

**Supplementary Information**

**How functional groups to influence ROS generation and cytotoxicity of graphene quantum dots**

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**Experimental Section**

**Materials.** Graphite was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Phenylhydrazine, benzoic anhydride and 2-bromo-1-phenylethanone were obtained from Sigma-Aldrich. Sulfuric acid, nitric acid, NaOH, and Na\textsubscript{2}CO\textsubscript{3} were purchased from Beijing Chemicals (Beijing, P.R. China). Dialysis bags (retained molecular weight: 1000 Da) were ordered from Shanghai Sangon Biotechnology Development. Ultrapure water was prepared by using a Milli-Q-Plus system (18.2 MΩ cm) and was used in all experiments. All reagents were used as received without any further purification.

**Synthesis of GQDs.** GQDs were prepared according to previously published methods,\textsuperscript{1} with some modifications. Specifically, 5 g of graphite was mixed with 80 mL of fuming HNO\textsubscript{3}, followed by adding 240 mL of H\textsubscript{2}SO\textsubscript{4} (98%). This mixture was
sonicated for 3 h and stirred for 48 h at 110 °C. After cooling to room temperature, the mixture was placed under mild ultrasonication for few minutes, and the pH was adjusted to 6 with Na$_2$CO$_3$ and NaOH in an ice bath. The suspension was filtered through a 0.22 μm microporous membrane to remove the large tracts of GO, and a deep yellow solution (yield ca. 30%) was separated. The mixture was further dialyzed in a dialysis bag, and greenish-yellow fluorescent GQDs were obtained.

**Chemical titration of oxygen functional groups on GQDs.** The titration of carbonyl groups on GQDs was prepared as followed. 1 g of phenylhydrazine (PH, 99%) and 50 μL of HCl acid (38 %) were dissolved in 50 ml CHCl$_3$, and then 100 mg of GQDs was added into the solution. After stirring under N$_2$ protection for 72 h, the precipitate was filtered out and washed with CHCl$_3$ in Soxhlet extractor for 20 h to remove the physical adsorbed PH molecules until the PH molecules couldn’t be detected in the supernatant by UV-Vis spectra (Fig. S16). The precipitate is dried in vacuum at 60 °C for 24 h to give yellow-brown GQD derivative (GQDs-PH)$_2$.

The titration of phenol groups on GQDs was prepared as followed. 5 g of benzoic anhydride (BA, 98%) and 100 mg of GQDs was dissolved in 50 ml of CHCl$_3$. After stirring under N$_2$ protection at 60 °C for 24 h, the precipitate was filtered out and washed with CHCl$_3$ to remove the physical adsorbed BA molecules until the BA molecules couldn’t be detected in the supernatant by UV-Vis spectra (Fig. S16). The precipitate is dried in vacuum at 60 °C for 24 h to give yellow-brown GQD derivative (GQDs-BA)$_2$.

The titration of carboxylic acid groups on GQDs was prepared as followed. 2 g of
2-bromo-1-phenylethanone (BrPE, 99%) and 100 mg of GQDs was dissolved in 50 ml of CHCl₃. After stirring under N₂ protection at room temperature in dark for 5 h, the precipitate was filtered out and washed with CHCl₃ to remove the physical adsorbed BrPE molecules until the BrPE couldn’t be detected in the supernatant by UV-Vis spectra (Fig. S16). The precipitate is dried in vacuum at 60 °C for 24 h to give yellow-brown GQD derivative (GQDs-BrPE).²

**Apparatus and characterization.** UV absorbance measurements were carried out on a JASCO V-550 UV-vis spectrophotometer, equipped with a Peltier temperature control accessory. Fluorescence spectra were measured on a JASCO FP-6500 spectrofluorometer equipped with a temperature-controlled water bath. All spectra were recorded in a 1.0 cm path length cell. FT-IR characterization was carried out on a BRUKE Vertex 70 FT-IR spectrometer. The samples were thoroughly ground with exhaustively dried KBr. AFM measurements were performed using Nanoscope V multimode atomic force microscope (Veeco Instruments, USA). TEM images were recorded using a FEI TECNAI G2 20 high-resolution transmission electron microscope operating at 200 kV. Zeta potential measurements were performed on a Nano-ZS Zetzsozer ZEN3600 instrument (Malvern Instruments Ltd., UK). XPS measurement was performed on an ESCALAB-MKII spectrometer (VG Co., United Kingdom) with Al Kα X-ray radiation as the X-ray source for excitation. Fluorescence lifetime was measured with a time-resolved spectrofluorometer (FLSP920, Edinburgh Photonics) under the excitation of a 375 nm laser.

