Supporting Information

Nitric Oxide-Dependent Biodegradation of Graphene Oxide Reduces Inflammation in the Gastrointestinal Tract

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Experimental Section

GO characterization

Graphene oxide (GO) produced by Hummers' method was obtained from Graphenea (Spain) and characterized as described.[1] The structural properties for the GO in E3 medium were determined by transmission electron microscopy (TEM) and atomic force microscopy (AFM). Specifically, carbon film coated TEM grids were pre-treated with a glow discharge using a current of -25 mA and for a duration of 30 min. Solutions of 50 μg/mL were drop-casted on the grids, left to stand for 2 min before removal of excess sample leaving a thin film which was left to dry for at least 30 min before imaging. TEM images were acquired at 80 kV, and the size distribution analysis was determined using ImageJ. AFM images were acquired using a Bruker Multimode 8 AFM in tapping mode with an OTESPA probe. Additionally, the hydrodynamic diameter and zeta potential were determined using a ZetaSizer Nano instrument (Malvern Instruments Ltd., UK) at a concentration of 50 μg/mL.

Endotoxin assessment

Endotoxin content was determined based on TNF-α secretion in primary human monocyte-derived macrophages in the presence or absence of the specific LPS inhibitor, polymyxin B, as previously reported.[2] GO samples were found to be endotoxin-free (data not shown).

Zebrafish experiments

The wild-type and transgenic adult zebrafish (*Danio rerio*) were maintained at 28 ± 0.5℃ on a 14 h:10 h light/dark cycle in the fish breeding circulatory system at the KI zebrafish core facility. Two pairs of male/female fish were placed in a single mating tank with a divider one day prior to spawning. Spawning was triggered by removing the divider in the morning. Embryos were collected after 2 h, washed, and then transferred to E3 medium in a petri-dish. The E3 medium was prepared following a standard recipe (0.29 g/L NaCl, 0.0133 g/L KCl, 0.0483 g/L $CaCl_2 \cdot 2H_2O$, 0.0815 g/L $MgCl_2 \cdot 6H_2O$; pH=7). Healthy and fertilized embryos at the same developmental stages were selected and raised up to 5 dpf for further experiments. Zebrafish larvae (conventional fish and fish with knockdown of *nos2a*) were exposed at 5 dpf to LPS and GO (5 μg/mL) for 24 h. LPS at 10 μg/mL was

used as positive control. L-NAME (15 mM) was used to inhibit NOS in conventional fish. After exposure, larvae were used for further assays as described below. All the procedures were carried out in compliance with national ethical guidelines and the study was approved by the regional committee for animal experiments in Stockholm (permit no. 14049-2019).

Knockdown of *nos2a*

The zebrafish genome contains one *nos1* gene and two *nos2* genes (*nos2a* and *nos2b*), and a previous study has shown that *nos2a* displays the inducible properties of a classical inducible nitric oxide synthase (iNOS).[3] To knock down *nos2a*, morpholino oligonucleotides (MO*nos2a*, ACAGTTTAAAAGTACCTTAGCCGCT) (Gene Tools, Philomath, OR) were injected at non-toxic concentrations (0.6 mM) into one-cell stage zebrafish embryos as described.[4] Healthy and fertilized embryos were selected and raised to 5 dpf for further experiments. Validation of the *nos2a* knockdown was evaluated by RT-qPCR.

RT-qPCR

RNA was extracted using a commercial total RNA extraction kit (RNeasy Mini Kit, QIAGEN, Sweden). RNA concentration was quantified by NanoDrop spectrophotometer. Total RNA (500 ng) was reverse transcribed using the iScript Advanced cDNA Synthesis Kit (Bio-Rad, Sweden) following the manufacturer's protocol. Transcription of *nos2a* (forward primer: ATGGGAAGACAAGCACAAACC, reverse primer: CATCTGGGGAAGGTGTGATT) was quantified by relative RT-qPCR using a QuantStudio 5 Real-Time PCR System. Reaction mixtures were formulated using Absolute qPCR SYBR Green Mix (ThermoScientific). Thermal cycling conditions were: 95°C for 10 min, 40 cycles of 3-step amplification of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. The transcription level was normalized to zebrafish *β-actin* and then calculated relative to control using the $2^{-\Delta\Delta Ct}$ method. All qPCR reactions were conducted in triplicate for each sample.

