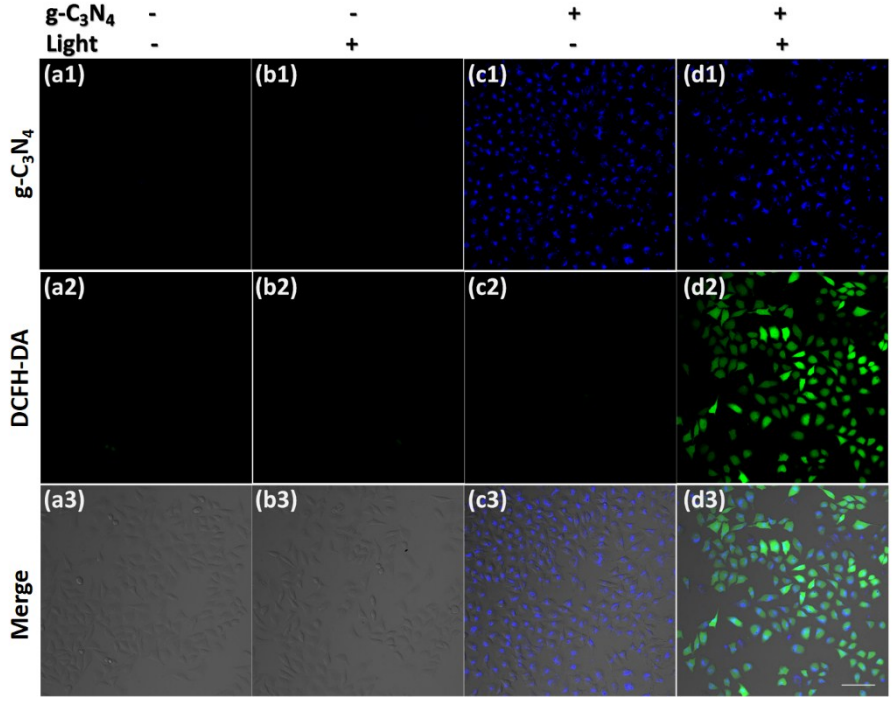

Fig. S9. Fluorescence and DIC overlay images of LED light-triggered ROS generation of HeLa cells after treating with HP1/HP2@g-C_3N_4 nanoassembly. (a1, a2, a3) Cells stained with ROS green fluorescence probe DCFH-DA alone for 30 min. (b1, b2, b3) Cells stained with DCFH-DA for 30 min and subsequently treated with LED light irradiation for 10 min. (c1, c2, c3) Cells incubated with HP1/HP2@g-C_3N_4 nanoassembly (140 μg/mL) for 6 h and subsequently stained with DCFH-DA for 30 min. (d1, d2, d3) Cells incubated with HP1/HP2@g-C_3N_4 nanoassembly (140 μg/mL) for 6 h and subsequently stained with DCFH-DA for 30 min followed by LED light irradiation for 10 min. (LED light irradiation: λ=450 nm; Power density: 20 mW cm^{-2}; irradiation time: 10 min.) Scale bars are 100 μm.

Reference:

1. Y. Zeng, C. Liu, L. Wang, S. Zhang, Y. Ding, Y. Xu, Y. Liu, S. Luo, *J. Mater. Chem. A*, 2016, **4**, 19003-19010.