Supporting Information

pH and Thermal Dual-Responsive Graphene Oxide Nanocomplexes for Targeted Drug Delivery and Photothermal-Chemo/Photodynamic Synergetic Therapy

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1.1 Materials.

Graphite powder, sulfuric acid (98%), sodium nitrate and potassium permanganate were obtained from Merck Chemical Co. (Germany). Doxorubicin hydrochloride (DOX.HCl) was purchased by Macklin Biological Co. Ltd. Methylene blue (MB), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ADMA), folic acid (FA), dimethyl sulfoxide (DMSO), 2',7'-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. Chloroacetic acid was obtained from Shanghai J&K Scientific Co., Ltd. N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) were provided by Aladdin. An acridine orange (AO)/ethidium bromide (EB) staining kit was purchased from Shanghai Sangon Biotech Co., Ltd. Rabbit red blood cells (RBC, 4%, v/v) were obtained from Shanghai Ming Jing Biological Technology Co., Ltd. All other solvents and chemicals were of analytical grade and used as received. The pure water used in all the experiments was produced by a Milli-Q-purification system (Millipore, USA).

1.2 Preparation and Carboxylation of NGO

GO was synthesized from pristine graphite power by a modified Hummers' method according to our previous report.[36] One gram of graphite powder and 25 mL of H_2SO_4 were placed in a 250 mL three-necked flask, and the mixture was stirred for 1 hour in an ice water bath by electromagnetic stirring to achieve a uniform mixture. Then, 0.5 g of $NaNO_3$ was added, and the mixture was further reacted in the ice water bath for 2

h. Then, 4 g of $KMnO_4$ was slowly added into the pre-oxidized product, and the reaction was continued for 2 h in the ice water bath. Thereafter, the flask was carefully transferred to an oil bath and heated at 38 °C for 0.5 h, and then, 50 mL of deionized water was slowly added to the mixture. After, the mixture was further stirred and reacted for 20 min at 98 °C in the oil bath, and a solution containing 30 mL of deionized water and 5 mL of H_2O_2 was added to terminate the reaction. The resulting solution was filtrated through a 0.22 μm membrane, and the GO solid was washed separately with 0.5 M hydrochloric acid and distilled water and finally resuspended to obtain the GO suspension. Finally, the GO suspension was treated by an ultrasonic cell crusher (Scientz-IID, Ningbo, China) at a power of 630 W for 3 h to obtain the NGO.

The carboxylated graphene oxide was synthesized using a method based on previous reports.[38-39] The previously obtained NGO was dispersed and ultrasonicated in distilled water to create a uniformly dispersed solution. Then, 2.4 g of NaOH and 2.0 g of chloroacetic acid were added into the aqueous NGO suspension (1 mg mL^{-1}, 10 mL), and the resulting suspension was sonicated for 2 h to assure the conversion of the hydroxyl groups (-OH) on the surface of GO to epoxy groups (-CH(O)CH-). The resulting suspension was first purified by dialysis at room temperature for 24 h to obtain the intermediate product, denoted as NCGO, which has greater water solubility and more carboxylic acid groups available for subsequent conjugation.

1.3 Preparation of NCGO-FA.

A certain amount of NHS, EDC and NCGO were mixed and ultrasonicated to

obtain a uniform solution. Then, 20 mL of a folic acid solution (0.5%, pH=8.0 in $NaHCO_3$ solution) was added into the mixture, and the resulting mixture was stirred overnight at room temperature. The final suspension was purified by dialysis for three days to remove excess NHS, EDC, and unreacted folic acid.

1.4 Characterizations of NGO, NCGO and NCGO-FA.

The morphologies of the NGO nanosheets were studied using a transmission electron microscope (TEM, JEM-1230EX, Japan). The infrared spectra of the NGO, NCGO, NCGGO-FA and FA were acquired using a Fourier transform infrared (FT-IR) spectrophotometer (Bruker Tensor 27, Germany) in the range from 4000 to 500 cm^2. The X-ray diffraction (XRD) patterns of the NGO, NCGO and NCGGO-FA were carried out using a Rigaku Ultima IV diffractometer with Cu Kα radiation, and the patterns were collected from 5° to 80° 2θ. Raman spectra were obtained using a JY Horiba HR-800 spectrophotometer from 1000 to 2000 cm^{-1} at room temperature. In addition, X-ray photoelectron spectroscopy (XPS) was performed on a Perkin-Elmer PHI 5900 spectrometer by using Al Kα radiation.

1.5 Cell Culture.

Human cervical cancer cell lines (HeLa cells) and human breast adenocarcinoma cell lines (MCF-7 cells) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The HeLa cells and MCF-7 cells were dispersed in DMEM containing 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin) and cultured in an incubator containing 5% CO_2 at a temperature of 37 ℃.

1.6 Evaluation of Cellular Uptake.

To image the intracellular drug distribution and cellular uptake behavior of the NCGO@DOX-FA and NCGO@MB-FA complexes, the HeLa and MCF-7 cells (with a density of 1×10^5 cells/well) were placed into a 35-mm glass-bottomed petri dish and cultured overnight to ~80% confluence. Subsequently, the original culture medium was removed, and the cells were carefully washed twice with PBS. Then, 2 mL of fresh culture medium containing NCGO@DOX-FA and NCGO@MB-FA were added to the petri dish, and the cells were further incubated for 6 h at 37 °C. Afterward, the culture medium containing the nanomaterial was removed again, the cells were carefully washed three times with PBS buffer solutions, and the cells were then co-incubated with DAPI (100 ng mL^{-1}) for 30 s at 25 °C in the dark to stain the cell nuclei. Finally, the cells were, again, carefully washed three times with PBS buffer solution, and then, the cellular uptake and intracellular distribution were qualitatively observed by a confocal laser scanning microscope (CLSM, C2si, Nikon).

1.7 *In vitro* Cell Cytotoxicity Assay.

An MTT assay was used to evaluate the cytotoxicity of NGO, NCGO and NCGO-FA. The HeLa and MCF-7 cells (1×10^4 cells per well) were placed into 96-well plates and incubated overnight to allow cell attachment. Then, the original culture medium was withdrawn and replaced with fresh medium containing NGO, NCGO and NCGO-FA at various concentrations (10, 20, 50, 100 and 200 μg mL^{-1}), and the wells were incubated for 24 h. Afterwards, 20 μL of an MTT (5 mg mL^{-1} dissolved in PBS) solution was added into each well. After 4 h of incubation, 150 μL

of dimethyl sulfoxide (DMSO) was added to each well to dissolve the bluish-violet formazan crystals, and the absorbance of the medium solution at a wavelength of 492 nm was measured by a microplate reader (Beijing Putian New Bridge Technology Co., Ltd, Beijing, China).

In addition, the synergetic antitumor efficiency of the NCGO@DOX-FA and NCGO@MB-FA complexes was also evaluated by an MTT assay with HeLa and MCF-7 cells. After the HeLa and MCF-7 cells were incubated overnight, the original culture medium containing dead cells was removed, and fresh medium containing various concentrations of the NCGO@DOX-FA and NCGO@MB-FA complexes was added. After further incubation for 16 h, the cells were subjected to a PTT treatment (808 nm-irradiation at 1.5 W cm^{-2} for 10 min) and a combined PTT and PDT treatment (808 nm-irradiation at 1.5 W cm^{-2} for 5 min + 660 nm-irradiation at 50 mW cm^{-2} for 5 min), and then, these cells were further incubated at 37 °C for 8 h. Afterwards, 20 μL of an MTT solution was added to each well. After incubation for 4 h, 150 μL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the bluish-violet formazan crystals, and the absorbance of the medium solution at a wavelength of 492 nm was measured by a microplate reader.

1.8 Intracellular Reactive Oxygen Species (ROS) Detection.

The HeLa and MCF-7 cells were placed into 35 mm culture dishes at a density of 5×10^4 cells/dish and incubated overnight. Then, the original culture medium containing dead cells was removed, and the cells were carefully rinsed three times with PBS. Fresh medium containing NCGO@MB-FA (50 μg mL^{-1}) was added to the

petri dishes, and the cells were further incubated for 24 h at 37 °C. Afterward, the cells were irradiated by a 660 nm-laser at a power of 50 mW cm^{-2} for 10 min or not. Subsequently, 10 μM (final concentration) of a DCFH-DA solution was added to each dish, and the cells were stained at 37 ℃ for 30 min in the dark. After that, the cells were carefully washed three times with PBS, and the fluorescence signals of the DCF inside the cells were recorded by a confocal laser scanning microscope. As abovementioned, the cells were centrifuged and washed to obtain a PBS suspension of the cells, and then the intracellular average fluorescence intensity was measured by flow cytometry (BD Accuri C6, USA).

1.9 Efficacy of the Synergetic Therapy Determined by Visual Observation.

The HeLa and MCF-7 cells (2×10^4 cells/dish) were seeded into a 35 mm dish and incubated overnight to allow cell attachment. Next, the cells were incubated with either 50 μg mL^{-1} NCGO@DOX-FA or 50 μg mL^{-1} NCGO@MB-FA for 16 h, and the cells were subjected to a PTT treatment (808 nm-irradiation at 1.5 W cm^{-2} for 10 min), or a combined PTT and PDT treatment (808 nm-irradiation at 1.5 W cm^{-2} for 5 min + 660 nm-irradiation at 50 mW cm^{-2} for 5 min), or not. After that, the cells were further incubated at 37 °C for an additional 8 h after the laser treatment. Subsequently, the cells were carefully washed three times with PBS, and AO/EB was added to stain the live and dead/apoptotic cells. Then, the cells were observed by a confocal laser scanning microscope to analyze cell apoptosis.

1.10 Hemolysis Assay.

The RBC (2%, v/v) suspensions were obtained after the cells were washed with

phosphate buffer saline and centrifuged five times. After that, 0.6 mL of the blood erythrocyte suspension was mixed with 0.4 mL of PBS (used as a negative control), 0.4 mL of deionized water (used as a positive control) and 0.4 mL of the complex suspensions at various concentrations (0.01, 0.05, 0.1, 0.25 and 0.5 mg mL^{-1}), and then, the mixtures were incubated at 37 ℃ in a water bath and maintained for 2 h. Subsequently, all the mixtures were centrifuged at 2500 rpm for 5 min, and the absorbance of the upper supernatants was measured by a UV-vis spectrometer at a wavelength of 540 nm. Herein, the hemolytic ratio (HR) was calculated as follows:

$$\mathrm{HR}\ (\%) = \frac{A_{sample} - A_{negative}}{A_{positive} - A_{negative}} \times 100\%. \quad (5)$$

The data are presented as the mean ± the standard deviation (SD), and the measurement was repeated in triplicate for each of the samples.

Table S1. The statistical analysis of the fluorescence microscopy images (Figure 12) by Image J

	Live cell	**Apoptotic cell**		**Live cell**	**Apoptotic cell**
A1	100%	0	**B1**	100%	0
A2	47.80%	52.20%	**B2**	50.00%	50.00%
A3	38.64%	61.36%	**B3**	49.95%	50.05%
A4	21.67%	78.33%	**B4**	30.77%	69.23%
A5	29.60%	71.40%	**B5**	30.06%	69.93%

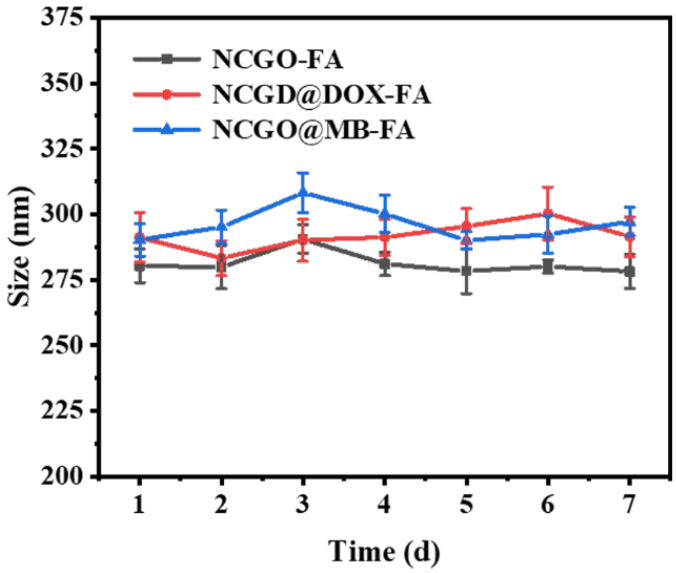

Figure S1. The sizes of NCGO-FA, NCGO@DOX-FA and NCGO@MB-FA dispersing in water for 7 days (SD, n=3).

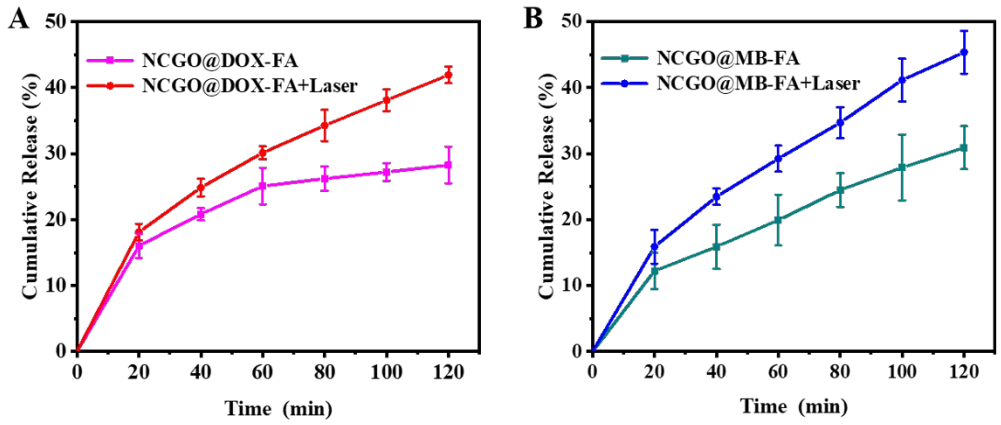

Figure S2. The drug release profiles of (A) NCGO@DOX-FA and (B) NCGO@MB-FA complexes with and without NIR laser irradiation (SD, n=3).

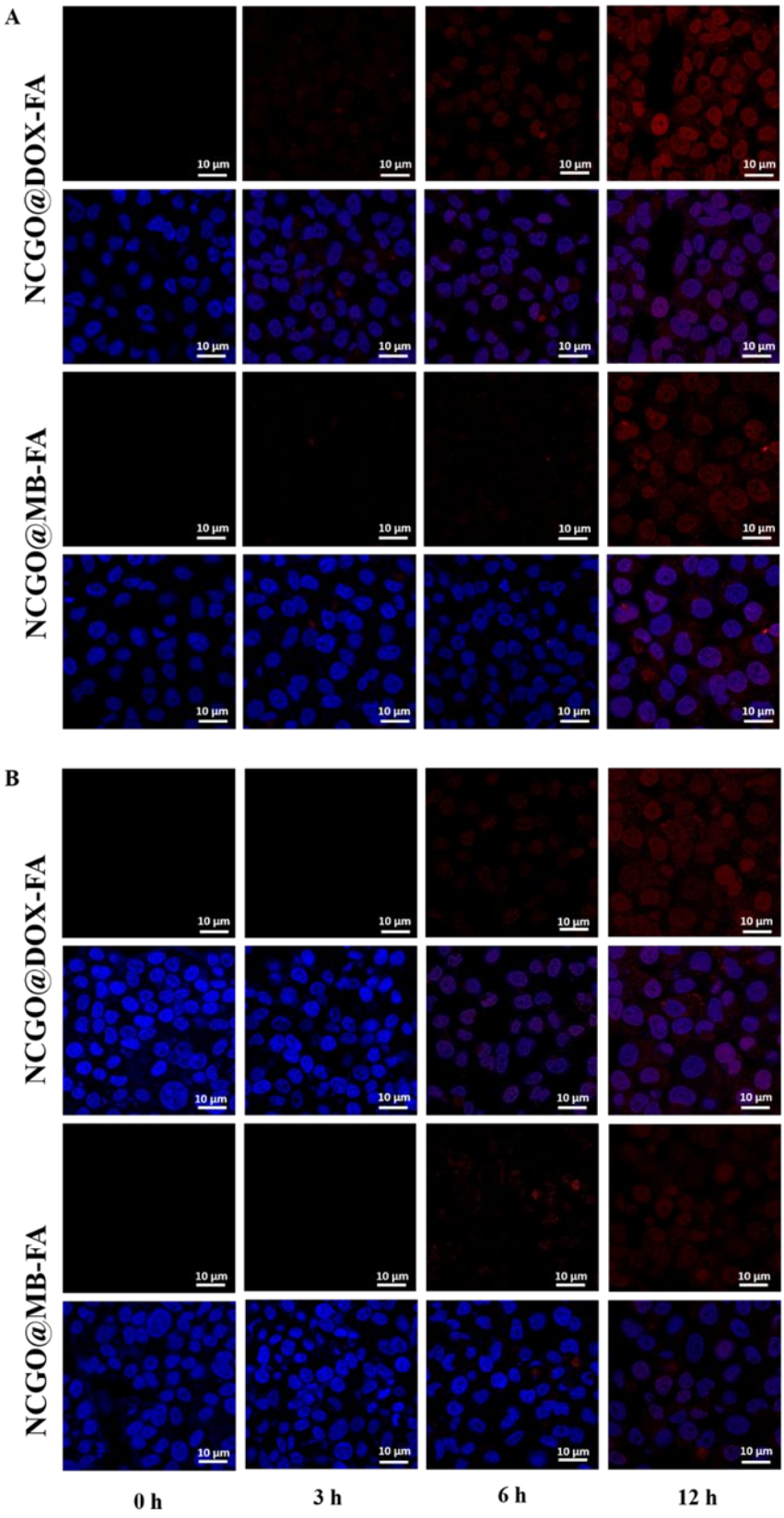

Figure S3. Fluorescence microscopy images of (A) the HeLa cells and (B) the MCF-7 cells after incubation with NCGO@DOX-FA or NCGO@MB-FA nanoplatforms (20 μg mL^{-1}) for 0, 3, 6 and 12 h.