**Determination of ROS formation from photoexcited GQDs.** Experiments were
carried out using 200 μg mL⁻¹ GQDs or GQD derivatives in a reaction volume of 3 mL buffer solution (25 mM Na₂HPO₄, pH 7.0, r.t.) with 2.5 mM ABTS as substrate. The mixture was exposed to light (450 nm, 1 W cm⁻²) for a series of time and the absorption of ABTS at 417 nm was measured. For DCFH-DA, 0.5 mL of DCFH-DA in DMSO was chemically hydrolyzed to DCFH with 2 mL of 0.01 M NaOH in the dark for 30 min at room temperature. The reaction was stopped by adding 10 mL of the phosphate buffer (25 mM Na₂HPO₄, pH 7.0). The stock solution of DCFH was wrapped by aluminum foil and kept on ice before use. ¹ 700 μg mL⁻¹ GQDs or GQD derivatives and 10 μM DCFH were added to 500 μL buffer solution (25 mM Na₂HPO₄, pH 7.0, r.t.), then the mixture was exposed to light or stay in dark for 5 min and the fluorescence intensity of DCF was measured.³

**Photostability comparison of GQDs, GQDs-PH, GQDs-BA, and GQDs-BrPE.** To guarantee a reliable comparison, the photoluminescence (PL) intensity of GQDs, GQDs-PH, GQDs-BA, and GQDs-BrPE was adjusted to the same value. The excitation wavelengths were 330, 330, 330 and 333 nm for GQDs, GQDs-PH, GQDs-BA, and GQDs-BrPE, respectively. The emissions at 446, 448, 445 and 446 nm were recorded by using a JASCO FP-6500 spectrofluorometer equipped with a temperature-controlled water bath.⁴

**Cell culture.** HeLa cells were cultured in 25 cm² flasks in Dulbecco’s modified eagle medium (DMEM; Gibco) containing 10% (v/v) fetal bovine serum (Gibco) at 37 °C in an atmosphere of 5% (v/v) CO₂ in air. The media were changed every 24 h and the cells were passaged by trypsinization before confluence.
Intracellular Determination of ROS. The generation of reactive oxygen radicals was monitored using DCFH-DA, a nonfluorescent compound that reacts with intracellular free radicals and generates the fluorescent product dichlorofluorescein (DCF). The intensity of the DCF fluorescence correlates with the amount of intracellular reactive oxygen radicals. To perform the experiment, 100 μg mL⁻¹ GQDs solution was added to the Hela cells for 6 h, then 20 μM DCFH-DA was added and the mixture was incubated at 37 °C for 30 min. Controls were cultivated under the same conditions without the addition of the GQDs. After exposed to laser for 5 min or not exposed to laser treatment the cells were washed twice with PBS solution, and finally the fluorescence intensity was monitored by flow cytometric analysis and a fluorescence spectrofluorometer.

Cell Toxicity Assays. For the methylthiazolyl tetrazolium (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) assay, cells were plated at a density of 10,000 cells per well on 96-well plates for 24 h, followed by the introduction of a series of concentrations of GQDs, GQDs-PH, GQDs-BA and GQDs-BrPE for 6 h. After which the cultures were either exposed to laser (450 nm 1 W cm⁻²) for 5 min or not exposed to laser treatment. After irradiation, all groups of cells were incubated for another 24 h. Then the cells were treated with 10 μL MTT (5 mg mL⁻¹ in PBS) for 4 h at 37 °C and then lysed in DMSO for 10 min at room temperature in the dark. Absorbance values of formazan were determined at 570 nm with an automatic plate reader.

Cellular uptake efficiency. 100 μg mL⁻¹ GQDs or GQD derivatives solution was
added to the Hela cells and incubated at 37 °C for 6 h, the supernatant for cells was obtained. The PL intensity of the supernatant was measured and the concentration of GQDs or GQD derivatives in the supernatant was calculated by the concentration dependent PL intensity curve. The cellular uptake efficiency was decided to be the difference between origin concentration (100 μg mL⁻¹) and supernatant concentration of GQDs.

**Cell images.** Hela cells were seeded in 24-well plates and incubated at 37 °C in a 5% CO₂ atmosphere for 24 h. Next, the culture medium was replaced with fresh complete medium either with GQDs (200 μg mL⁻¹) or without them, after which the cultures were either exposed to laser for 5 min or not exposed to laser (450 nm 1 W cm⁻²) treatment. After irradiation, all groups of cells were incubated for another 24 h. Calcein/propidium iodide (PI) dye mix in PBS was then added to the cells, followed by 10 min of incubation in the dark. The cells were then washed twice with PBS and viewed with an Olympus BX-51 optical system microscope (Tokyo, Japan) with a blue filter. Pictures were taken with an Olympus digital camera.

