Supporting Information

Positively charged graphene oxide nanoparticle: precisely label the plasma membrane of live cell and sensitively monitor extracellular pH *in situ*

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S1. Materials and Preparation of GO and GO-PEG

I. Materials

$KMnO_4$ (A.R.), P_2O_5 (A.R.) , H_2SO_4 (A.R.), HCl (A.R.), H_2O_2 (30%, MOS level) were purchased from Beijing Chemical Works. Graphite powder, $K_2S_2O_8$ and Rhodamine 6G were obtained from Sigma-Aldrich. The carboxylic polyethylene glycol (PEG) was synthesized using a solid-phase method (Nanocs Inc. $M_r \approx 5000$). Cell culture medium RPMI1640 and fetal bovine serum (FBS) were purchased from Hyclone. Cell counting kit-8 (CCK-8) reagent was from DOJINDO Lab., Japan. All other materials were commercially available and used as received unless otherwise mentioned. Water with a resistivity of 18.2 MΩ·cm, used throughout the experiment, was purified with a Milli-Q system from Millipore Co., USA.

II. Preparations of positively charged GO by modified Hummers' method and negatively charged GO via normal Hummers' way, respectively

The positively charged GO was synthesized from natural graphite powder by the revised method of Hummers and Offeman[1]. Prior to the GO preparation, preoxidization of graphite was needed, in detail, the

graphite powder (1.5 g) was put into an 80 °C solution of concentrated H_2SO_4 (4 mL), $K_2S_2O_8$ (1 g), and P_2O_5 (1 g). After reacted for 2 h, the resultant dark blue mixture was allowed to cool to room temperature. Then, the mixture was resuspended in distilled water, filtered, and washed until the pH of rinse water became neutral. The product was dried in air at ambient temperature.

This preoxidized graphite was then subjected to oxidation by Hummers' method with revision. The oxidized graphite powder (1.5 g) was put into cold (0 °C) concentrated H_2SO_4 (50 mL). Then, $KMnO_4$ (4.5 g) was added gradually with stirring in ice-bath. It was worth mentioning that when each portion of $KMnO_4$ (about 200 mg) was added to the suspension, the temperature of suspension increased rapidly and even up to 50 °C sometimes. After the temperature of the suspension dropped to about 10 °C, another portion of $KMnO_4$ was added. After the addition of $KMnO_4$ was finished, the mixture was stirred at 35 °C for 2 h. The reaction was terminated by addition of a large amount of distilled water (500 mL) and 30% H_2O_2 solution (50 mL). Subsequently, the color of the mixture changed to bright yellow. Then the mixture was filtered and washed with 5% HCl solution (500 mL) in order to remove metal ions. Then the GO product was suspended in distilled water to give a viscous, brown dispersion, which was subjected to dialysis to completely remove metal ions and acids.[2] The resulting GO (3 mg mL^{-1}) viscous dispersion is stable for a period of months. Then, a fraction of viscous GO dispersion was ultracentrifuged at 10000 rpm for 30 min, and the lemon supernatant (GO hydrogel) with positive charge was stored for further all relevant experiment.

For comparison purposes, GO with negative charge was prepared by the conventional Hummers' method.[3] In detail, 0.5 g of graphite flakes was put into a glass beaker, then 0.6 g of KNO_3 and 23 mL of 98% (w/w) H_2SO_4 were added and the mixture was mixed under stirring in an ice bath. Then, 3 g of $KMnO_4$ was slowly added to the mixture under the ice bath so that the temperature of the mixture was not allowed to reach 20 °C. Then，the beaker was placed in a 35 °C water bath, and the mixture was stirred for about 1 h to form a thick paste. 50 mL of high-purity water was added to the formed paste, and stirred at 90°C for 35 min. Finally, 100 mL of high-purity water was added, followed by the slow addition of 3 mL of H_2O_2 (30%), meanwhile the color of the solution turned from dark brown to brownish yellow. The warm

solution was then filtered and washed with 100 mL high-purity water. The obtained GO product was suspended in distilled water to give a brown dispersion, which was also subjected to dialysis to completely remove metal ions and acids. The resulting GO suspension was stored for further use.

III. Preparation of GO-PEG

In a typical method, by optimizing reaction conditions, phosphate buffered saline (PBS) containing 36 mg mL^{-1} GO hydrogel and 15.3 mg mL^{-1} PEG was prepared, and the mixture was placed in an ice-bath and ultra-sonicated for 15 minutes under 40 W microwave output. Through electrostatic interaction, PEG-COOH, PEG-NH_2 molecules were attached to the GO nanoparticle non-covalently. Then, the GO-PEG suspension was centrifuged at 8000rpm for 30 min to discard the free-standing GO. The supernatant solution was collected and stocked for further studies.