NO/$ONOO^-$ production *in vivo*

NO production in zebrafish was determined with the DAF-FM-DA reagent[5] obtained from Sigma-Aldrich (Stockholm). Briefly, 10 larvae for each treatment were washed, incubated

with DAF-FM-DA according to the manufacturer's protocol, and incubated for 1 h in the dark at 28.5°C. The live samples were then washed, anaesthetized in 0.01% tricaine solution, and positioned in 1% low-melt agarose for imaging. Fluorescence images were captured using a fluorescence microscope operating with a FITC filter set (SMZ25 NIKON). The integrated fluorescence intensity of the gut was quantified and corrected with ImageJ. Peroxynitrite generation, in turn, was determined by using DAX-J2 PON Green,[6] according to the manufacturer's instructions (AAT BioQuest, Sunnyvale, CA). After exposure, the fish were incubated with the fluorescent probe for 2 h in the dark at 28.5°C. The live samples were then collected, and fluorescence images were captured as above.

Inflammation assessment *in vivo*

Transgenic zebrafish *Tg*(*mpx*:eGFP) expressing GFP under the neutrophil-specific myeloperoxidase (*mpo*) (also called *mpx*) promoter was used to visualize the movement of neutrophils.[7] After exposure as indicated, the live samples were washed, anaesthetized, and positioned for fluorescence and confocal images. Fluorescence images were captured using a fluorescence microscope operating with a FITC filter (SMZ25 NIKON) while confocal images were captured with a confocal microscope (Zeiss LSM880). The numbers of neutrophils migrated to the gut were quantified manually based on 10 fish per treatment group.

Raman confocal microspectroscopy

GO degradation was monitored by Raman confocal analysis as described previously for mammalian model systems.[8] In brief, for the *in vitro* samples, GO was prepared by drop-casting 20 µl onto a quartz slide and allowed to dry before the analysis. Raman analysis was performed using a confocal Raman microscope (WITec alpha300 system) with a laser of 532 nm wavelength set at an integration time of 0.5 s and 60× magnification. The scan area for each sample was adjusted to 50 × 50 µM. For determination of the D-band and G-band, an average of the whole scan (i.e., 2500 spectra per sample) was calculated and displayed. For the *in vivo* samples, zebrafish larvae exposed to GO for 24 h were washed, anaesthetized in 0.01% tricaine solution, and positioned in 1% low-melt agarose on glass slides. Samples were then dried on a plate heater at 50°C prior to loading onto the confocal

Raman microscope. The scan area was adjusted to 50 × 200 μM. GO spectra shown for each treatment represent the average of 10.000 spectra recorded across the whole area scan.

NO/ONOO$^-$ production *in vitro*

Real-time NO production was determined by a chemiluminescence NO analyzer (Eco Physics, Durnten, Switzerland) as previously described.[9] Briefly, after a period of equilibration (5 min), 2 mL of PAPA NONOate solution (300 μM) (Abcam, Cambridge, UK) was injected. Air was used as a carrier. Xanthine/xanthine oxidase (X/XO) (Sigma-Aldrich) were injected to investigate whether the NO signal would decline due to the reaction of NO with superoxide radicals. For SIN-1, nitrogen gas was used as the carrier. In brief, 2 mL of SIN-1 solution (300 μM) (Sigma-Aldrich) were injected and recorded for 5 min, followed by another injection of SIN-1 (900 μM). NO production was recorded throughout the experiment by using a data acquisition system (AcqKnowledge v3.9, Biopac MP150, Goleta, CA). Peroxynitrite generation was evaluated by the DAX-J2 PON Green assay,[6] following incubation with the indicated concentrations of SIN-1 for 2 h. PAPA NONOate (without X/XO) was used as a negative control. The fluorescence signal was captured every 20 min in a microplate reader (Infinite F200 Tecan, Männedorf, Switzerland).

