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Supporting Information

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Polyglycerol-Grafted Graphene Oxide with pH-Responsive Charge-Convertible Surface to Dynamically Control the Nanobiointeractions for Enhanced in Vivo Tumor Internalization

Yajuan Zou, Alberto Bianco* and Yuta Nishina**

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Polyglycerol-Grafted Graphene Oxide with pH-Responsive Charge-Convertible Surface to Dynamically Control the Nanobiointeractions for Enhanced *in Vivo* Tumor Internalization

Yajuan Zou, [a] * *Alberto Bianco,* [a] [b] * *Yuta Nishina*[a] *

[a] Dr. Y. Zou, Dr. A. Bianco, Prof. Y. Nishina

Research Institute for Interdisciplinary Science, Okayama University, 3-1-1 Tsushimanaka, Kita-ku, Okayama 700-8530, Japan

[b] Dr. A. Bianco, CNRS, Immunology, Immunopathology and Therapeutic Chemistry, UPR3572, University of Strasbourg, ISIS, Strasbourg, 67000, France

Methods

Materials: Glycidol was purchased from Kanto Chemical Co. Inc. (Japan). The following reagents were supplied by Wako Pure Chemicals (Japan): sodium azide (NaN_3), pyridine, *N*,*N*-dimethylformamide (DMF), sodium dodecyl sulfate (SDS), antibiotics penicillin/streptomycin (100 U/mL), 20% glutaraldehyde, paraformaldehyde (PFA), triethylamine (TEA), dimethylsulfoxide (DMSO), fluorescamine, Cy5- and Cy5.5-NHS ester, isoflurane, and formalin. The following reagents were purchased from Tokyo Chemical Industry (Japan): 4-dimethylaminopyridine (DMAP), triphenylphosphine (PPh_3), dimethylmaleic anhydride (DMMA) and succinic anhydride (SA). The following reagents were purchased from Nacalai Chemicals (Japan): *p*-toluenesulfonyl chloride (TsCl), potassium dihydrogenphosphate (KH_2PO_4), sodium hydroxide (NaOH),

SDS-PAGE running buffer. FBS and BCA protein assay kit (T9300A) were supplied by Biosera, Inc. (France) and Takara, Bio., Inc. (Japan), respectively. Precast polyacrylamide gel (5-20%, E-R520L) and protein loading buffer (AE-1430) were purchased from ATTO, Japan. Hoechst (33342) was supplied by Thermo Fisher Scientific. Potassium phosphate dibasic trihydrate ($K_2HPO_4 \cdot 3H_2O$), Dulbecco's modified Eagle medium (DMEM), RPMI-1640 Medium and 0.25% trypsin-EDTA solution were supplied by Sigma-Aldrich. RIPA Lysis Buffer was supplied by Cruz Credit™. Mouse macrophage J774A.1 cells (JCRB9108) and mouse colon CT26 cells (RCB2657) were purchased from Japanese Collection of Research Bioresources Cell Bank and RIKEN BioResource Research Center, respectively.

Equipment: SEM was observed on a Hitachi SU9000 field emission microscope. AFM images were acquired on a SPM-9700HT (Shimazu, Japan) at tapping mode with excitation frequency range of 204-497 kHz. TGA was run from room temperature to 800 °C at a heating rate of 10 °C/min under N_2 on a DTG-60AH apparatus (Shimazu, Japan). The IR spectra was recorded on an Irtracer-100 (Shimazu, Japan) in the wavenumber range of 4000 to 500 cm^{-1} with a resolution of 4 cm^{-1} for 40 scans. The DLS and zeta potentials were recorded on an ELSZ-2000N analyzer (Otsuka Electronics, Japan). Raman spectra were recorded within the range of 800-4000 cm^{-1} on a Jasco NRS3100 spectroscopy using a laser of 532 nm. The surface chemistry of GO, GOPG, and $GOPGNH_2$ was analyzed by XPS (JPS9030, JEOL). UV spectra were recorded on a V-670ST spectrophotometer (Jasco, Japan). The BCA quantification was measured on a MPR-A100 microplate reader (AS ONE, Japan) at 570 nm. The SDS-PAGE images were acquired on an ImageQuant 800 scanner (Cytiva, GE Healthcare, USA). TEM observation of cell samples was performed on field emission transmission electron microscopes (H-7650, Hitachi, Japan). The confocal images were acquired on a confocal scanning laser microscope (FV3000, Olympus, Japan). GOPGNHDMMA-Cy5 and cell nucleus were observed at Cy5 mode (λex = 640 nm, λem = 665 nm) and DAPI mode (λex = 350 nm, λem = 461 nm), respectively. The *in vivo* live animal imaging and *ex vivo* imaging were conducted on a Lumazone imaging system (λex = 650 nm, λem = 710 nm).

Synthesis of GOPG. GO was synthesized from graphite *via* a modified Hummers' method, as we reported previously.[1] To prepare GOPG, GO (~100 mg) in Milli-Q H_2O was washed with glycidol by ultracentrifugation (67, 000g, 90 min) three times for solvent exchange. Then, the GO precipitate was

resuspended in glycidol (10 mL) and bath-sonicated at room temperature for 1h. Afterwards, the resulting dispersion was stirred at 75 °C for 20 h. The reaction product was dispersed in Milli-Q H_2O, and GOPG was collected by centrifugation (20, 000g, 1 h) and further washed with Milli-Q H_2O by repeated redispersion/centrifugation cycles for three additional times.

Synthesis of GOPGNH$_2$. The GOPGNH$_2$ was prepared by converting a part of hydroxyl groups in GOPG to amino groups *via* OTs→N_3→NH_2 transformations, according to our previous report.[2] GOPGNH$_2$ with amino groups of *l*, *m*, and *h* densities were obtained by controlling the degree of tosylation in GOPGOTs. Briefly, GOPGOTs-*l*, GOPGOTs-*m*, and GOPGOTs-*h* were obtained by reacting GOPG (100 mg) with TsCl of 20, 50 and 80 mg, respectively, in pyridine (10 mL) in the presence of DMAP (10 mg).

Synthesis of GOPGNH-DMMA. As for the synthesis of GOPGNH-DMMA, GOPGNH$_2$ (10 mg) was dispersed in 0.5 M phosphate buffer (pH 8.0, 10 mL), and DMMA (100 mg) was added to the dispersion in several portions. The pH of the reaction system was maintained in the range of 8.5-9.0 with the addition of NaOH solution (0.1 N). After the completion of the reaction, GOPGNH-DMMA was collected by centrifugation (20, 000g, 20 min) and further washed with 0.1 M phosphate buffer (pH 8.0) by repeated redispersion/centrifugation cycles. GOPGNH-SA was prepared in a similar procedure by using SA instead of DMMA.

Preparation of GOPGNH-DMMA-Cy5/-Cy5.5. Freshly prepared GOPGNH-DMMA (3 mg) was dispersed in 0.1 M PB buffer (pH 8.0, 3 mL), and Cy5-NHS ester (6.0 μg) was added to the dispersion. After stirring at RT for 30 min, the as-prepared GOPGNH-DMMA-Cy5 was collected with Vivaspin Turbo 15 (MWCO 100, 000 Da). GOPGNH-DMMA-Cy5.5 was prepared in a similar procedure by using Cy5.5-NHS ester instead of Cy5-NHS ester.

Stability of GOPGNH-DMMA at pH 7.4 and 6.5. The freshly prepared GOPGNH-DMMA was incubated in 0.01 M phosphate buffer (pH 7.4 or 6.5) at 37 ℃ at a concentration of 1.0 mg/mL. The amount of exposed amines in GOPGNH-DMMA was quantified *via* the fluorescamine method[3] with slight modification. Briefly, at predetermined time intervals (2, 6, 12, 24, 48 and 96 h), an aliquot of dispersion from each incubation (200 μL) was taken out and incubated with 2% TEA (3 mL in DMSO) and 1 mg/mL fluorescamine (300 μL in DMF) at RT for 10 min. The fluorescence intensity was measured on a RF-5300 PC Fluorophotometer

(Shimazu, Japan) at excitation and emission of 400 and 465 nm, respectively. The ones of phosphate buffer and the corresponding $GOPGNH_2$ precursors were also recorded at each time point and regarded as blank and positive control, respectively. For the zeta potential measurement, 150 μL of GOPGNH-DMMA dispersion was taken at identical time points and centrifuged at 20, 000g to remove buffer solution. The resulting precipitates were resuspended in Milli-Q H_2O for measurement. The zeta potentials of GOPGNH-SA incubated at pH 7.4 and 6.5 for 24, 48 and 96 h were recorded in similar procedures. In the above assays, the results of GOPGNH-DMMA and GOPGNH-SA just after preparation were referred to as the time point of 0 h.

