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COMMUNICATION

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

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Polyethylene glycol modified graphene oxide (GO-PEG) nanoparticle was prepared, which was positively charged and fairly stable in buffer solution. The GO-PEG nanoparticle possesses a special affinity to plasma membrane of live cell, and its yellowish-green fluorescence is sensitively responsive to the extracellular physiological solution in situ.

Labeling the plasma membrane of live cells can define extra/intracellular environment and assist in exploring the plasma membrane correlated processes. It plays a key role in biological diagnosis and relevant study on the molecular mechanisms.¹ Although organic fluorescence dyes,² fluorescent proteins,³ are well-synthesized and widely used in staining the plasma membrane, it still remains a great challenge to develop advanced probe with specific targeting ability to adequately detect the underlying membrane function at the level of live cells. As a representative biological event, alterations in homeostasis of the extracellular environment occurring very early in the disease cascade can be used for the early diagnosis of many diseases, including cancer.⁴ In particular, a low extracellular pH can be considered as one of the significant indications of malignant tumors because in tumor cells, the increased glucose catabolism results in significant production of lactate and H⁺.⁵ On this basis, monitoring the pH of extracellular microenvironment with non-invasive may provide important clues to assess cells' physiological or pathological state and their response to different therapeutic interventions.

Recently, graphene oxide (GO) has aroused much interest in a wide landscape of investigation, especially in biomedical field owing to its unique electronic, optical, mechanical and electrochemical properties.^{6,7} In particular, developing GO for cell imaging has shown its excellent performance as tags.^{8,9} However, for live cells, exploring functionalized GO for subcellular marking precisely and analyzing specific molecule sensitively in situ still remains at very early stage. In this communication, we attempt to demonstrate that the localized peroxidation of sp² hybridized carbon atoms results in the cationic surface charge of GO. The positively charged property, together with large two-dimensional structure,^{10,11} significantly enhance the biocompatibility and marking capability of GO to plasma membrane. In addition, for the sake of improving stability and dispersity of GO in physiological solution, we modified GO

nanoparticles with carboxylic polyethylene glycol (PEG-COOH) non-covalently through electrostatic interaction (Scheme 1a). Meanwhile, considering GO's tunable fluorescence is sensitive and reversible response to pH,^{12,13} the obtained GO-PEG may become an effective fluorescent nanoprobe for labeling plasma membrane precisely and detecting extracellular pH variation during the growth and metabolism of cancer cells (Scheme 1b).



Scheme 1 (a) Non-covalent decoration of positively charged GO by PEG-COOH; (b) GO-PEG behaves as an effective fluorescent nanoprobe for labeling plasma membrane and monitoring extracellular pH change (from pH 7.6 to 6.5) during the growth and metabolism of cancer cells.

Constructing the nanoprobe started from preparation of GO by modified Hummers' method.¹⁴ (see ESI, S1, Part II). The as-obtained GO dispersion was mixed with PEG-COOH and the mixture was fully ultra-sonicated to get a well-dispersed GO-PEG dispersion (see ESI, S1, Part III). The morphology and dispersity of as-prepared GO and GO-PEG were studied by atomic force microscopy (AFM). As shown in Figure 1a, the sizes of the resulting GO and GO-PEG nanosheets range from 100 to 200 nm. The results are in good agreement with those from relevant dynamic light scattering (DLS) measurement (see ESI, Figure S1), which reveals the mean diameter of GO and GO-PEG are about 149 nm (magenta histogram) and 109 nm (blue histogram), respectively. The decrease of size distribution can be ascribed to the cutting function of ultrasound. Moreover, the AFM result from random measurement in Figure 1a depicts the thickness of GO nanosheets ranges from 1 to 1.5 nm, disclosing the exfoliated nanosheets are single layer.¹⁵ As a comparison, after the

decoration of PEG on the surface of GO, we observed the apparent increase of average thickness. Notably, compared with that of GO, AFM images in Figure 1a also directly show the improved dispersibility of GO-PEG, suggesting that surface modification with PEG might serve as an effective means to improve the dispersibility of nanostructure. The as-prepared GO-PEG nanoparticle is substantial stable in phosphate buffered saline (PBS) for a long storage time (see ESI, Figure S2). Furthermore, we found for the first time that the surface charges of as-prepared GO and GO-PEG are about 30 and 15 mV, respectively, while GO prepared by the conventional Hummers' method was negatively charged (-50 mV) (see Figure 1b). Our results indicate that there are special cationic chemical groups in GO prepared by modified Hummers' method.