GO degradation analysis *in vitro*

GO (50 μg per sample) suspended in PBS was incubated with peroxynitrite for 5 days. Peroxynitrite was generated by SIN-1, simultaneously producing nitric oxide and superoxide, or a NO donor (PAPA NONOate), combined with superoxide-generating xanthine/xanthine oxidase (X/XO).[10] PAPA NONOate and X/XO alone were used as controls. SIN-1, PAPA NONOate and xanthine were added 5 times per day (every 1.5 h) and the final concentration was 300 μM. Additionally, 1.5 μL of solution containing 0.125 mU of xanthine oxidase was added in the morning and in the evening. After 5 days of treatment, samples were stored at -20ºC for further analysis by Raman (see above), TEM, and AFM. *Transmission electron microscopy.* For TEM analysis, 20 μL of the GO suspension were dropped on a carbon-coated copper grid and dried overnight. Thereafter, the grids were washed by dipping in Milli-Q® water three times to remove the salts from the samples. The samples were analyzed by JEOL 2100 TEM with 200 kV accelerating

voltage. *Atomic force microscopy*. For AFM, GO samples were spin-coated on a clean mica sheet and measurements were performed under a nitrogen atmosphere with the PSIA XE 150 SPM/AFM instrument (Park System, South Korea). Topographical images were acquired using large scale scans and images were analyzed using XEI software (Park Systems).

Statistical analysis

The *in vivo* assays were conducted three times using 10 fish per treatment and statistical analysis of the data was performed using Student's t-test (GraphPad, Prism 8). Results shown are mean values ± S.D. Statistically significant differences were considered when $p < 0.05$.

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Table S1. Summary of the physicochemical characterization of GO sheets.

Physicochemical properties	***Technique***	***GO***
Lateral dimension (90% of particles)	*TEM*	0.8 -5 µm
	SEM	0.1 -3 µm
	Optical microscopy	0,8 - 15 µm
	AFM	0.2 -2 µm
Thickness	*AFM*	1 - 2 nm
Optical properties	*Absorbance*	A_{230}=0.059 * C_{GO} (µg/mL)
	Fluorescence, λ_{exc}*=525nm*	F_{600}=0.643 * C_{GO} (µg/mL)
Degree of defects (*I_D/I_G*)	*Raman spectroscopy*	1.33 ± 0.03
Surface charge	*ζ-Potential*	-40.6 ± 1.5 mV
Functionalization degree	*TGA*	45%
C% & O% Composition		C: 66.6±0,3; O: 31.0±0,2
Impurities (%)		S: 2,0±0,1; B: 0,4±0,2
Purity (%C + %O)		97,6±0,2
C:O ratio		2,1 ± 0,0
C-C & C=C	*XPS (n=3)*	42,8 ± 0,1 % (284,6 eV)
C-O		41,9 ± 2,5 % (286,6 eV)
C=O		8,4 ± 2,2 % (287,3 eV)
O=C-O		5,7 ± 0,4 % (288,6 eV)
π-π*		1,2 ± 0,1 % (290,2 eV)