S2. Characterization of GO and GO-PEG

GO and GO-PEG were deposited on mica and dried in air. Then, their morphologies were examined by atomic force microscope (AFM, PicoView1.8, Agilent, America) operating at the tapping mode, respectively. The size distribution and zeta pontential of GO and GO-PEG were documented by dynamic light scattering (DLS, Particle sizing systems, USA) studies by the NICOMP 380/ZLS (PSS) and the data were analyzed by ZPW388 software, respectively. FTIR spectra were obtained using a Nicolet iN10 MX infrared spectrometer (Thermo Scientific, US). A MCT/A detector, KBr/Ge beam splitter, 4 cm^{-1} resolution and 4000–500 cm^{-1} measurement window were used to record 5 times for each sample. Samples were dried on a diamond plate before measurement. Absorption spectra of GO and GO-PEG were obtained using a UV-visible (UV-1800 SHIMADZU, Japan) spectrometer equiped with a deuterium lamp, at scanning regions of 200–900 nm in triplicate studies, respectively. All fluorescence measurements were performed on RF-5301PC fluorescence spectrometer (SHIMADZU, Japan) equiped with a Xe arc lamp. Excitation and emission slits were set to 10 nm. All data were obtained in triplicate. pH measurement were performed on FE20 pH meter (METTLER TOLEDO). Statistical analysis was performed with SPSS 13.0.

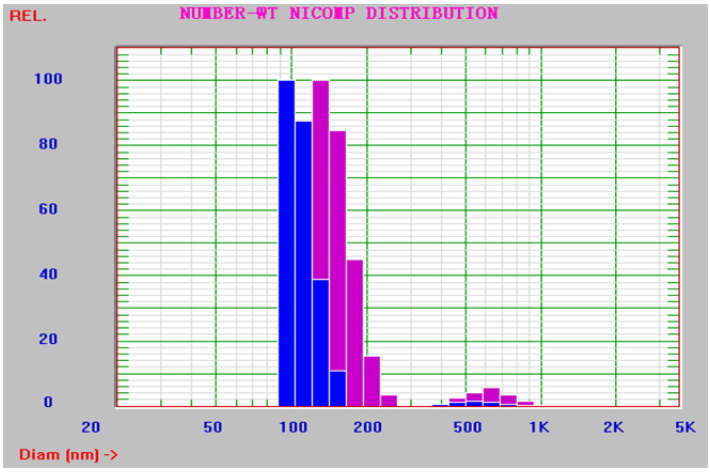

Figure S1. Size distribution of GO (magenta histogram) and GO-PEG (blue histogram) *(prepared by modified Hummers' method)* evaluated from DLS results.

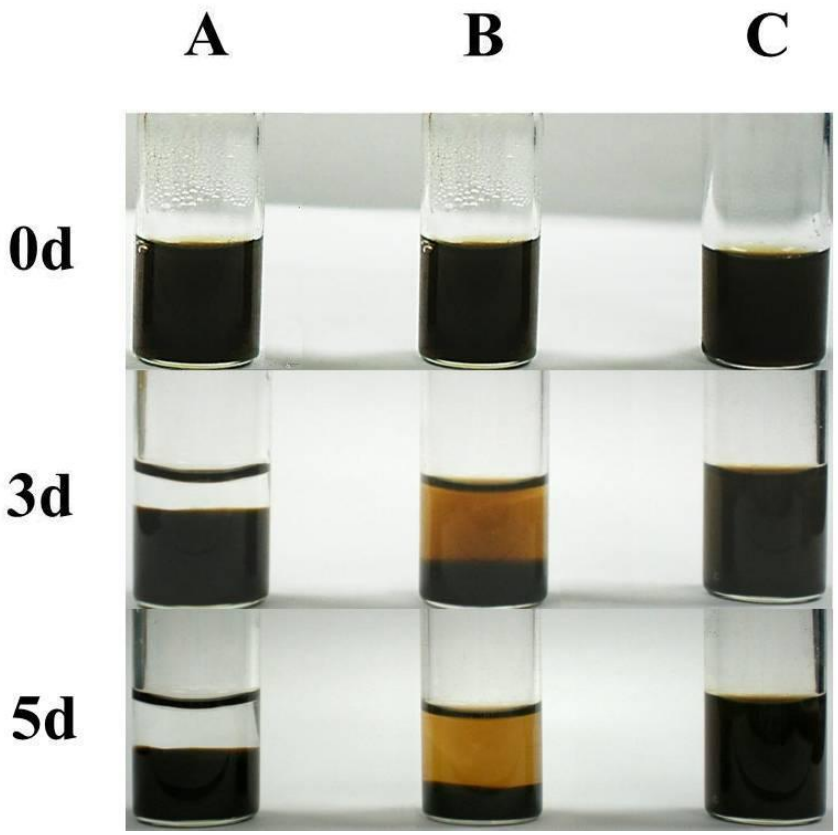

Figure S2. Stability of GO (sample A), GO-PEG-NH_2 (sample B), and GO-PEG-COOH (sample C) in PBS for 0 day, 3 day, and 5 day, respectively. Obviously, GO-PEG sample is more stable and the products are homogenous solution.

S3 XPS spectra of GO

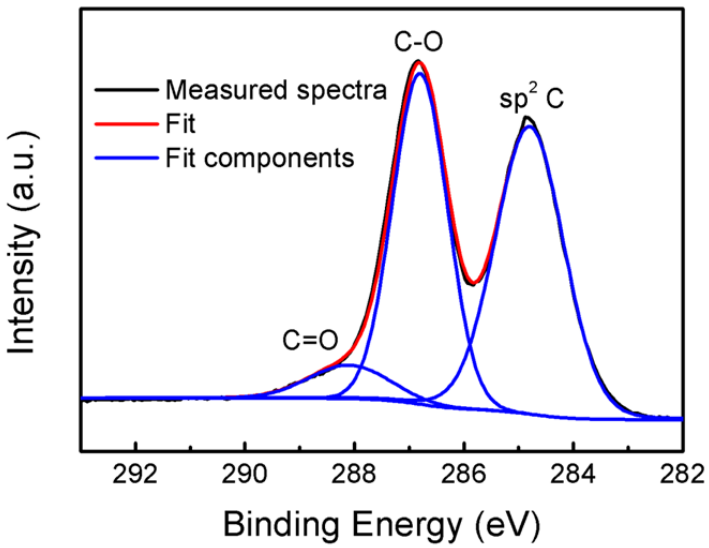

Figure S3.XPS spectra for the C1s of positive charged GO.

X-ray photoelectron (XPS) was performed on an ESCALAB 250Xi electron spectrometer (Thermo Scientific, US) using 300 W Al Kα radiation at about 3×10^{-9} mbar, and the binding energies were referenced to the C1s line at 284.8 eV from adventitious carbon. XPS pattern of the C1s spectra of pristine GO consists of three different chemically shifted components, which can be assigned to sp^2 carbons in aromatic rings (284.8 eV), epoxide (C–O–C, 286.8 eV) and carbonyl groups (>C=O, 288.1 eV) [3, 4] (see Figure S3). It has been reported that C1s spectrum of carbonium species exhibit two separate peaks. Besides, the electron-deficient carbenium center (287-289 eV) increases the C1s electron binding energy of the remaining carbon atoms (284 eV) by 3-5 eV with the integrated area ratio of two Gaussian peaks ranging between 1:3 and 1:5.[5] Hence, it is hardly to pick up the carbonium peak because the characteristic peak of carbonium might be covered by that of carbonyl groups.

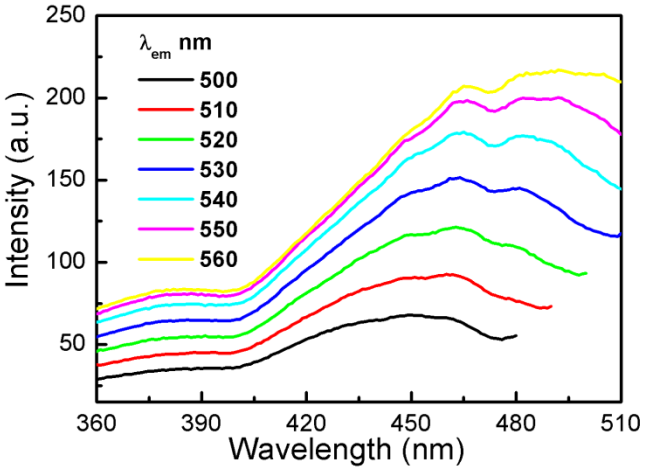

Figure S4. Emission-dependent excitation wavelength of GO obtained under fixed emission wavelength in the range from 500 to 560 nm.

