

***p*-Sulfonated Calix[6]arene modified Graphene as a 'Turn on' Fluorescent Probe for L-Carnitine in Living Cell**

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Experimental sections

Materials

All chemicals were A.R. grade and were purified by standard procedures. Graphite, hydrazine solution (≥ 85 wt %), and ammonia solution (25-28 wt %) was purchased from Sinopharm Chemical Reagent Beijing Co., Ltd. (SCRC, China). All solutions were prepared in MilliQ water.

Instruments.

The UV source was a 250 W high-pressure fluorescent Hg lamp with the strongest emission at 365 nm (Institute of Electrical Light Source, Beijing). Fluorescence spectra were taken on a FluoroMax-P luminescence spectrometer (HORIBA JOBIN YVON INC). The FT-IR spectra were detected by a Thermo Nicolet NEXUS IR spectrometer with KBr disks. The ultrasonic bath was a SB120D Supersonic instrument. The transmission electron microscopy images of graphene were measured by a Philips TecnaiG2 TEM using an accelerating voltage of 200 kV. Tapping mode atomic force microscopy (AFM) characterizations were conducted on a Nanoscope III (Digital Instrument) scanning probe microscope. X-ray photoelectron spectroscopic (XPS) images were gained by PHI Quantera SXM.

1. Preparation of Graphite Oxide.

GO was prepared by oxidation of natural graphite powder (325 mesh, Tianjing) according to the method developed by Hummers and Offemann.^[11] Briefly, native graphite flake (2 g) was mixed with concentrated H₂SO₄ (12 mL), K₂S₂O₈ (4 g), and P₂O₅ (4 g), and then incubated at 80 °C to preoxidize the graphite. The resultant dark blue mixture was thermally isolated and allowed to cool to room temperature over a period of 6 h. The mixture was diluted to 150 mL and then filtrated. The product was then dried in air at ambient temperature overnight, after washing with distilled water until neutral. This preoxidized graphite was then subjected to oxidation by Hammer's method. The preoxidized graphite powder (1 g) was added to 46 mL of cold H₂SO₄ (0 °C), and 6 g of KMnO₄ was gradually added under stirring in an ice bath with stirring while keeping the temperature of the mixture below 20 °C. After 1 g of NaNO₃ was added to the mixture stirred for 15 min, and the resulting mixture was further stirred at 35 °C for 2 h and distilled water (100 mL). The reaction was ceased by the addition of a large amount of distilled water (280 mL) and 5 mL of 30% H₂O₂ was added, after which the color of the mixture changed to bright yellow. The mixture was filtered and washed with 1:10 HCl aqueous solution (250 mL) to remove metal ions followed by washing with 200 mL of water to remove the acid. The resulting solid was dried in air and diluted to make a GO aqueous dispersion (0.5 mg/mL), which is stable for a period of years, was used to prepare exfoliated GO. The purified GO dispersion was sonicated for 1.5 h at 300W to exfoliate GO, and unexfoliated GO was removed by centrifugation (3000 rpm, 5min). Eventually, it was further purified dialysis for one

week to remove the remaining metal species. The GO solution was stable for a period of months and was used for film preparation.

2. Preparation of conjugation of *p*-Sulfonated Calix[6]arene with graphene.

p-sulfonated calix[6]arene was synthesized according to Shinkai's method¹. ¹HNMR was performed at ambient temperature in D₂O, which is in accordance with previous literature. To preparation of conjugation of *p*-sulfonated calix[6]arene modified graphene (CMG), the GO dispersion (0.5 mg/mL) 20.0 ml was mixed with 20 mg *p*-sulfonated calix[6]arene, 150.0 μL of ammonia solution, and 20.0 μL of hydrazine solution in a 100-ml glass vial. After being stirred for a few minutes, the vial was put in a water bath (95 °C) for 1 h. The obtained CMG dispersion was filtered with a nylon membrane (0.22 μm) to obtain water-soluble CMG complexes. Successively, CMG samples was treated by centrifugalization method and washed with water 3 times to remove the excess of CX6. The as-prepared CMG can be redispersed readily in water by ultrasonication. The obtained product was characterized by UV-visible spectra and X-ray photoelectron spectra (XPS) and atomic force microscopy (AFM).