Dynamic protein corona formed on GOPGNH-DMMA. The freshly prepared GOPGNH-DMMA was incubated in 0.01 M phosphate buffer (pH 6.5 or 7.4) containing 10% FBS at a concentration of 1.0 mg/mL at 37 °C. To avoid the interference of protein aggregates in FBS, the freshly thawed FBS solution was centrifuged at 20, 000g for 20 min before use. At predetermined time intervals (2, 6, 12, 24, 48 and 96 h), an aliquot of dispersion from each incubation (350 μL) was taken out and centrifugation at 20, 000g for 20 min to pelletize the complexes of GOPGNH-DMMA and FBS. The complexes were further washed with Milli-Q H_2O (1.0 mL) three times to remove free unbound proteins. The resulting pellets were treated with 10% SDS (100 μL) to detach the adsorbed proteins. After centrifugation at 20, 000g for 20 min, the supernatant containing the adsorbed proteins was collected and used for further analysis. The amounts of adsorbed proteins were quantified *via* BCA according to the manufacture's instruction.

Cell culture. J774A.1 and CT26 cells were maintained in DMEM and RPMI 1640 culture medium, respectively, and supplemented with 10% FBS and 1% antibiotics penicillin/streptomycin. All the cells were grown in a humidified incubator (APC-30D, Astec) supplied with 5% CO_2 at 37 °C.

Uptake of GO and GOPG by J774A.1. J774A.1 cells were seeded in petri dishes (Ø 10 cm) at a density of 1.0×10^6 cells per dish and incubated at 37 °C overnight to allow cell adhesion. Then, the culture medium was discarded and replaced with fresh medium containing GO and GOPG (100 μg/mL, adjusted based on the mass of GO). After a further incubation of 24 h, cell pellets were collected, washed with PBS, and fixed with 2% glutaraldehyde/4% paraformaldehyde in 0.1 M PBS buffer (1 mL). In the next step, the cell pellets were embedded in epoxy resin and sliced for TEM observation.

Dynamic cellular uptake of GOPGNHDMMA-Cy5. In line with the stability and dynamic protein affinity assays, the cellular uptake of GOPGNH-DMMA was examined at time points of 2, 6, 12, 24, 48 and 96 h, and the uptake under conditions of pH 7.4 and 6.5 were assayed with J774A.1 and CT26 cells, respectively. Take the condition of pH 7.4 as an example, the J774A.1 cells were seeded in glass bottom dishes (Ø 35mm) at a density of 2.0×10^5 cells per dish and incubated overnight to allow cell adhesion. For early time points of 2 and 6 h, the culture medium was discarded and replaced with DMEM medium containing freshly prepared GOPGNH-DMMA-Cy5 (100 μg/mL). After further incubation for 2 or 6 h, the cells were washed with PBS and stained with Hoechst (0.5 μg/mL) for 30 min at 37°C. Then, the cells were washed with PBS three times, fixed with 2% PFA for 10 min at RT and subjected to CLSM observation. For the time points of 12, 24, 48 and 96 h, 200 μL of GOPGNH-DMMA-Cy5 dispersion (1.0 mg/mL) at pH 7.4 was blended with 1.8 mL of DMEM medium 6 h prior to each indicated time points and added to adhered J774A.1 cells. After the cells were incubated with GOPGNH-DMMA-Cy5 till indicated time, they were treated with Hoechst staining and PFA fixation as mentioned above. The assay with CT26 cells was performed in similar procedures, except for that the culture medium was replaced with RPMI 1640 medium containing GOPGNHDMMA-Cy5 (100 μg/mL) at pH 6.5.

Animals and tumor models. ICR mice aging 5 weeks and BALB/c nude mice aging 5 weeks were supplied by Japan SLC, Inc. (Kyoto, Japan). All animal experiments were performed in accordance with the protocol approved by the Animal Care and Use Committee of Okayama University (OKU-2024619). All the animals were maintained under specific pathogen-free conditions and allowed to access food and water freely. Allograft tumor models were established by subcutaneously implanting CT26 cells (5×10^5 in 100 μL PBS) into the right flank of mouse body.

Pharmacokinetics studies. ICR mice (n = 3 for each group) were intravenously administered with PBS dispersion of GOPGNH-DMMA (200 μL at material concentration of 10 mg/mL for each mouse). At 15 min, 30 min, 1 h, 1.5 h, 2 h, 6 h, 12 h, 24 h, 48 h and 96 h post-injection, blood samples from tail vein were collected and lysed with RAPI buffer. GOPGNH-DMMA in the lysis solution was collected by centrifugation and GO content was determined *via* a label free method.[4] The half-life of GOPGNH-DMMA was calculated by GraphPad Prism10.

***In vivo* and *ex vivo* imaging.** BALB/c nude mice bearing CT26 tumors (around 100 mm^3) were intravenously administered with GOPGNH-DMMA-Cy5.5 (200 μL at material concentration of 5 mg/mL for each mouse). Under anesthesia with 2% isoflurane, the *in vivo* fluorescence images were acquired at pre-injection and 2, 6, 12, 24, 48 and 96 h post-injection on a Lumazone imaging system. After the final *in vivo* imaging at 96 h, the mice were euthanized, major organs including heart, lung, liver, spleen, and kidney, along with tumor nodules were harvested and subjected to *ex vivo* imaging.

Analysis of tissue samples. Just after *ex vivo* imaging, the specimens of tumor and major organs were fixed in 10% formalin, embedded in paraffin, and sliced into thin sections. The sections of tumor tissue were stained with Hoechst (2.0 μg/mL) and analyzed *via* CLSM. The sections of other tissues were stained with hematoxylin & eosin (H&E) and observed on a light microscopy for histopathological analysis.

Statistical analysis. All the results were expressed as mean ± standard deviation (SD, $n = 3$). The data of BCA quantification (Figure 3d, 3e, 3f) and *ex vivo* fluorescence intensity (Figure 5e) were further analyzed by two-tailed Student's T-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ($n = 3$).

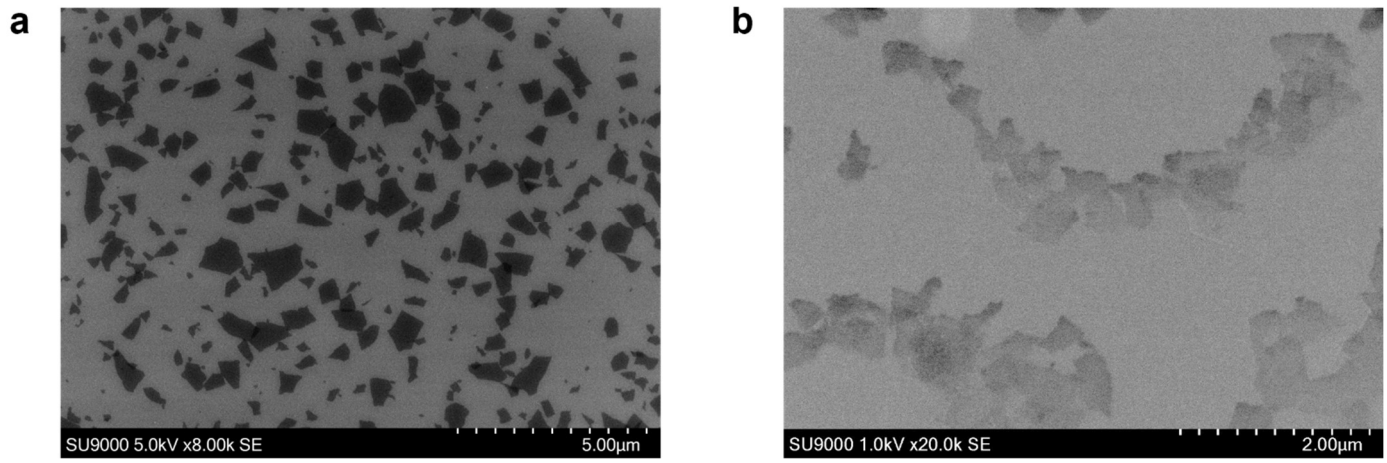


Figure S1. SEM images of (a) GO and (b) GOPG**.**

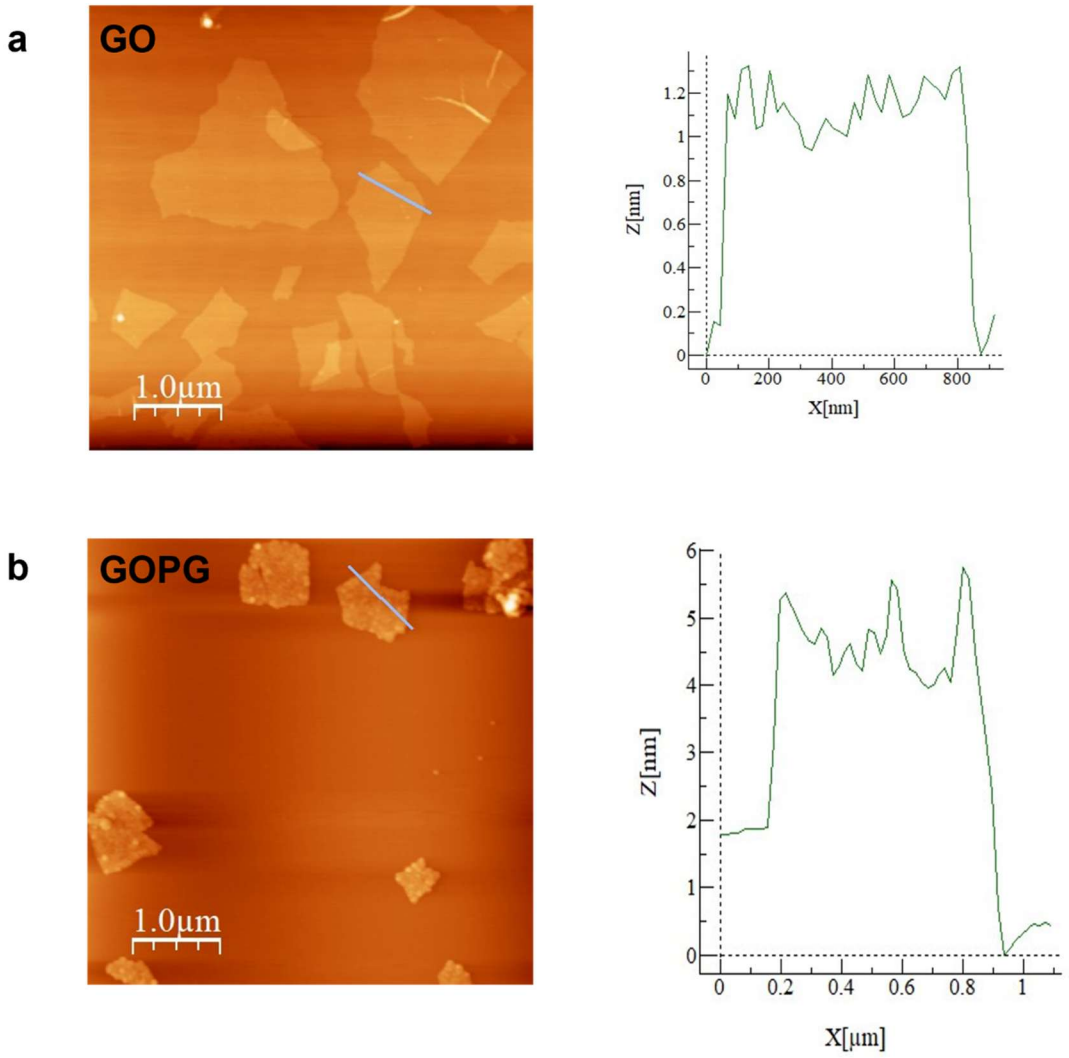


Figure S2. AFM analysis of (a) GO and (b) GOPG.

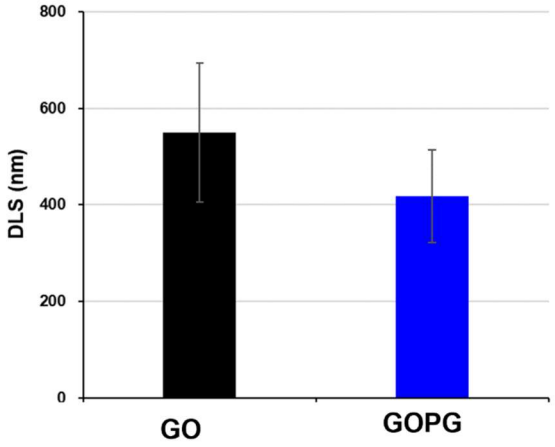


Figure S3. DLS size of GO and GOPG.

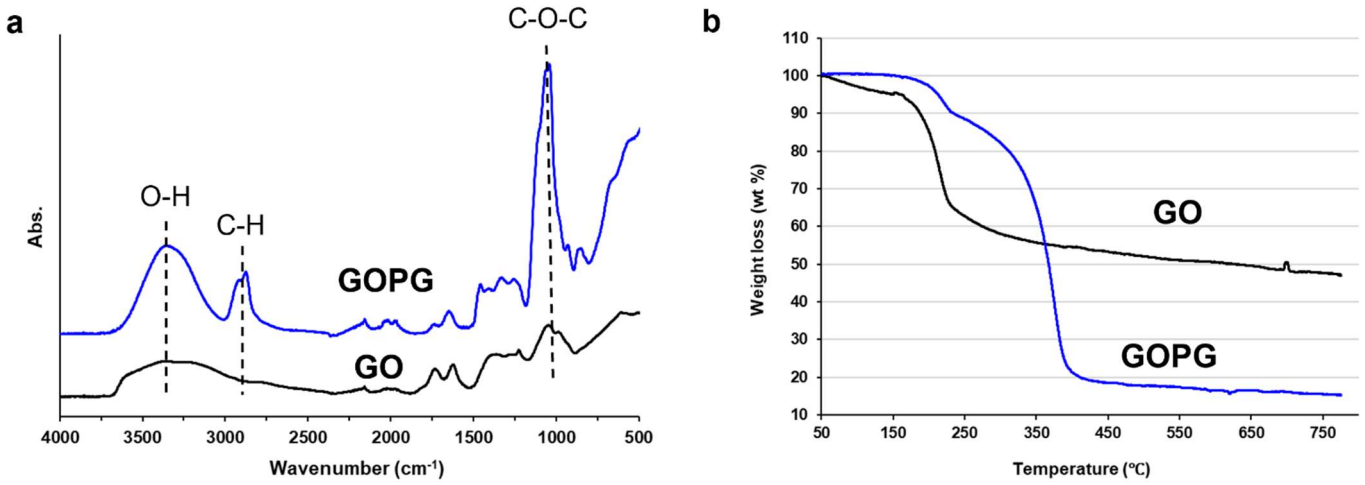


Figure S4. IR spectra (a) and TGA weight loss curves (b) for GO and GOPG.

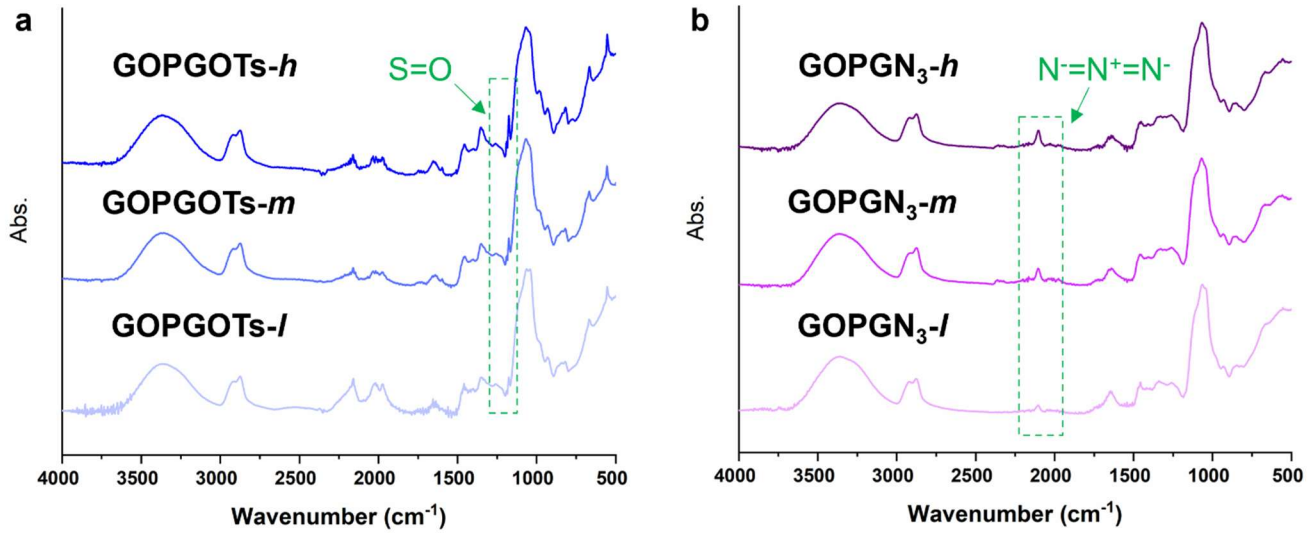


Figure S5. IR spectra of GOPGOTs (a) and $GOPGN_3$ (b) with functional groups of different densities.

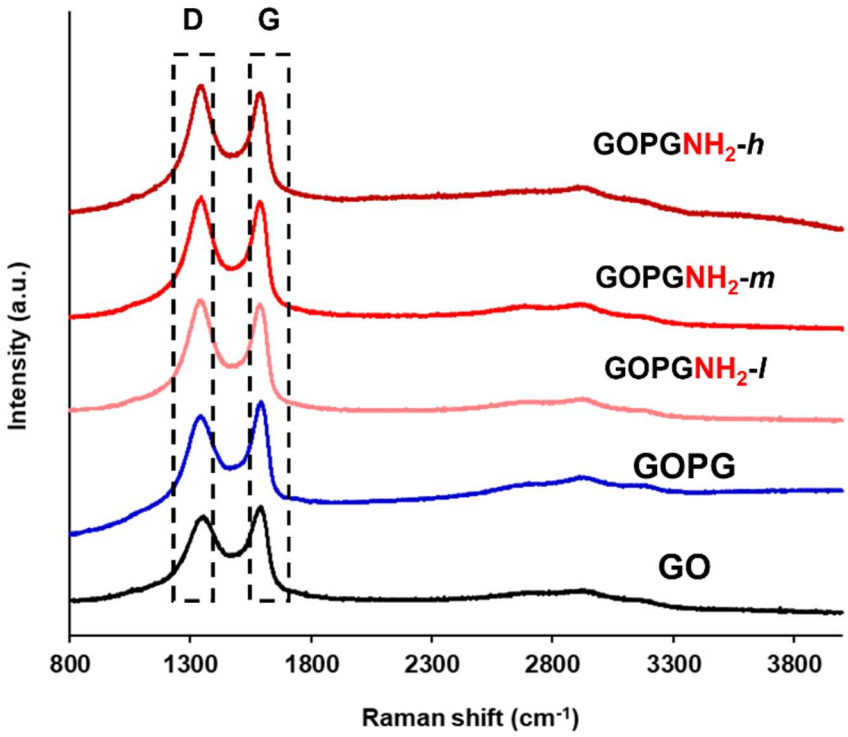
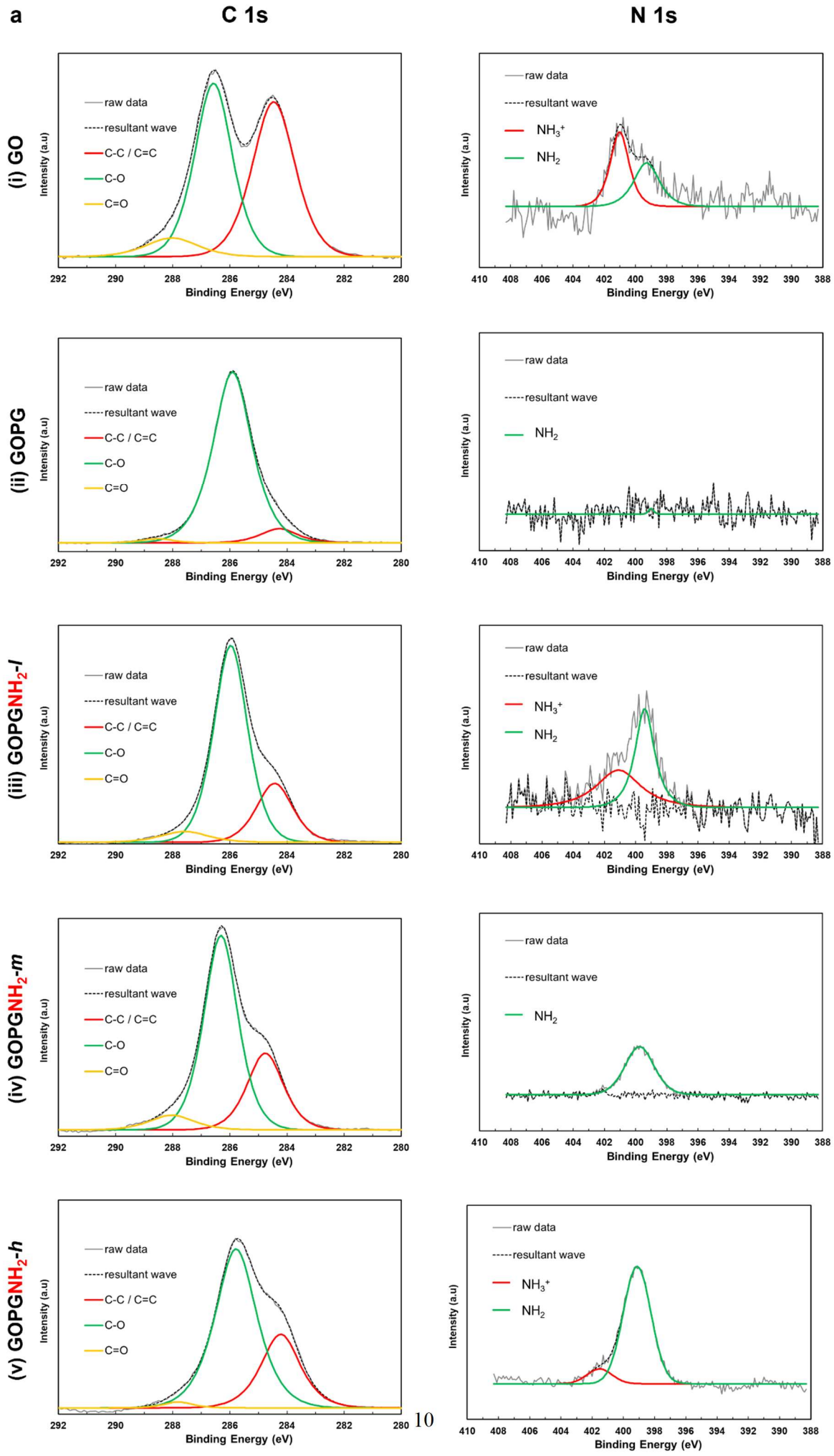


Figure S6. Raman spectra of GO, GOPG, GOPGNH$_2$-*l*, GOPGNH$_2$-*m*, and GOPGNH$_2$-*h*.


a
C 1s
N 1s
(i) GO
(ii) GOPG
(iii) GOPGNH2-l
(iv) GOPGNH2-m
(v) GOPGNH2-h
raw data
resultant wave
C-C / C=C
C-O
C=O
NH3+
NH2
Intensity (a.u)
Binding Energy (eV)
292
290
288
286
284
282
280
410
408
406
404
402
400
398
396
394
392
390
388

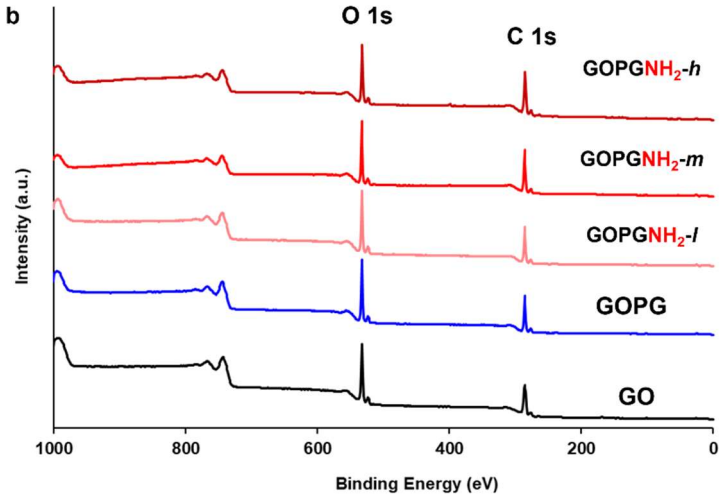


Figure S7. (a) XPS survey scan of GO, GOPG, GOPGNH$_2$-*l*, GOPGNH$_2$-*m*, and GOPGNH$_2$-*h*. (b) High resolution C 1s (left panel) and N 1s (right panel) spectra of (i) GO, (ii) GOPG, (iii) GOPGNH$_2$-*l*, (iv) GOPGNH$_2$-*m*, and (v) GOPGNH$_2$-*h*.