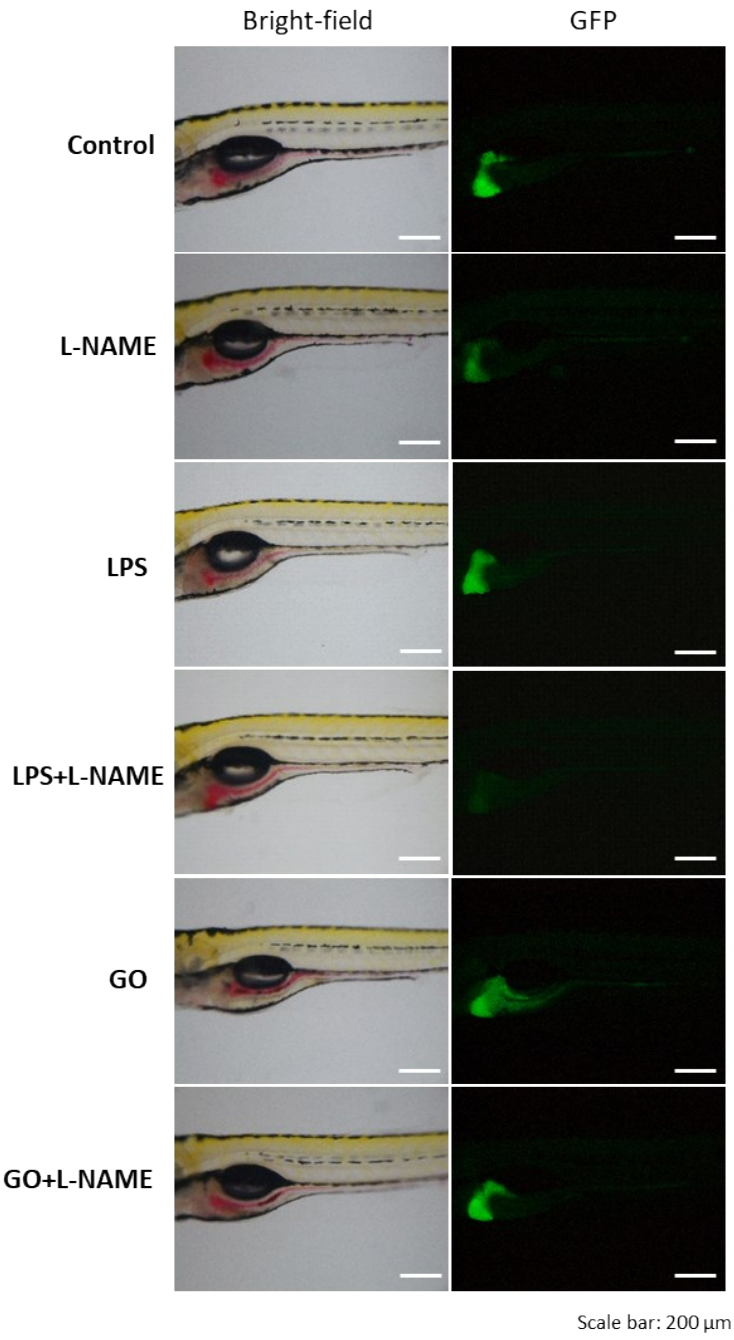

Figure S1. Peroxynitrite generation in zebrafish. Zebrafish larvae were exposed to LPS, LPS+L-NAME, GO, GO+L-NAME, L-NAME for 24 h, and then incubated with DAX-J2 PON Green for 2 h. Fluorescence images with a FITC filter set were captured using a fluorescence microscope. Fluorescence indicative of $ONOO^-$ was observed in GO-exposed fish.