3. Cell culture.

HepG2 cells were grown in dulbecco's modified eagles medium (DMEM, Invitrogen, USA) supplemented with 10% FBS. The cells were seeded in tissue culture plates and incubated in a fully humidified atmosphere at 37 °C containing 5% CO₂. For MTT assays, the cells were seeded in 96-well plates in 200 mL culture medium containing the CMG with different concentrations (3~51 μg/L) and maintained for 24 h. 20 mL

of 5 mg/mL MTT (Sigma-Aldrich, USA) solution was then added to each well of the 96-well plates. Following incubation for 4 h at 37 °C, the cells were lysed with dimethyl sulphoxide (DMSO; Sigma-Aldrich, USA) solution. Then the absorbance of the supernatants was measured at 490 nm using a microplate reader (Bio-Rad 680, USA).

4. Confocal fluorescence microscopy.

The cells were grown on glass coverslips placed at the bottom of 24-well culture plates. In this experiment, the medium was replaced with fresh medium containing the materials safranin T (0.06 mg/mL). After 1 h of treatment, the coverslips were removed, washed three times with phosphate-buffered saline (PBS), fluorescence images were taken (Figure 6A and D). HepG2 cells incubated with safranin T for 1 h, being washed with cold PBS, then incubated with CMG at a concentration of 0.5 mg/mL for 2 h and washed with PBS and observed under a fluorescence microscope (Figure 6B and E). Being treated with safranin T and CMG, cells was incubated in medium containing L-caritine(0.6 mg/mL), fluorescence images were taken with a confocal fluorescence microscope (Olympus, Japan) (Figure 6C and G). The same condition was applied when rhodamine B serve as indicator except cells incubated with rhodamine B (0.06 mg/mL) for 3 h.

5. Data Analysis and Fitting.

All fittings were performed in a nonlinear manner according to spectrofluorometric titrations.² For the direct host-guest titrations, the complexation process of the CMG

host (H) with the dye indicator (In) or competition substrates guest (G) was expressed by eq 1 and eq 2, respectively, according to a 1:1 host-guest binding stoichiometry:



where $\Delta\varepsilon$ denote molar extinction coefficient of the chromophoric CMG derivative upon inclusion complexation of the dyes, and K_d is dissociation constant for HIn, K_a' is the binding constant of host to indicator based on an directly binding model.

$$K_d = \frac{[\text{H}][\text{In}]}{[\text{H} \cdot \text{In}]} = \frac{1}{K_a'} = \frac{([\text{H}]_0 - \Delta F / \Delta\varepsilon)([\text{In}]_0 - \Delta F / \Delta\varepsilon)}{\Delta F / \Delta\varepsilon} \quad (3)$$

where ΔF the changes in the fluorescence intensity of the chromophoric CMG derivative upon inclusion complexation of the dyes. Solving the simultaneous equations, we obtained eq 4.

$$\Delta F = \frac{\Delta\varepsilon ([\text{H}]_0 + [\text{In}]_0 + K_d) \pm \sqrt{\Delta\varepsilon^2 ([\text{H}]_0 + [\text{In}]_0 + K_d)^2 - 4\Delta\varepsilon^2 [\text{H}]_0 [\text{In}]_0}}{2} \quad (4)$$

The initial and equilibrium concentrations were designated by a_0 and a for In, b_0 and b for G, c_0 and c for H, respectively. Since H, G, and HG are spectrophotometrically transparent in a visible region, the change ΔF in intensity of an dye with the addition of H and G is related to the equilibrium concentration ($a_0 - a$) of HIn by eq.5.