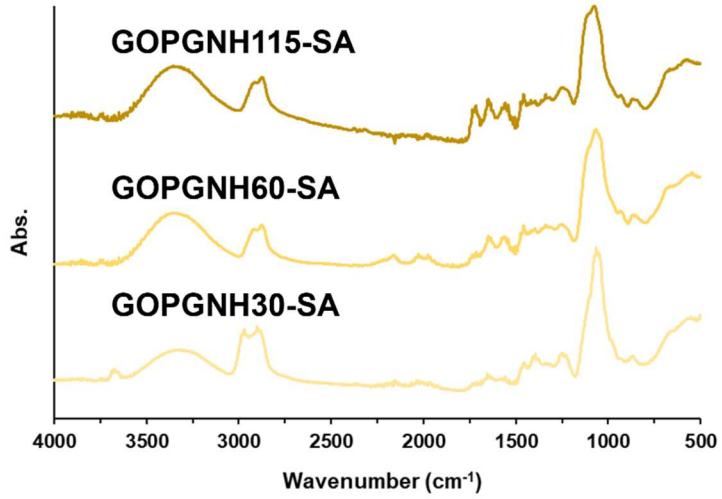


Figure S8. IR spectra of GOPGNH-SA. GOPGNH115-SA, GOPGNH60-SA and GOPGNH30-SA were prepared from GOPGNH$_2$ bearing amino group of 33, 61 and 115 μmol/g, respectively.

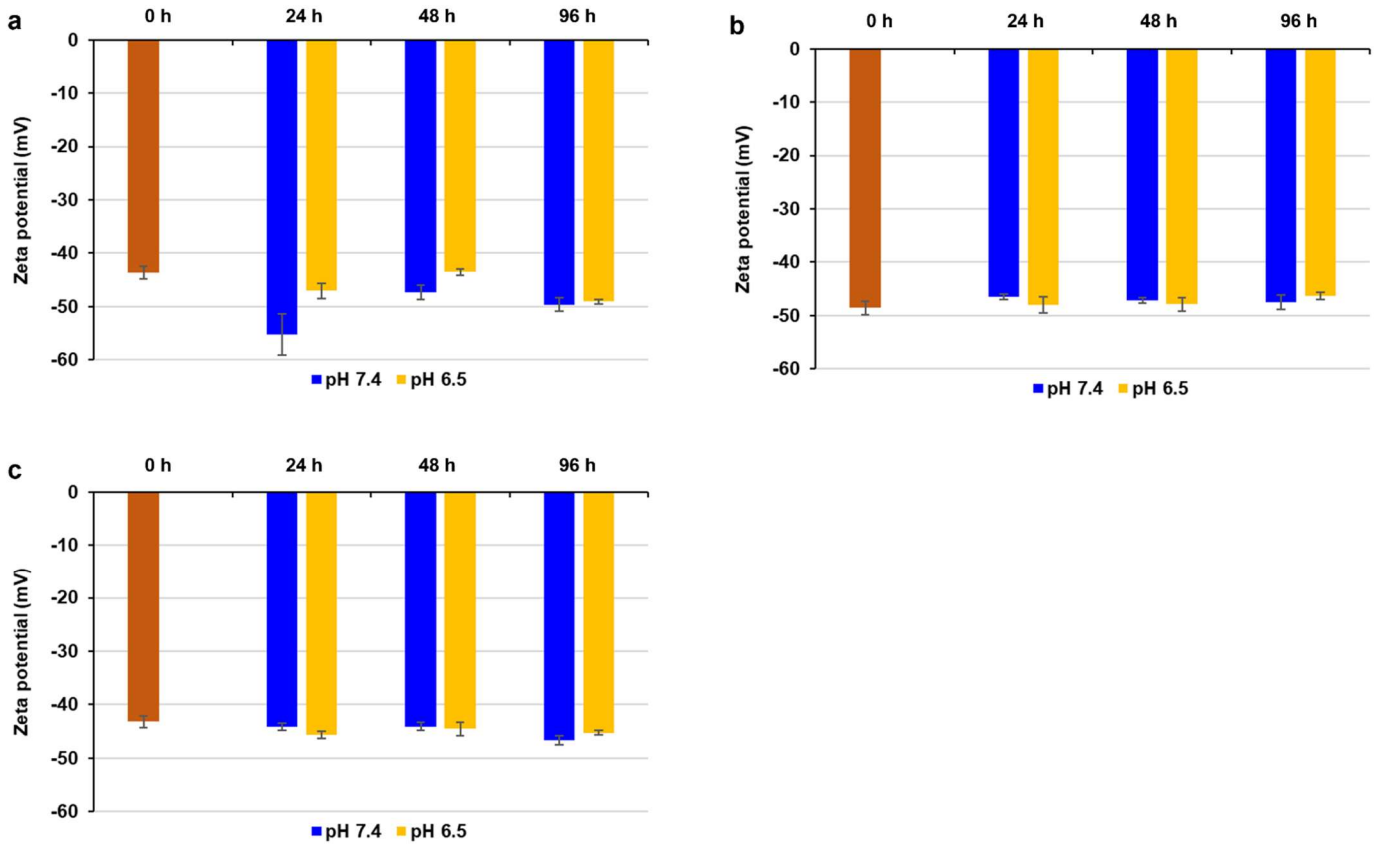


Figure S9. Zeta potentials of (a) GOPGNH115-SA, (b) GOPGNH60-SA and (c) GOPGNH30-SA just after synthesis (0 h) and after their incubation in phosphate buffer at pH 7.4 and 6.5 for 24, 48 and 96 h.

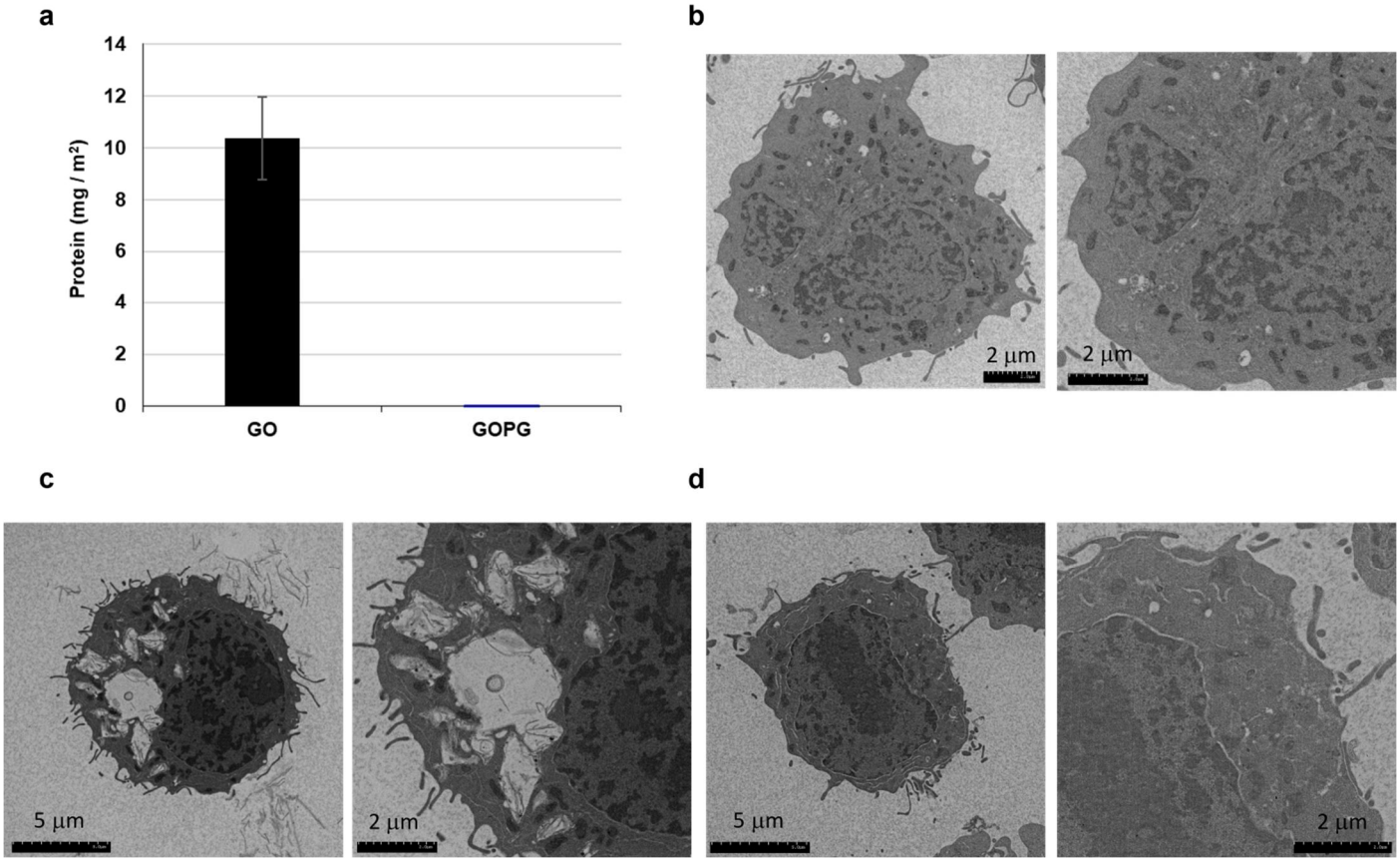


Figure S10. Analysis of protein corona formed on GO and GOPG and their uptake by J774A.1 macrophages. (a) BCA quantification of proteins adsorbed onto GO and GOPG. (b-d) TEM images of J774A.1 macrophages after their treatment with (b) PBS, (c) GO and (d) GOPG for 24 h. The pristine GO can absorb high amounts of proteins (>10 mg m^{-2}), which consequently leads to the extensive association of GO sheets with the membrane of J774A.1 cells and their cellular internalization (c). In contrast to pristine GO, almost no protein was detected on the surface of GOPG, resulting in non-observable GO sheets surrounding J774A.1 or inside the cells. Indeed, the cells treated with GOPG (d) exhibited similar morphologies to control cells (b), *e.g.*, intact structures with several small vesicles.

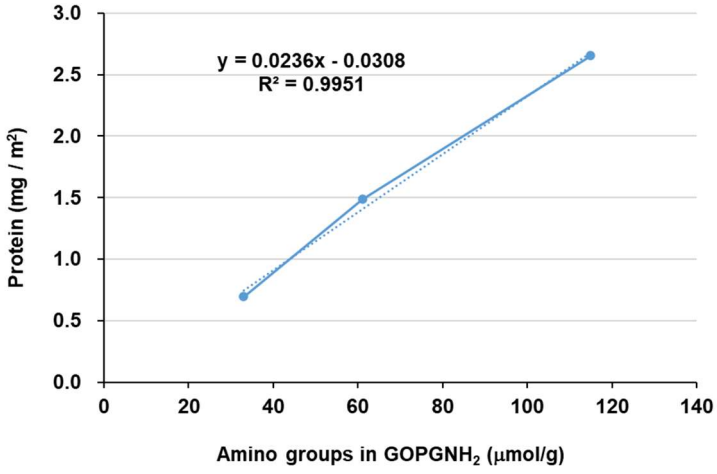


Figure S11. The linear correlation of proteins adsorbed on GOPGNH$_2$-*l*, -*m* and -*h* against the content of amino group.

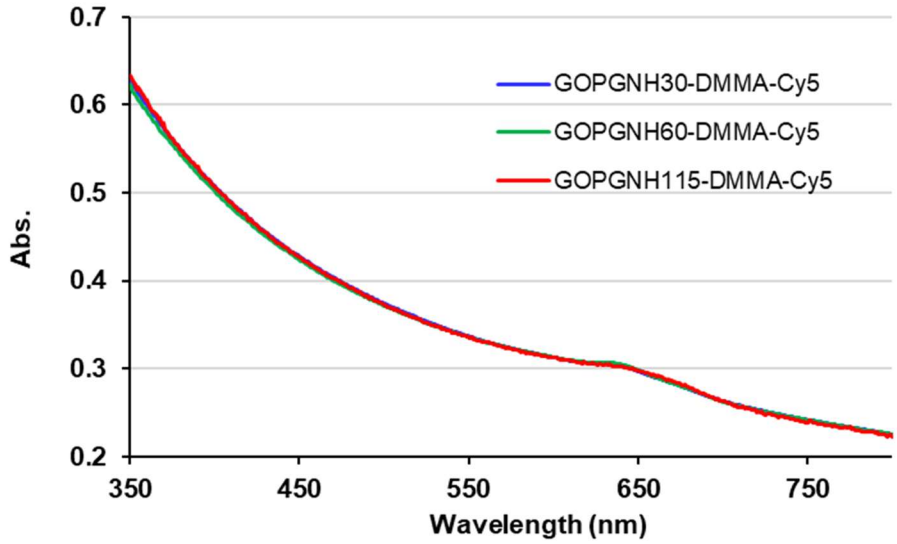


Figure S12. UV spectra of the different GOPGNH-DMMA-Cy5 conjugates recorded at material concentration of 0.1 mg/mL. Since the amount of free unreacted amines in GOPGNH-DMMA (around 3.3, 6.1 and 11.5 μmol/g in GOPGNH30-DMMA, GOPGNH60-DMMA and GOPGNH115-DMMA, respectively) is higher than the amount of added Cy5-NHS ester (3 μmol/g GOPGNH-DMMA), the Cy5 molecules are assumed to fully react to afford similar fluorescence intensities in all the GOPGNH-DMMA-Cy5 conjugates.

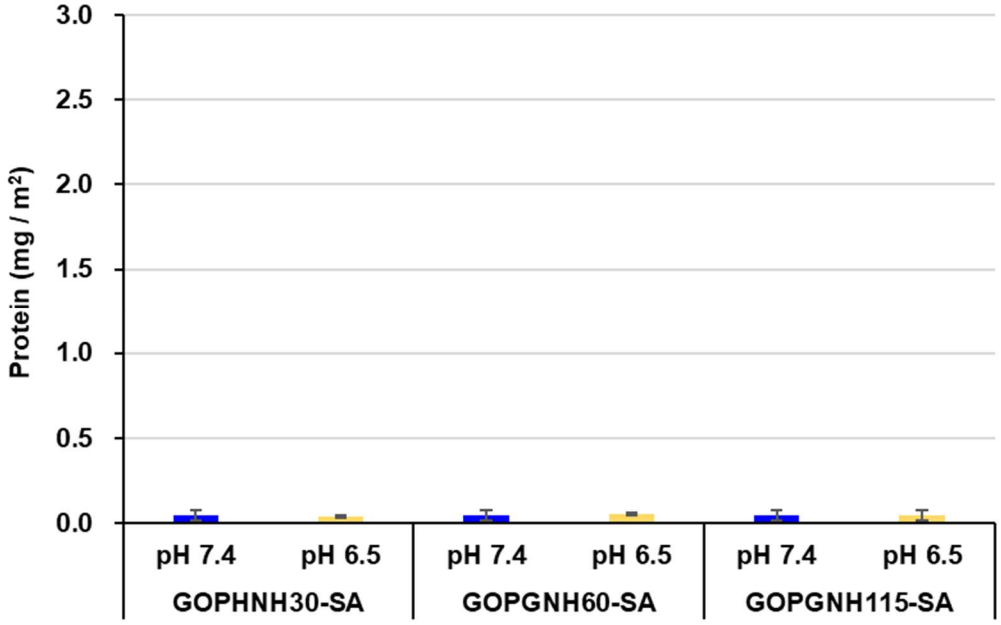


Figure S13. BCA quantification of FBS proteins adsorbed onto GOPHNH-SA at pH 7.4 and 6.5 (96 h).

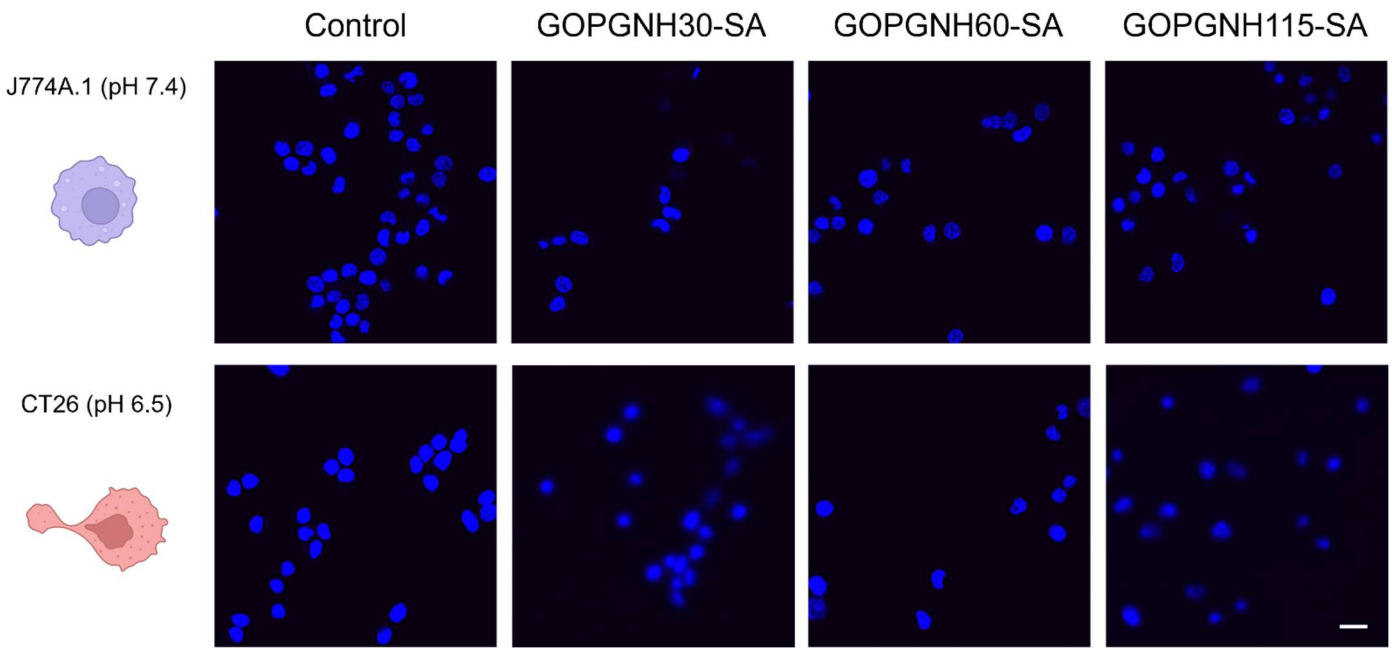


Figure S14. Uptake of GOPGNH-SA by J774A.1 macrophages (upper row) and CT 26 cells (lower row) at 96 h. Scale bar, 20 μm.

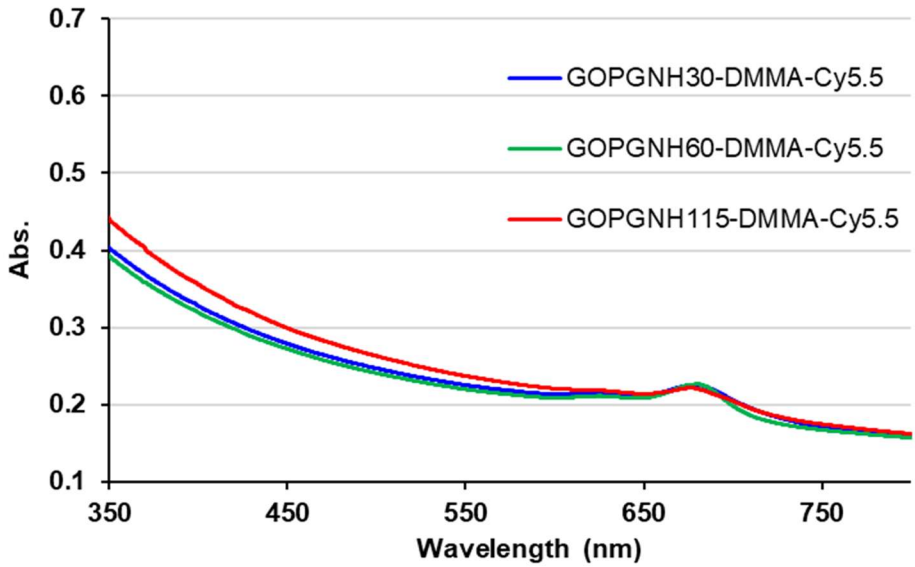


Figure S15. UV spectra of the different GOPGNH-DMMA-Cy5.5 conjugates recorded at material concentration of 0.075 mg/mL.

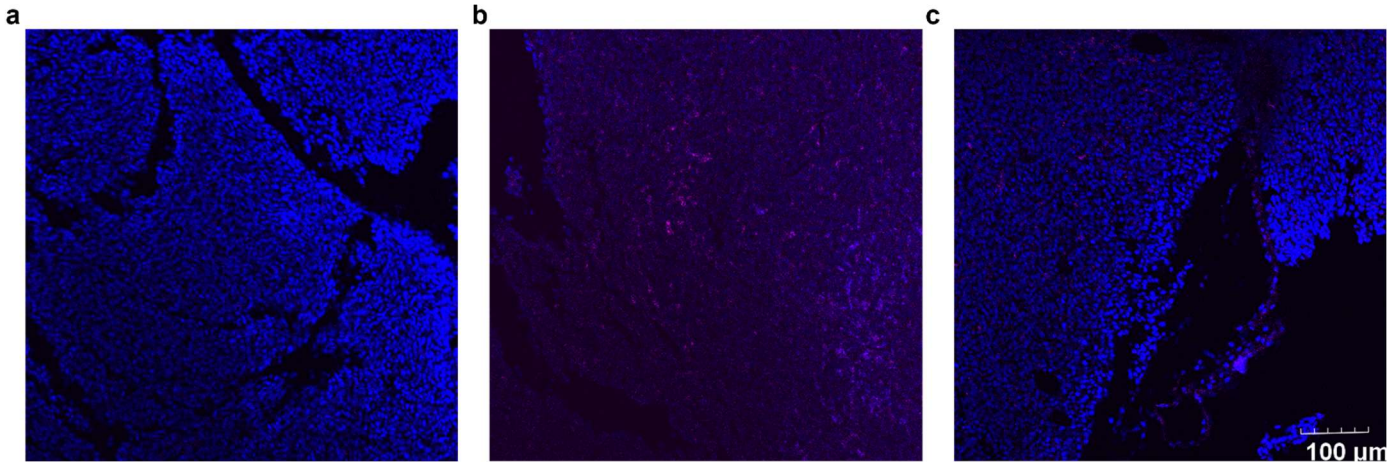


Figure S16. CLSM images of tumor tissue sections collected from mice injected with (a) GOPGNH115-DMMA-Cy5.5, (b) GOPGNH60-DMMA-Cy5.5 and (c) GOPGNH30-DMMA-Cy5.5. Scale bar, 100 μm.

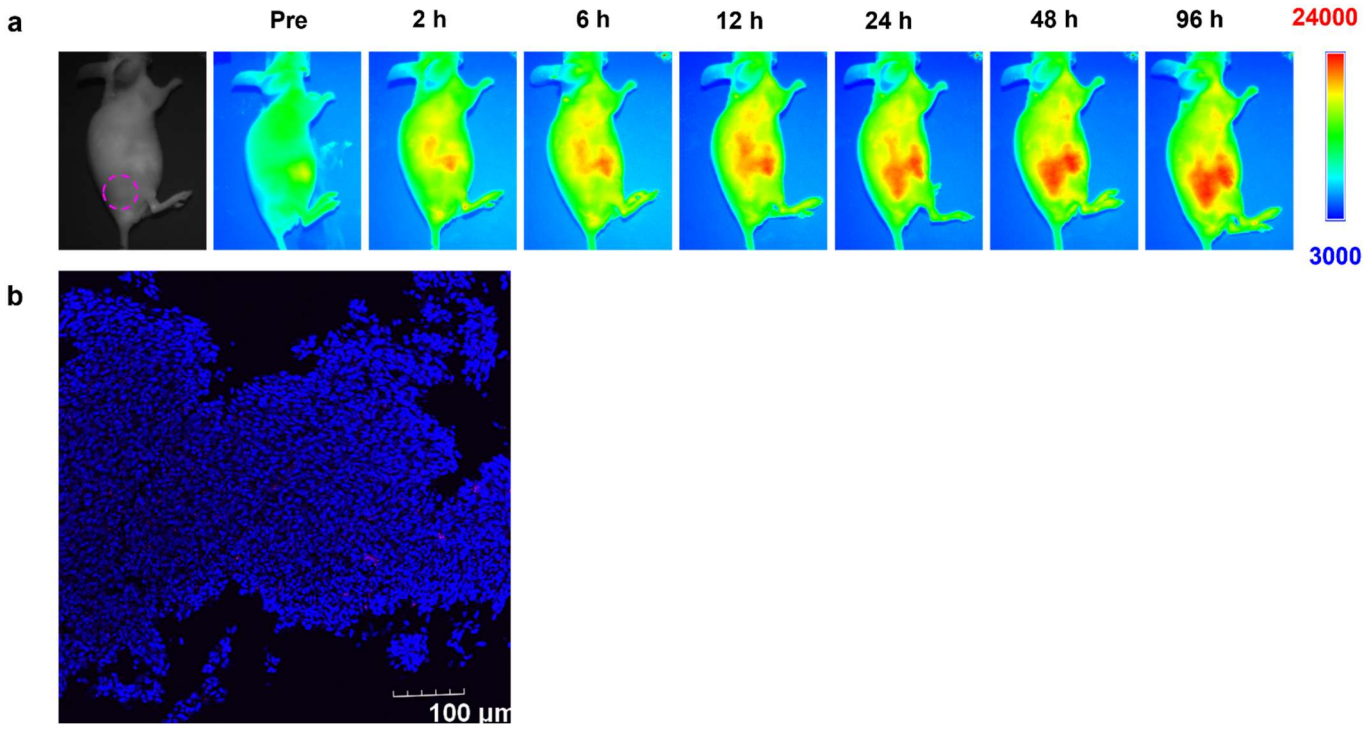


Figure S17. *In vivo* distribution and tumor cellular internalization of GOPGNH60-SA-Cy5.5. (a) Live animal imaging of CT26 tumor bearing BALB/c nude mice intravenously administrated with GOPGNH60-SA-Cy5.5 at pre injection, and 2, 6, 12, 24, 48 and 96 h post injection. (b) CLSM images of tumor tissue sections. Scale bar, 100 μm.

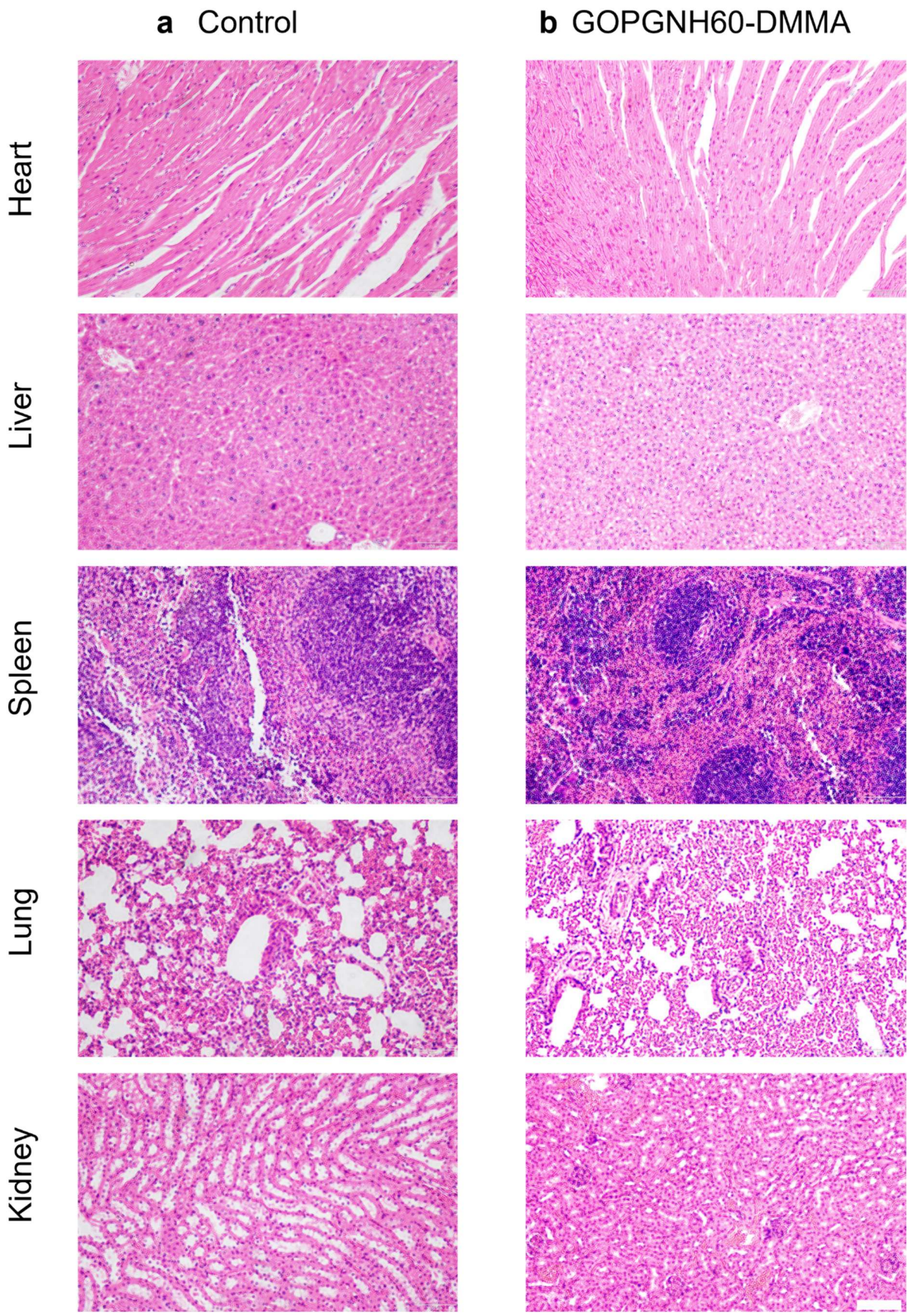


Figure S18. H&E staining of tissue sections of mice at 96 h-post injection with (a) PBS and (b) GOPGNH60-DMMA. Scale bar, 100 μm.

Table S1. XPS analysis of the atomic content of carbon, oxygen, and nitrogen on the surface of GO, GOPG, $GOPGNH_2$-*l*, $GOPGNH_2$-*m*, and $GOPGNH_2$-*h*.

GO conjugates	C 1s (at%)	O 1s (at%)	N 1s (at%)
GO	66.33	32.09	0.35
GOPG	62.80	37.03	0.06
$GOPGNH_2$-*l*	67.88	31.5	0.58
$GOPGNH_2$-*m*	66.79	32.36	0.80
$GOPGNH_2$-*h*	64.66	33.54	1.59

Table S2. Content of amino groups in $GOPGNH_2$ determined by Kaiser test.

GO conjugates	Average content of amino group (μmol/g)	Standard derivation (μmol/g)[a]
$GOPGNH_2$-*l*	33.06	1.53
$GOPGNH_2$-*m*	60.66	5.21
$GOPGNH_2$-*h*	115.20	2.51

[a]From three independent measurements.

The calculation processes of the total surface area of GOPG (350 μg) in $GOPGNH_2$ and GOPGNH-DMMA samples for SDS-PAGE and BCA analyses.

1. The weight of a single GO nanosheet (500 nm lateral size) can be calculated as follows.

In the basal plane of GO, one carbon atom is shared by three hexagonal rings. Therefore, one hexagonal ring occupies two carbon atoms, and its weight can be calculated from the molar mass of carbon (12.01 g mol^{-1}) and Avogadro constant (6.023×10^{23} mol^{-1}).

$$W = 2\times\frac{12.01\ \mathrm{g}\ mol^{-1}}{6.023\times10^{23}\ mol^{-1}} = 3.988\times10^{-5}\ \mathrm{ag} \quad (S1)$$

The area of a hexagonal ring in the basal plane of GO can be calculated *via* following equation, given the distance of 0.142 nm between two neighboring carbon atoms.

$$S = 3\times d^2\frac{\sqrt{3}}{2} = 3\times(0.142\ nm)^2\times\frac{\sqrt{3}}{2} = 5.246\times10^{-2}\ nm^2 \quad (S2)$$

The number of hexagonal rings in GO nanosheet (500 nm lateral size) is calculated based on the area of GO nanosheet and the one of hexagonal ring.

$$\mathrm{N}= \frac{S_{GO}}{\mathrm{S}} = \frac{500\ nm \times 500\ nm}{5.246\times10^{-2}\ nm^2} = 4.766 \times 10^6 \qquad (S3)$$

The weight of carbon atoms in a single GO nanosheet (500 nm lateral size) can be derived from the weight and number of hexagonal rings in the basal plane of GO.

$$W_C = W \times \mathrm{N} = 3.988 \times 10^{-5}\ \mathrm{ag} \times 4.766 \times 10^6 = 190\ ag \qquad (S4)$$

The mass of a single GO nanosheet W_{GO} is determined to be around 380 ag, based on the weight of carbon and its content in GO (~50 wt%, TGA, Figure S4b).

2. The weight of a single GOPG nanosheet was calculated based on the mass of a GO nanosheet and its content in GOPG (C_{GO}~30 wt%, according to TGA, Figure S4b).

$$W_{GOPG} = \frac{W_{GO}}{C_{GO}} = \frac{380\ ag}{30\%} = 1267\ \mathrm{ag} \qquad (S5)$$

3. Number of GOPG in 350 μg samples.

$$N_{GOPG} = \frac{350\ ug}{W_{GOPG}} = \frac{350\ ug}{1267\ ag} = 2.76 \times 10^{11} \qquad (S6)$$

4. Surface area of a single GOPG nanosheet with lateral size of 500 nm (assumed to be a flat 2D structure).

$$S_{GOPG} = 500\ nm \times 500\ nm \times 2 = 5.0 \times 10^5\ nm^2 \qquad (S7)$$

5. Total surface area of GOPG nanosheets in 350 μg samples.

$$S_{GOPG,\ Total} = S_{GOPG} \times N_{GOPG} = 5.0 \times 10^5\ nm^2 \times 2.76 \times 10^{11} = 1.38 \times 10^{-2}\ m^2 \qquad (S8)$$

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