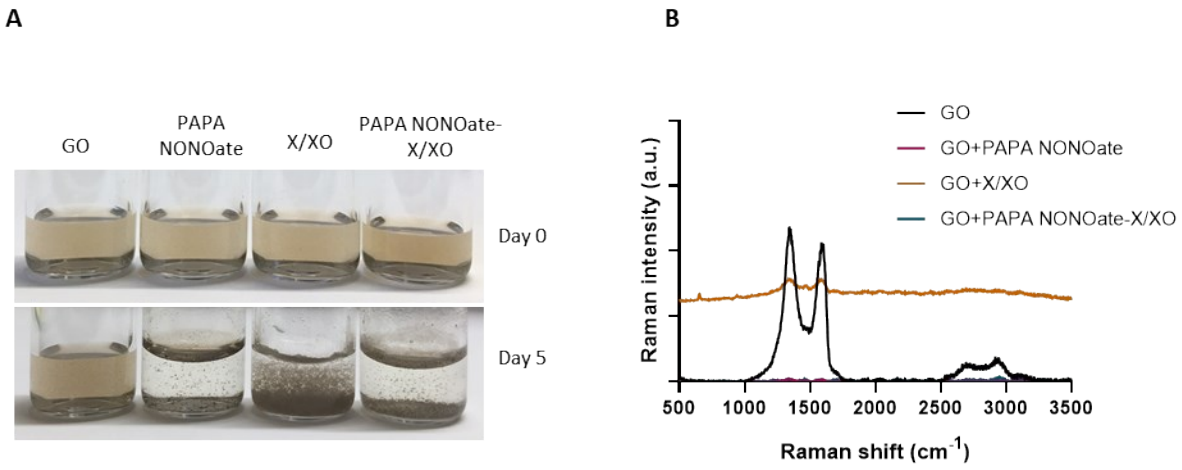

Figure S2. Peroxynitrite-dependent degradation of GO. (a) Visual evidence of degradation upon incubation of GO with PAPA NONOate and PAPA NONOate-X/XO for 5 days. X/XO alone did not exert observable effects. (b) Raman analysis of GO after incubation with PAPA NONOate, X/XO, and PAPA NONOate-X/XO. Degradation of GO was observed after incubation with PAPA NONOate and PAPA NONOate-X/XO as evidenced by the loss of the D and G bands. However, D and G bands remained unaffected after incubation with X/XO.

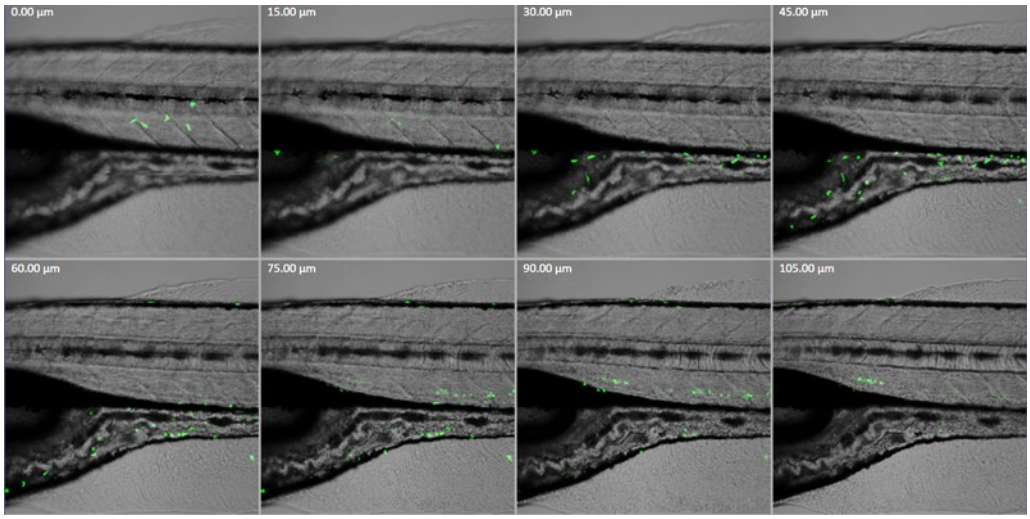

Figure S3. Neutrophil migration in zebrafish following oral uptake of GO. The transgenic zebrafish strain, Tg*(mpx:eGFP)* was utilized to visualize the movement of neutrophils. Zebrafish were exposed to GO and L-NAME (to inhibit NOS) for 24 h. Z-stack images were captured by confocal microscopy. Few neutrophils were observed in the gut lumen, with most cells infiltrating to the lamina propria, the thin layer of tissue beneath the epithelium.