$$a_0 - a = \frac{\Delta F}{\Delta\varepsilon} \quad (5)$$

Where $\Delta\varepsilon$ is different from molar absorbance between H and In, similarly, a is given by

$$a = \frac{(\Delta F_\infty - \Delta F)}{\Delta\varepsilon} \quad (6)$$

Where ΔF_{∞} is different from molar absorbance between H and In ($\Delta F_{\infty} = \Delta \varepsilon$). The dissociation constant K_d for HIn is represented by $K_d = c \cdot a / (a_0 - a)$, so that the equilibrium concentration of H is given by

$$c = \frac{K_d(a_0 - a)}{a} \quad (7)$$

On the other hand, the dissociation constant (K_a) for HG is represented by

$$K_a = \frac{c_0 - c - (a_0 - a)}{c[b_0 - c_0 + c + (a_0 - a)]} \quad (8)$$

Since the value of $a_0 - a$ is very small compared with either $c_0 - c$ or $b_0 - c_0 + c$ under the experimental condition of $a_0 < b_0, c_0$, eq.8 is simplified as eq.9:

$$K_a = \frac{c_0 - c}{c(b_0 - c_0 + c)} \quad (9)$$

The value of K_d and $\Delta \varepsilon$ were easily determined, and introduce these values into eq.5, 6, 7, we obtained the value of c , which is in turn introduced into eq.9 to give the K_a value.

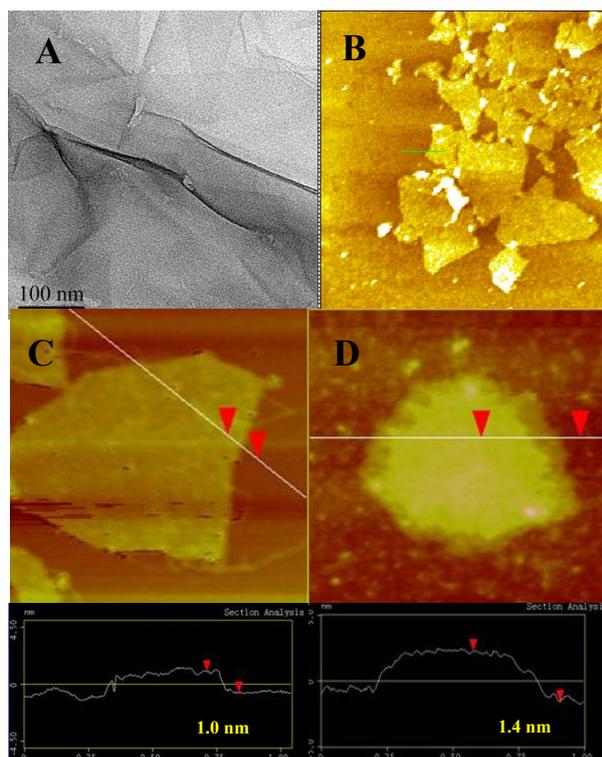


Figure S1. A) TEM images of GO; B),C) AFM images of GO, and D) CMG

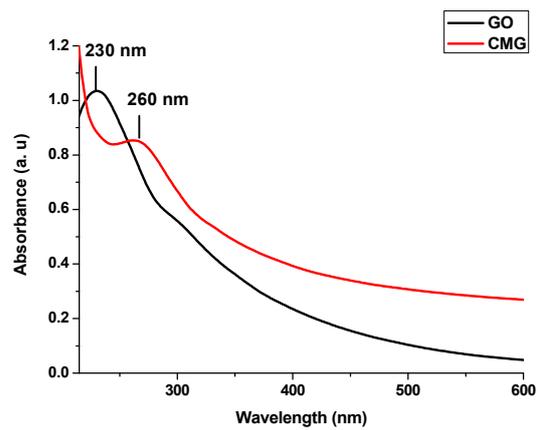


Figure S2. A) UV/Vis spectra of the GO (curve red) and CMG (curve black) in aqueous solution;

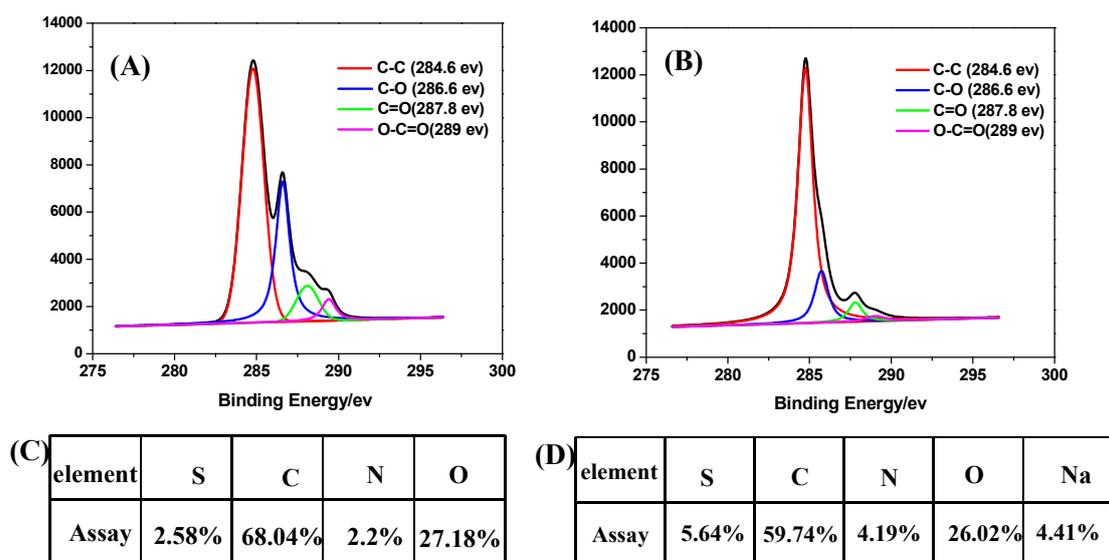


Figure S3. The C1s XPS spectra of (A) GO; and (B) CMG; Element content analysis of GO (C); and CMG (D).

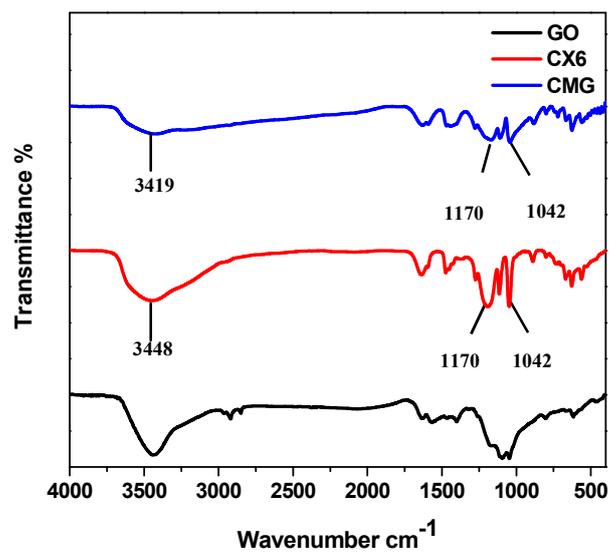


Figure S4. FT-IR spectra of the reduced graphene oxide (curve black), CX6 (curve red), and CMG (curve blue).

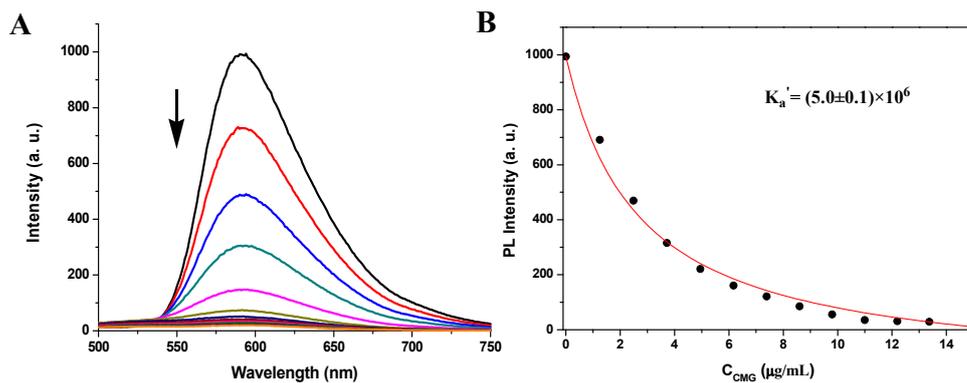


Figure S5. A) The effect of increasing concentrations of CMG (concentrations ranging from 0 $\mu\text{g/mL}$ to 13 $\mu\text{g/mL}$) on the fluorescence intensity of safranin T ($\lambda_{\text{ex}} = 588 \text{ nm}$) dispersion. Safranin T concentration is 10^{-4} M . B) The dependence of intensity values of safranin T on the increasing concentration of CMG, and the combined solution mixed well for 3 minutes then tested. All fittings were performed in a nonlinear manner according to directly complexation model (eq.4).

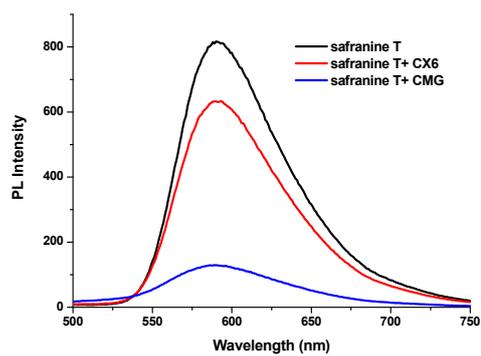


Figure S6. Fluorescence spectra of safranine T (10^{-4} M), and safranine T in the presence of 100 μ L of CX6 (10^{-3} M), CMG (0.5 mg/mL) in sequence.

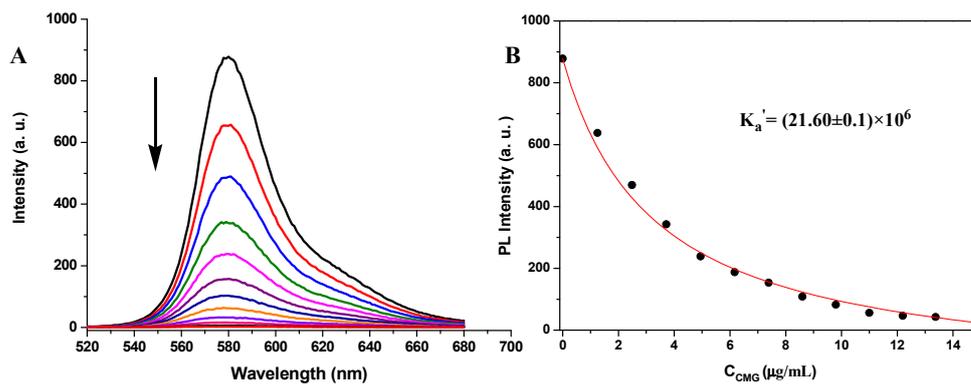


Figure S7. A) The effect of increasing concentrations of CMG (concentrations ranging from 0 $\mu\text{g/mL}$ to 13 $\mu\text{g/mL}$) on the fluorescence intensity of rhodamine B ($\lambda_{\text{ex}} = 577$ nm) dispersion. Rhodamine B concentration is 10^{-5} M, and the combined solution mixed well for 3 minutes then tested. B) The dependence of I values of rhodamine B on the increasing concentration of CMG based on nonlinear fitting using directly binding model (eq.4).

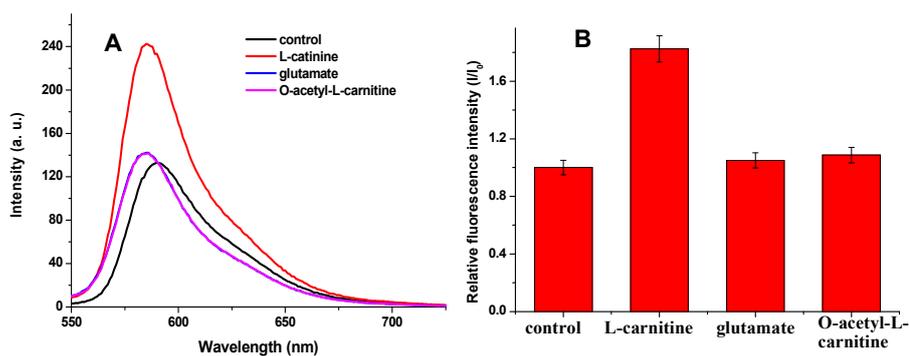


Figure S8. A) Fluorescence spectra of CMG/rhodamine B receptor solution without and with L-carnitine, glutamate, and O-acetyl-L-carnitine. B) Fluorescence ratio I/I_0 of CMG/rhodamine B receptor upon addition of L-carnitine, glutamate, and O-acetyl-L-carnitine.

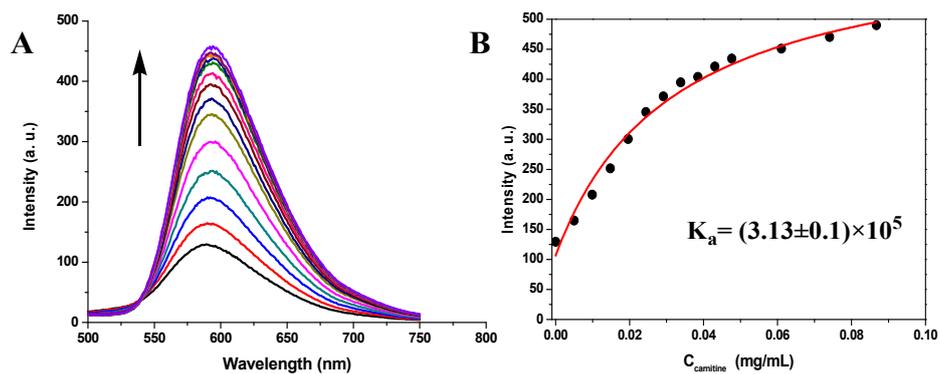


Figure S9.A) Fluorescence spectra of the CMG/safranin T complexes via different concentrations of L-carnitine (0~86 $\mu\text{g/mL}$). B) The increase in relative fluorescence intensity at the same CMG/safranin T concentration, fitted according to the competitive binding model (eq. 9). Successive fluorescence recovery was observed as the concentration of L-carnitine increased.

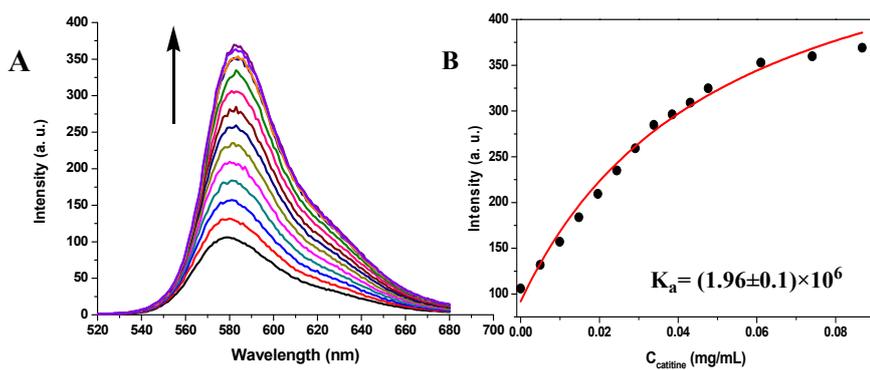


Figure S10. A) Fluorescence spectra of the CMG/rhodamine B complexes *via* different concentrations of L-carnitine (0~86 μ g/mL). B) Typical curve-fitting plots are shown, which based on nonlinear fitting relationship using a competitive binding model (eq. 9), and successive fluorescence recovery was found as the concentration of L-carnitine increased.

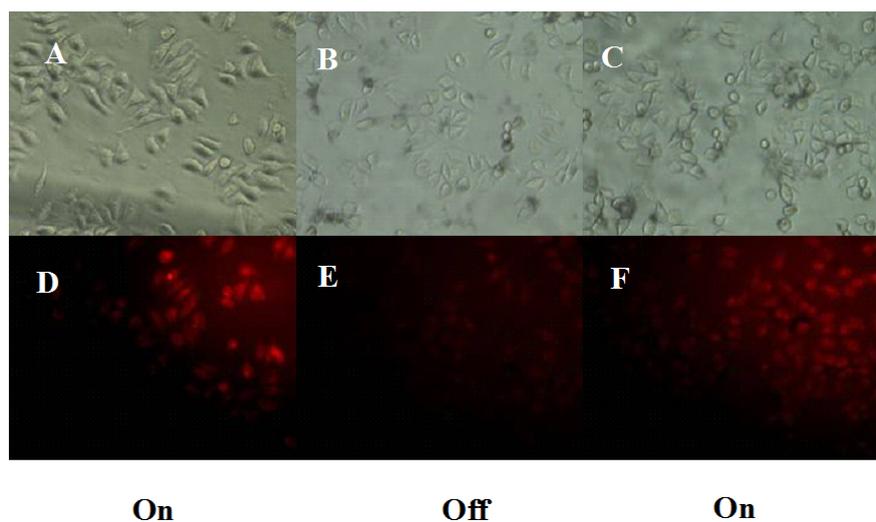


Figure S11. Cellular uptake of CMG hybrid shown by Bright-field (top) and wide-field fluorescence images (bottom). HepG2 cells were incubated with rhodamine B without CMG (A, D) or with CMG (B, E); and Rhodamine B, CMG, L-Carnitine (C, F) for 2~3 h at 37 °C. Images were obtained after extensive washing of cells with PBS.

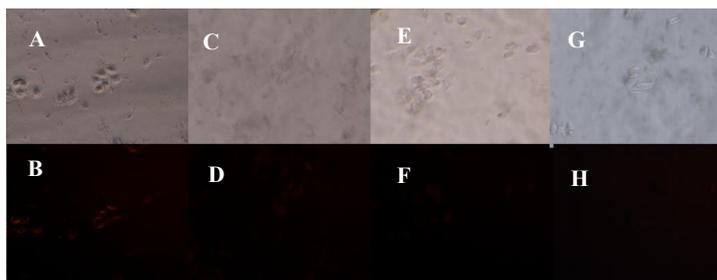


Figure S12. HepG2 cells, incubated with CMG/safranin T and then O-acetyl-L-carnitine (A, B), or incubated with CMG/safranin T and then glutamate (C, D), or incubated with CMG/rhodamine B and then O-acetyl-L-carnitine (E, F), or incubated with CMG/rhodamine B and then glutamate (G, H) was shown by confocal fluorescence microscopy images (bottom) and wide-field fluorescence images (top).

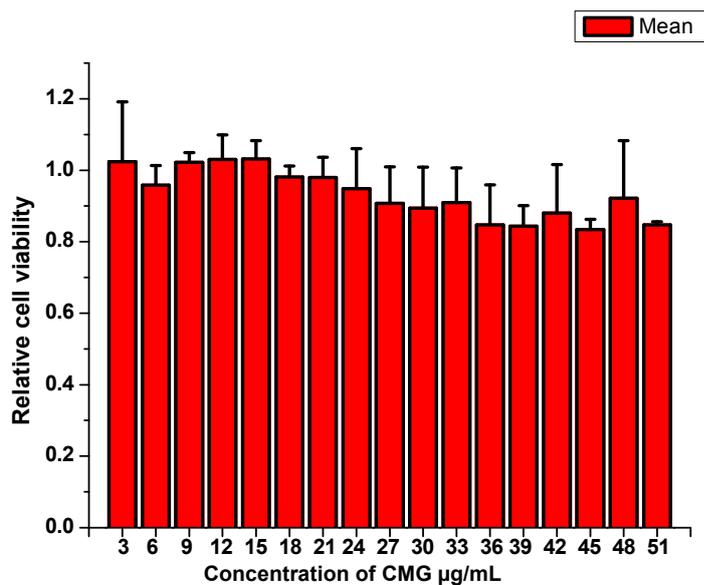


Figure S13. Relative cell viability of HepG2 treated with CMG. HepG2 cells were incubated with different concentrations of CMG for 24 h in fresh medium .

- 1 a) C. D. Gutsche, B. Dhawan, K. H. No, R. Muthukrishnan, *J. Am. Chem. Soc.* 1981, **103**, 3782–3792; b) S. Shinkai, S. Mori, H. Koreishi, T. Tsubaki, O. Manabe, *J. Am. Chem. Soc.* 1986, **108**, 2409–2416.
- 2 a) Y. J. Zhang, W. X. Cao and J. Xu, *Chin. J. Chem.*, 2002, **20**, 322–326; b) Y. Liu, B. H. Han, S. X. Sun, T. Wada, Y. Inoue, *J. Org. Chem.* 1999, **64**, 1487–1493; c) Y. Matsui, K. Mochida, *Bull. Chem. Soc. Jpn.*, 1979, **52**, 2808–2814.