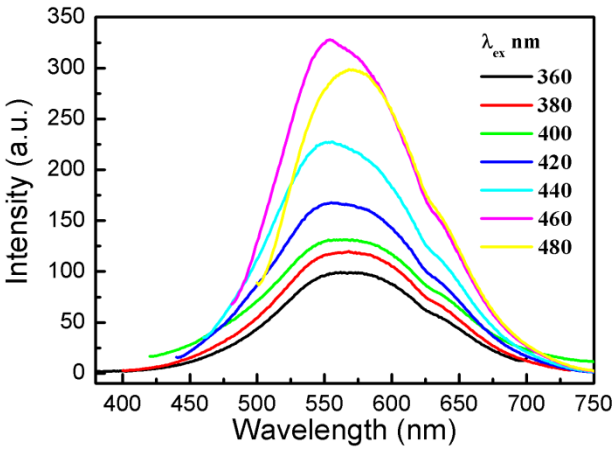

Figure S5.Excitation-independent emission wavelength of GO obtained under fixed excitation wavelength in the range from 360 to 480 nm.

S4. Quantum Yield (QY) of GO and GO-PEG

The quantum yield of GO and GO-PEG were calibrated with Rhodamine 6G. According to the emission peak area and absorbance of GO, GO-PEG and references, we calculated the QY of GO and GO-PEG by the following equation.[6]

$$\varphi_{sample} = \varphi_{ref} \times \frac{F_{sample}}{F_{ref}} \times \frac{A_{ref}}{A_{sample}}$$

In the equation, φ_{ref} is the known quantum yield of the reference compound (Rhodamine 6G, reported

QY=95%), F_{sample} and F_{ref} are the area integral of fluorescence emission curves of the sample and reference, respectively. A_{ref} and A_{sample} are the corresponding UV-visible absorbance of the reference and the sample. According to the aforementioned results, wavelengths of excitation at 460 nm for the fluorescence quantum yields are selected.

S5. Cell experiment

I. Cell culture

HeLa cells (Human cervical cancer cell line) were cultured in RPMI1640 and 10% FBS supplemented with 1% penicillin and streptomycin, reseeded every 2-4 days to maintain subconfluency. The cells were cultured in a humid incubator at 37 °C, under an atmosphere containing 5% CO_2.

II. Cell viability assay

5×10^4 cells mL^{-1} HeLa cells were split into 96-well plates and incubated for 24 h to allow cells to attach. Except from control wells, the contents in the remaining wells were added into medium with GO-PEG and the final concentrations were 9, 18, 36 μg mL^{-1}, next continued to be cultured for different times (1, 2, 4 h), respectively. Then the non-effective nanosheets were removed and the fresh growth medium was added, respectively. After cultured for an additional 48 h, the cells were washed with PBS and then incubated with fresh medium containing 10% (v/v) CCK-8 reagent for 4 h at 37°C for cytotoxicity assay. Then the absorbance at 450 nm was measured using a microplate reader (SpectraMAX M2, Sunnyvale, California). Survival of untreated cells was set as 100%, and that of test cells was expressed as a percentage of untreated cells. Data are shown as mean± the standard error from five independent experiments.

III. Confocal imaging of live HeLa cells

HeLa cells were grown in 35 mm glass-bottom Petri dish, supplemented with culture medium at concentration to allow 90% confluence in 24 h. After GO-PEG with a final GO concentration of 18 μg mL^{-1} was delivered and co-cultured with HeLa cells for 2 h, cells were thoroughly washed and imaged using an UltraVIEW Vox (PerkinElmer) confocal system attachment and a Nikon Ti-e microscope with 60×1.4 NA plan apochromat oil immersion lens. Excitation wavelength was set at 488 nm and HeLa cells

marked by GO-PEG were detected mainly in the green channel (500-550 nm). To confirm the labeling ability of GO-PEG for other cell line, Ramos cells were also selected for the assays (see Figure S6). The image further denmonstratd that the GO-PEG nanoparticle possesses the special affinity to plasma membrane of live cell.