**Quantum Yield (QY) Measurements.** Quinine sulfate in water (QY = 0.577) was chosen as a standard. The quantum yields of GQDs, GQDs-PH, GQDs-BA and GQDs-BrPE in water were calculated according to the formula:

$$\Phi = \Phi_s (I/I_s) (A/A_s) (n_s/n)^4$$

Where $\Phi$ is the quantum yield, $I$ is the measured integrated emission intensity, $n$ is the refractive index of the solvent (1.33 for water), and $A$ is the optical density. The subscript “s” refers to the reference standard with known quantum yield. To minimize
reabsorption effects, absorbencies in the 10 mm fluorescence cuvette were kept under 0.1 at the excitation wavelength (GQDs and GQD derivatives at 330 nm).

**Fig. S1** Excitation and emission PL spectra of GQD derivatives and GQDs, the concentration of GQD derivatives and GQDs: 100 μg mL⁻¹.

**Fig. S2** PL intensity of GQD derivatives and GQDs obtained from their emission spectra.
Fig. S3 PL spectra of the (a) GQDs, (b) GQDs-PH, (c) GQDs-BA and (d) GQDs-BrPE at different excitation wavelengths. The concentration of GQD derivatives and GQDs: 100 μg mL⁻¹.

Fig. S4 Absorption spectra of GQD derivatives and GQDs, the concentration of GQD derivatives and GQDs: 50 μg mL⁻¹.
Fig. S5 TEM images of (a) GQDs, (d) GQDs-PH, (g) GQDs-BA and (j) GQDs-BrPE. HRTEM images of (b) GQDs, (e) GQDs-PH, (h) GQDs-BA and (k) GQDs-BrPE. The AFM images of (c) GQDs, (f) GQDs-PH, (i) GQDs-BA and (l) GQDs-BrPE, respectively. Insets of (c), (f), (i) and (l) are corresponding height profiles of GQDs,
GQDs-PH, GQDs-BA and GQDs-BrPE, respectively.

Fig. S6 Particle size distributions of (a) GQDs, (b) GQDs-PH, (c) GQDs-BA and (d) GQDs-BrPE; Height distributions of (e) GQDs, (f) GQDs-PH, (g) GQDs-BA and (h) GQDs-BrPE.

The typical transmission electron microscope (TEM) images showed that GQDs and GQD derivatives were well dispersed (Fig. S5a, d, g, j) with similar size distribution and the average size (Fig. S6). The typical atomic force microscopy (AFM) images showed their height distributions and the average heights of GQDs and GQD derivatives were also similar (Fig. S5 and S6). Furthermore, high-resolution TEM (HRTEM) images showed the four kinds of GQDs had crystallinity with lattice of 0.21 nm, which was consistent with the (102) diffraction planes of sp² graphitic carbon, and indicated that the four kinds of GQDs kept the similar crystallinity with graphene.4,6 Fig. S5 and S6 showed the dimension and height of GQDs had no obvious change, indicating that the change of ROS formation activity for GQD derivatives could be attributed to their chemical structural change rather than their morphology variation.
The results of Zeta potential measurements for GQD derivatives and GQDs.

Fig. S7

FT-IR spectra of GQD derivatives and GQDs.

The vibration band at 1848 cm\(^{-1}\) in the FT-IR spectrum of GQDs-PH could be assigned to the C=N bonds which formed during reacting with PH. For the GQDs-BA, a small peak at 1730 cm\(^{-1}\) could be observed owing to the formation of ester group. The FT-IR spectrum of GQDs-BrPE displayed increased vibrations at 2924 cm\(^{-1}\) and 2845 cm\(^{-1}\), which originated from the BrPE substituents.\(^{2b}\)
**Fig. S9** Luminescence decay curves of (a) GQDs, (b) GQDs-PH, (c) GQDs-BA and (d) GQDs-BrPE (excitation=375 nm, monitored at 480 nm).