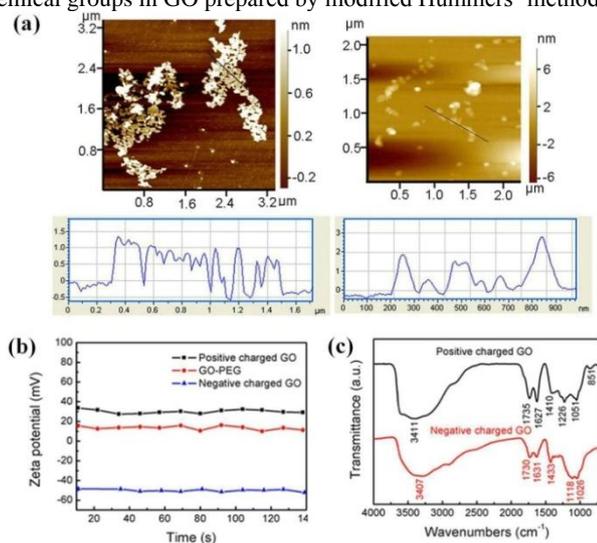


Figure 1 (a) AFM images of synthesized GO (left) and as-obtained GO-PEG (right) nanosheets; (b) Zeta potential of positively charged GO (black curve), GO-PEG (red curve) and negatively charged GO (blue curve). (c) FTIR spectra of positively charged (black curve) and negatively charged GO (red curve)

To preliminarily explore the underlying principle of positively charged surface and elucidate the successful preparation of GO and GO-PEG, Fourier transform infrared (FTIR) and X-ray photoelectron spectroscopy (XPS) were performed. As shown in Figure 1c, the black curve reveals series of functional groups which are structure characteristics of GO.¹² It should be noted that a peak centered at 851 cm^{-1} is assigned to the O–O vibration of peroxide ligand.¹⁶ However, for GO with negative charge, the peak at 851 cm^{-1} is nonexistent (see Figure 1c, red curve). And the vibrations of the carboxyl groups retain in both positively and negatively charged GO (C–O stretch at $1026\sim 1051\text{ cm}^{-1}$, C–OH stretch at $1118\sim 1126\text{ cm}^{-1}$; adsorbed water & skeletal vibrations of unoxidized graphitic domains at $1627\sim 1631\text{ cm}^{-1}$, C=O stretch at $1730\sim 1735\text{ cm}^{-1}$). The peroxide bond has been reported in the form of epoxide or oxygen bridges between multiple layers of GO.¹⁷ Considering the exfoliated nanosheets are comprised of single layer in our case, one can deduce that the peroxide bond may serve as dangling bond or bridging bond in the single layer of GO and the peroxidation of sp^2 hybridized carbon atoms may give rise to positive carbon atoms—carbonium (see Scheme 1a), which leads to the cationic surface charge of GO. XPS patterns of the C1s spectra of pristine GO also demonstrated that carbonium may be existent (see ESI, S3, Figure S3).

The optical properties of GO and GO-PEG were studied by combined analysis of absorption and photoluminescence (PL) spectra. As illustrated in Figure 2a, the UV-vis absorption spectra of GO and GO-PEG exhibit one broad band with a maximum at 232

nm and a shoulder at 300 nm which originate from the $\pi\text{-}\pi^*$ transition of C=C and $n\text{-}\pi^*$ transition of C=O, C–O–C or O–O groups, respectively.^{11,15} The results of emission-dependent excitation and excitation-independent emission wavelength confirm that the optimal excitation and emission peaks are at 460 nm and 542 nm, respectively (see ESI, Figure S3, Figure S4 and Figure 2a); these match the radiative recombination of electron-hole pairs in localized finite-sized sp^2 clusters within a sp^3 matrix.^{12,18} Moreover, a transparent solution of GO exhibits yellowish-green fluorescence when excited at 488 nm (see Figure 2b). Besides, the as-synthesized GO and GO-PEG possesses the fluorescence quantum yield at about 0.15% and 0.11%, respectively (see ESI, S4). From the comparison of absorbance and relevant excitation spectra (see ESI, Figure S4 and Figure 2a), only a small fraction of absorption contributes to the luminescence while the major fraction does not. GO's comparatively inhomogeneous characteristics may be the reason for its low fluorescence quantum yield.

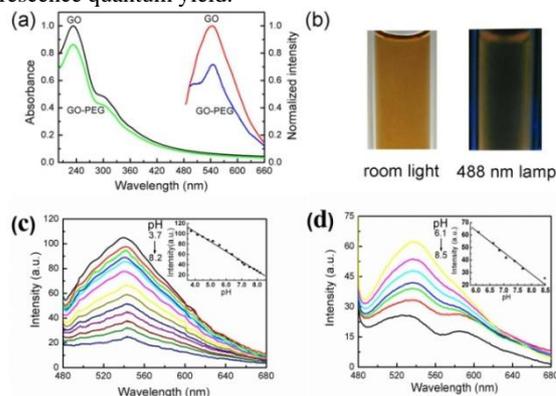


Figure 2 (a) Absorbance (left) and fluorescence emission (right, $\lambda_{\text{ex}}=460\text{ nm}$) spectra of GO and GO-PEG; (b) Photo and fluorescent images of GO obtained under room light and uv excitation at 488 nm, respectively; (c) pH-dependent fluorescence intensity behavior of GO in PBS (inset shows a good linearity in the pH range of 3.7-8.2, $R^2=0.99$); (d) pH-dependent fluorescence intensity behavior of GO-PEG in pH adjusted culture medium (inset shows a good linearity in the pH range of 6.1-8.5, $R^2=0.98$).

Interestingly, GO displays pH-dependent fluorescence behavior. As present in Figure 2c, GO dispersion shows higher fluorescence intensity under acidic conditions but the intensity drops intensively under alkaline conditions when pH changed from 3.7 to 8.2 by adding NaOH to PBS dispersed GO. A more unambiguous result was displayed in the inset of Figure 2c that a sigmoidal plot of the fluorescence intensity at 542 nm against pH behaved a linear decrease ($R^2=0.99$). In order to confirm whether the fluorescence of GO-PEG nanocomposite presents a similar sensitive response to pH variation in the biological culture medium, the fluorescence intensity of GO-PEG against pH change in RPMI1640 culture medium was investigated. As expected, it behaved a similar manner to that of GO against pH change when pH was varied from 6.1 to 8.5 ($R^2=0.99$, Figure 2d). Since it is not the first time to report linear pH-sensitive fluorescence on carbon nanomaterials,¹⁸ a conceivable mechanism can be proposed as follows. As discussed above, the absorption spectrum of GO (see Figure 2a) is a typical species where numerous transitions occur in parallel, including $n\text{-}\pi^*$ and $\pi\text{-}\pi^*$ transitions. Protonation and deprotonation have a great effect on the dissociation of oxygen-containing functional groups on GO, including carboxy group and aromatic hydroxyl group. At high pH condition, the electronic transition from the highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO) is of $n\text{-}\pi^*$ type, the corresponding fluorescence is relatively weak in intensity, whereas at low pH, protonation removes the lone pair electrons on

oxygen and HOMO-LUMO electronic transition becomes π - π^* type, significantly enhanced the fluorescence intensity.¹² Taking advantage of this special property, it may open opportunity to enable GO-PEG as a potential effective pH-sensitive probe at the level of live cells.

Although the aforementioned studies disclose GO-PEG have the potential ability to detect extracellular pH variation,¹³ it is imperative to clarify their toxicity and location to cells. Herein, HeLa cells were selected as model cells. The biocompatibility of GO-PEG was evaluated by studying the viability of cells after incubation with different doses of nanoprobe for different times, respectively. From the cell viability studies (see ESI, S5 and Figure 3a), we get the optimized concentrations ($18 \mu\text{g mL}^{-1}$ of GO-PEG for HeLa cells) and co-cultured time (2 h) for the nanoprobe in the following cell imaging and pH detecting assays.

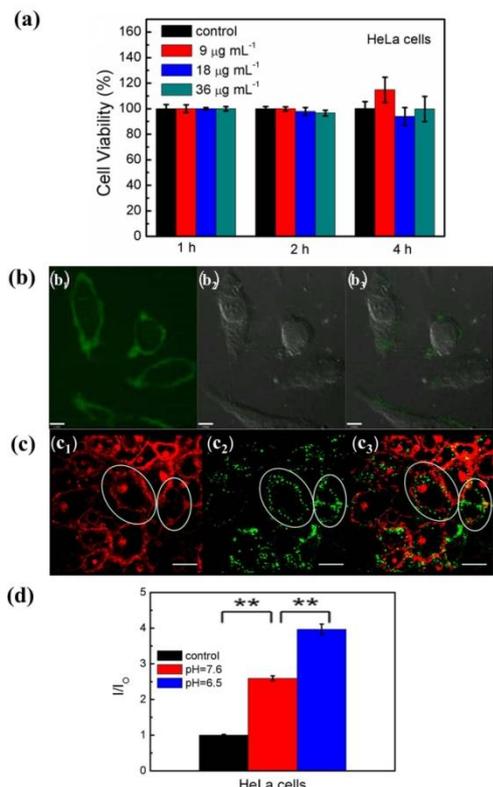


Figure 3 (a) Viability of HeLa cells after 1, 2 and 4 h incubation with 9, 18, 36 $\mu\text{g mL}^{-1}$ GO-PEG. Data are shown as mean \pm standard error from five independent experiments. (b) Overlay images of confocal fluorescence image ($\lambda_{\text{ex}}=488 \text{ nm}$) and bright field image of the HeLa cells incubated with $18 \mu\text{g mL}^{-1}$ GO-PEG for 2 h, respectively; scale bars is 11 μm ; (c) Images of HeLa cells incubated with $18 \mu\text{g mL}^{-1}$ GO-PEG and Dil dye for 2 h, several cells were circled with ellipses: (c₁) one-photon fluorescent image of well-distributed Dil in the whole plasma membrane (red fluorescence); (c₂) two-photon luminescence image of GO-PEG scattered on the cells' plasma membranes (green photoluminescence); (c₃) the overlapped image. The scale bars are 20 μm . (d) Relative emission intensity of HeLa cells marked by nanoprobe as a function of extracellular pH variation (pH 7.6 to 6.5) based on a standard flow cell cytometry with excitation light at 488 nm. I and I_0 represent cells fluorescent intensity with and without probe labeling, respectively. Error bars represent variation between three measurements (** $p < 0.005$)

Based on the well-defined PL and good biocompatibility of GO-PEG, we carried out the bioimaging test by confocal laser scanning microscopy (CLSM) with an excitation wavelength at 488 nm, to match the maximum excitation wavelength of GO (see ESI, S5, part

III). From the bright field channel in Figure 3b₂, GO-PEG incubated with HeLa cells does not weaken the activity of cells and the cells maintain their normal morphology. More importantly, the fluorescence (Figure 3b₁) and overlapped images (Figure 3b₃) vividly disclose that the yellowish-green nanoparticle mainly distribute on the cells' plasma membranes. Two-photon luminescence microscopy images provide further evidence that the GO-PEG targets the plasma membrane with dotted distribution (see ESI, S5, part IV and Figure 3c).⁸ The highly affinity of GO-PEG to plasma membrane might be ascribed to their comparability in structure. On one hand, the similar curvature between the GO and plasma membrane would facilitate their holding together. On the other hand, there might be multiple binding forces between GO and plasma membrane,¹⁰ including electrostatic, hydrophobic interactions, hydrogen-bonding or van der Waals interactions. In detail, the adsorption between cationic GO-PEG and negatively charged plasma membrane seems to be governed primarily by electrostatics interaction.¹¹ In addition, it is probably that sp^2 conjugated network of GO contribute to its interaction with plasma membrane through hydrophobic interactions. Last but not at least, other driving forces, like hydrogen bonds or van der Waals interactions of functional groups on GO (for example, $-\text{OH}$ or $-\text{COOH}$ groups) to the membrane bounded polysaccharides and glycoproteins might play a role in attraction. An accurate understanding on the driving forces requires further studies.

Inspired by GO-PEG's accurate location for plasma membrane and pH-sensitive features, the nanoprobe was successfully employed for mapping the pH change in the extracellular microenvironment of tumor cells via flow cytometry (FCM) (see ESI, S5, part V). To detect the pH variation of extracellular environment during the growth and metabolism of tumor cells, HeLa cells were incubated with $18 \mu\text{g mL}^{-1}$ GO-PEG for 2 h at pH 7.6 and 6.5, respectively. It is noteworthy that pH 7.6 and 6.5 were selected here to mimic the extracellular pH evolution of the tumor cells.⁵ Subsequently, the cells were rinsed and resuspended in the corresponding culture medium at different pH conditions (7.6 and 6.5) and the cells' mean fluorescence intensity were estimated by FCM (see ESI, Figure S7). The relative emission intensity of cells marked by nanoprobe is expediently defined as I/I_0 , where I and I_0 represent the emission intensity of cells with and without probe labeling, respectively. It should be noted that I/I_0 in the control cells without nanoprobe labeling is defined as 1. As can be seen from Figure 3d, compared with the control group, HeLa cells marked with GO-PEG exhibit the relative enhanced mean fluorescence intensity owing to GO-PEG's adsorption on the plasma membrane. Importantly, HeLa cells incubated at pH 6.5 behaves much higher mean fluorescence intensity than those incubated at pH 7.6 (I/I_0 changing from 2.59 to 3.97), probably resulting from the GO-PEG's sensitive response to pH which bounded on the plasma membrane of cells.

In conclusion, we have reported PEG decorated GO with positive charge which can be employed as plasma membrane marker of tumor cells in vitro. In particular, the fluorescence of GO-PEG is pH dependent, allowing for quantitatively monitoring the subtle evolution of extracellular acidic microenvironment during the growth and metabolism of cancer cells. We predict that GO-PEG will pave the way for development of novel fluorescence tags and biosensors to reflect the biological events about the plasma membrane in future.

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Notes and references

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Text: Graphene oxide nanoparticle with positive charge can be developed as fluorescent nanoprobe to detect extracellular pH variation of live cells