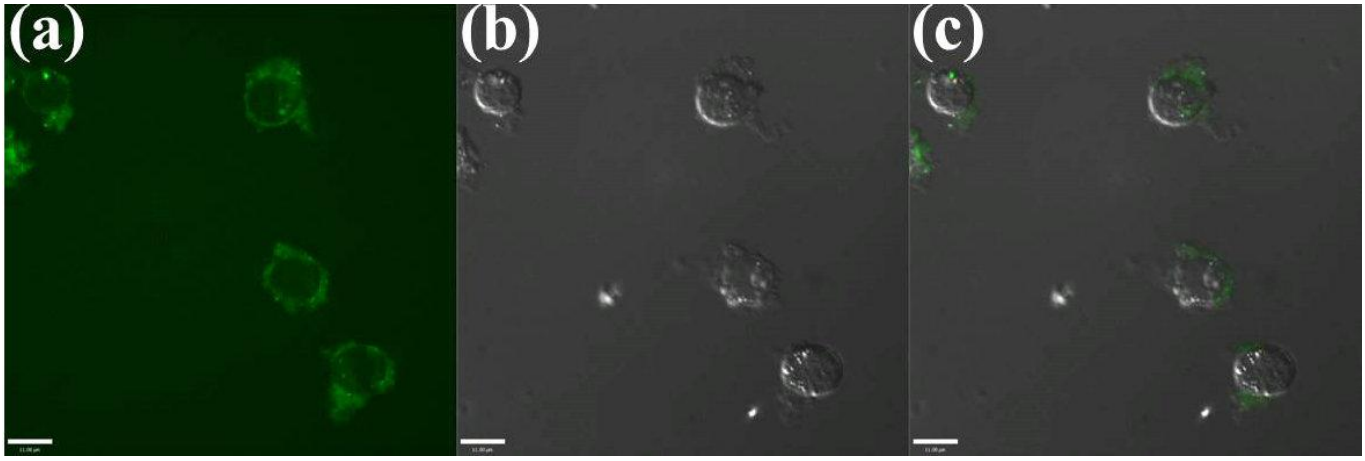

Figure S6. Overlay images of confocal fluorescence image (λ_{ex}=488 nm) and bright field image of the Ramos cells incubated with 35 μg mL^{-1} GO-PEG for 2 h; scale bars are 11 μm.

IV. Two-photon luminescence microscopy images of live HeLa cells

To further determine the cellular distribution of the nanoparticle in tumor cells, GO-PEG and HeLa cells were co-cultured at 37 °C for 2 h. Subsequently, free nanoparticles were removed and the cells marked by the nanoprobe were imaged by one-photon fluorescence and two-photon luminescence simultaneously. A mode-locked femtosecond Ti: sapphire laser (Olympus, attached a Nikon fluoview multiphoton microscopy (FV1000MPE)) tuned to 800 nm with a repetition rate of 80 MHz and pulse width of 100 fs was used as the two-photon illumination source, whereas one-photon fluorescence image was obtained by a 559 nm He-Ne laser. The fluorescence from Dil excited by a 559 nm laser exhibits a well-distributed red region in the whole plasma membrane as revealed in Figure $3c_1$. In contrast, in Figure $3c_2$, the green dots scattered in the cells' plasma membranes could be attributed to the two-photon photoluminescence of GO-PEG, which could be ascribed to the nonlinear optical property of GO.[7] Moreover, the overlapped fluorescence microscopy and two-photon luminescence microscopy images (Figure $3c_3$) provide further evidence for GO-PEG targeting location according to the distribution of green and red channels.

V. Flow cytometry of GO-PEG marked cells

After incubated with 18 μg mL^{-1} GO-PEG for 2 h at pH 6.5 and 7.6, HeLa cells were rinsed with different cultural medium and detached from the surface by treatment with trypsin-EDTA, respectively. Then the cells were harvested by different cultural medium at pH 6.5 and 7.6, respectively. The mean fluorescence were measured by flow cytometry (Accuri C6, BD). Data are shown as mean± the standard error from three independent experiments. The excitation wavelength is 488 nm, and the emission wavelength is 530±15 nm. Compared with that of Hela cells without nanoprobe labeling, the mean fluorescence intensity of Hela cells marked with 18 μg mL^{-1} GO-PEG increased at the same cell count. Additionally, with the acid of cultural medium enhanced, the mean fluorescence intensity of Hela cells labeled by GO-PEG increased(Figure S7).

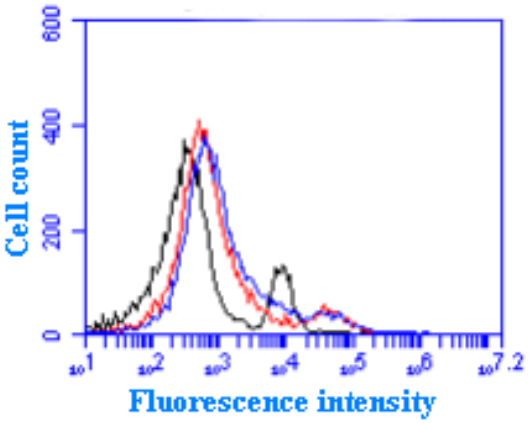

Figure S7 Flow cytometry results of Hela cells marked with 18 μg mL^{-1} GO-PEG at different pH value (pH 7.6,red line; pH 6.5,blue line). Hela cells without nanoprobe labeling (black line) served as control.

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