**Fig. S10** PL spectra of the buffer solution (25 mM Na₂HPO₄, pH 7.0) include DCF alone; DCF and GQDs; DCF and GQDs-PH; DCF and GQDs-BA; DCF and GQDs-BrPE. The concentration of GQD derivatives and GQDs: 100 μg mL⁻¹.
Fig. S11 Cells were treated with different GQDs for 6 h and exposed to the visible light irradiation (1 W cm\(^{-2}\), 5 min), and ROS generation was measured using DCF fluorescence. Flow cytometry analysis used to monitor the changes of intracellular ROS: (a) control; (b) GQDs; (c) GQDs-PH; (d) GQDs-BA; (e) GQDs-BrPE; (f) Quantification of the changes of intracellular ROS.
Fig. S12 Cells were treated with different GQDs for 6 h and stay in dark for 5 min, and ROS generation was measured using DCF fluorescence. Flow cytometry analysis used to monitor the changes of intracellular ROS: (a) control; (b) GQDs; (c) GQDs-PH; (d) GQDs-BA; (e) GQDs-BrPE; (f) Quantification of the changes of intracellular ROS.
Fig. S13 Cells were treated with different GQDs for 6 h and flow cytometry analysis was then conducted to monitor the fluorescence of (a) GQDs, (b) GQDs-PH, (c) GQDs-BA and (d) GQDs-BrPE used the same method as DCFH-DA, (f) is the quantification of the changes of fluorescence. GQDs possessed almost no fluorescence emission in flow cytometer, indicating the emission from GQDs won’t cause background for the test.
Fig. S14 Cells were treated with different GQDs for 6 h, the PL spectra of supernatant for cells with the addition of (a) GQDs, (b) GQDs-PH, (c) GQDs-BA and (d) GQDs-BrPE. Concentration dependent PL intensity of (e) GQDs, (f) GQDs-PH, (g) GQDs-BA and (h) GQDs-BrPE.

Fig. S15 Fluorescence microscopy images of Hela cell with different conditions. Viable cells were stained green with calcein, dead cells were stained red with propidium iodide (PI). (Scale bars: 100 μm.)

Fig. S16 UV-Vis spectra of PH (50 μg mL\(^{-1}\)), supernatant of GQDs-PH (a), BA (50 μg mL\(^{-1}\)), supernatant of GQDs-BA (b), BrPE (62.5 μg mL\(^{-1}\)) and supernatant of GQDs-BrPE (c) in CHCl\(_3\).
Fig. S17 TEM images of (a) GQDs, (b) GQDs-PH, (c) GQDs-BA and (d) GQDs-BrPE after laser irradiation. The morphology of four kinds of GQDs was kept after irradiation.

Table S1 The content of the carboxyl, hydroxyl and ketonic carbonyl groups in GQDs, GQDs-PH, GQDs-BA and GQDs-BrPE.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ketonic carbonyl (%)</th>
<th>Hydroxyl (%)</th>
<th>Carboxyl (%)</th>
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<tbody>
<tr>
<td>GQDs</td>
<td>37.4</td>
<td>31.6</td>
<td>31.0</td>
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<tr>
<td>GQDs-PH</td>
<td>8.2</td>
<td>43.1</td>
<td>48.7</td>
</tr>
<tr>
<td>GQDs-BA</td>
<td>40.4</td>
<td>8.0</td>
<td>51.6</td>
</tr>
<tr>
<td>GQDs-BrPE</td>
<td>49.6</td>
<td>25.3</td>
<td>25.1</td>
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Table S2 Quantum Yield of GQD derivatives and GQDs.

<table>
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<tr>
<th>Sample</th>
<th>Integrated emission</th>
<th>Abs at 330 nm</th>
<th>Refractive index of solvent (n)</th>
<th>Quantum Yield (%)</th>
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<tbody>
<tr>
<td>Quinine sulfate</td>
<td>10647.8</td>
<td>0.0811</td>
<td>1.33</td>
<td>57.7</td>
</tr>
<tr>
<td>GQDs</td>
<td>551.666</td>
<td>0.08742</td>
<td>1.33</td>
<td>2.77</td>
</tr>
<tr>
<td>Sample</td>
<td>(\tau_1) (ns)</td>
<td>(\tau_2) (ns)</td>
<td>(\tau_3) (ns)</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>GQDs-PH</td>
<td>0.69</td>
<td>2.15</td>
<td>7.16</td>
<td></td>
</tr>
<tr>
<td>GQDs-BA</td>
<td>0.68</td>
<td>2.11</td>
<td>7.54</td>
<td></td>
</tr>
<tr>
<td>GQDs-BrPE</td>
<td>0.69</td>
<td>2.08</td>
<td>7.37</td>
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</table>

**Table S3** Fluorescence lifetime obtained from luminescence decay curves of GQDs and GQD derivatives in Fig. S9.

**Table S4** Cellular uptake efficiency of GQDs and GQD derivatives obtained from Fig. S14.

<table>
<thead>
<tr>
<th>Sample</th>
<th>GQDs</th>
<th>GQDs-PH</th>
<th>GQDs-BA</th>
<th>GQDs-BrPE</th>
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<tbody>
<tr>
<td>Cellular uptake ((\mu g) mL(^{-1}))</td>
<td>19.1</td>
<td>21.8</td>
<td>19.9</td>
<td>20.5</td>
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</table>
Reference:


