# Shedding Plasma Membrane Vesicles Induced by Graphene Oxide Nanoflakes in Brain Cultured Astrocytes

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**Supplementary Experimental Section**

*Characterization of graphene oxide nanosheets*

The physicochemical characterization was performed as previously described in Rodrigues et al.[1]

The structural properties for the s-GO were determined by atomic force microscopy (AFM) and scanning electron microscopy (SEM). Specifically, AFM images were acquired at 80 kV using a Bruker Multimode 8 AFM in tapping mode with an OTESPA probe. SEM images were recorded on a Magellan 400L field emission scanning electron microscope (Oxford instruments) at the ICN2 Electron Microscopy Unit, which was equipped with an Everhart-Thornley as secondary electrons detector and using an acceleration voltage of 20 kV and beam current of 0.1 nA. Carbon film coated TEM grids were pre-treated with a glow discharge using a current of -25 mA and for a 30 min duration. 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 do dry for at least 30 min before imaging.

Additionally, Raman spectra were collected from DXR micro-Rman spectrometer (Thermo Scientific, UK) with a 50x objective. Zeta potential were determined using a ZetaSizer Nano ZS instrument (Malvern Instruments Ltd., UK) at a concentration of 20 μg/mL. TGA measurements were performed using a TGA 4000 thermogravimetric analyser (Perkin Elmer Ltd, UK) from 25°C

to 995°C at a rate of 10°C/min and using a nitrogen low rate of 20 mL/min. XPS measurements were performed at the National EPSRC XPS User's Service (NEXUS) facility using a Thermo Theta Probe XPS spectrometer with a monochromatic Al K-α source of 1486.68eV.

**References**

[1] A.F. Rodrigues, L. Newman, N. Lozano, S.P. Mukherjee, B. Fadeel, C. Bussy, K. Kostarelos, A blueprint for the synthesis and characterisation of thin graphene oxide with controlled lateral dimensions for biomedicine, 2D Mater. 5 (2018) 035020. https://doi.org/10.1088/2053-1583/aac05c.

**Supplementary Table**

| | *Technique* | ***s*-GO** |
|---|---|---|
| ***Lateral dimension*** | *SEM / AFM* | 0.050 - 0.5 μm |
| ***Thickness*** | *AFM* | 1-2 layers |
| ***Degree of defects ($I_D/I_G$)*** | *Raman* | 1.36 ± 0.03 |
| ***Surface charge*** | *ζ-potential* | -55.9 ± 1.4 mV |
| ***Functionalization degree*** | *TGA* | 41% |
| ***Chemical composition (Purity)*** | *XPS* | C: 67.6%, O: 32.2%, (99.8%)<br>S: 0.2% |
| ***C:O ratio*** | *XPS* | 2,1 |
| ***π-π*, O-C=O, C=O, C-O-C, C=C*** | *XPS* | 2.0%, 10.3%, 21.1%, 7.6%, 26.5% |

**Table S1.** Summary of the physicochemical characterization of s-GO nanosheets.

## Supplementary Figures

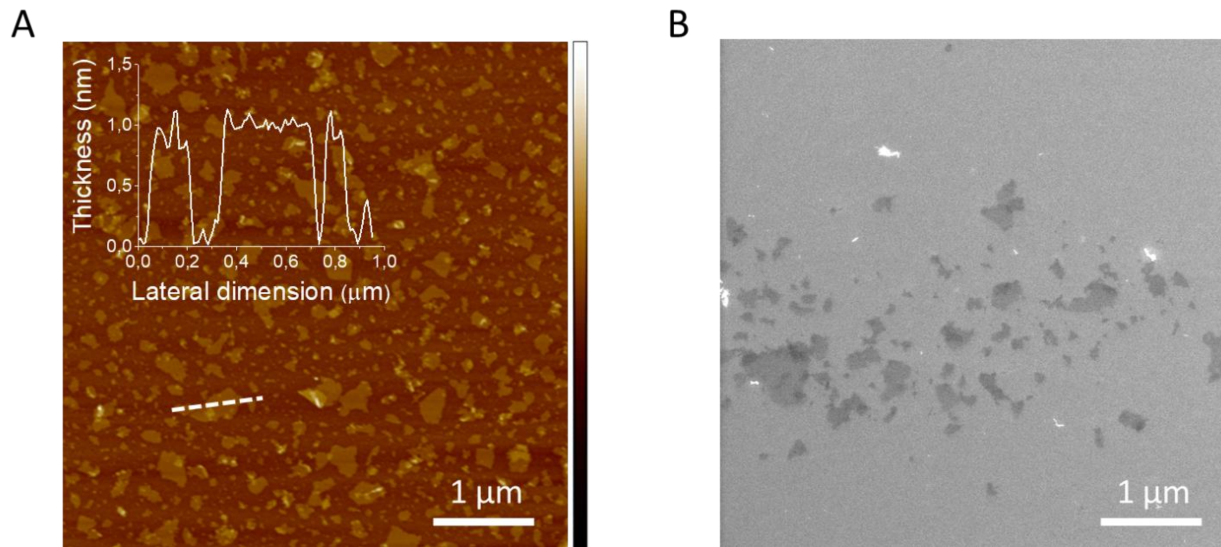


**Fig. S1.** Characterization of small-graphene oxide (s-GO) nanosheets. (A) Height image with insert of cross section analysis along the indicated dashed region and (B) SEM micrograph.

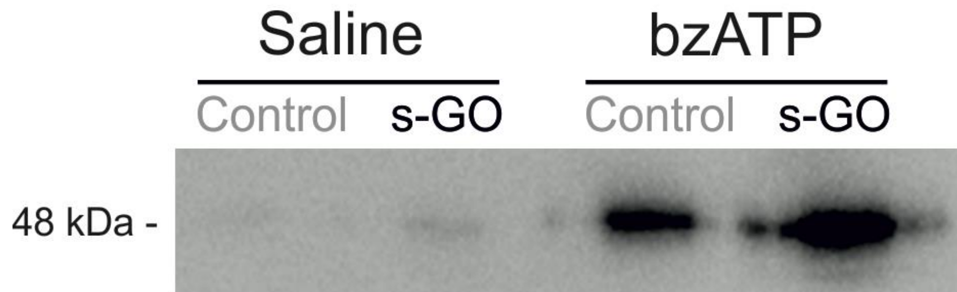


**Fig. S2.** Western blot analysis of pellets for the MV marker flotillin-1. Pellets were obtained from the medium of glial cultures treated or untreated (control) for 3 days with s-GO under two different conditions: stimulated and not stimulated (ringer) by 100 μM bzATP.

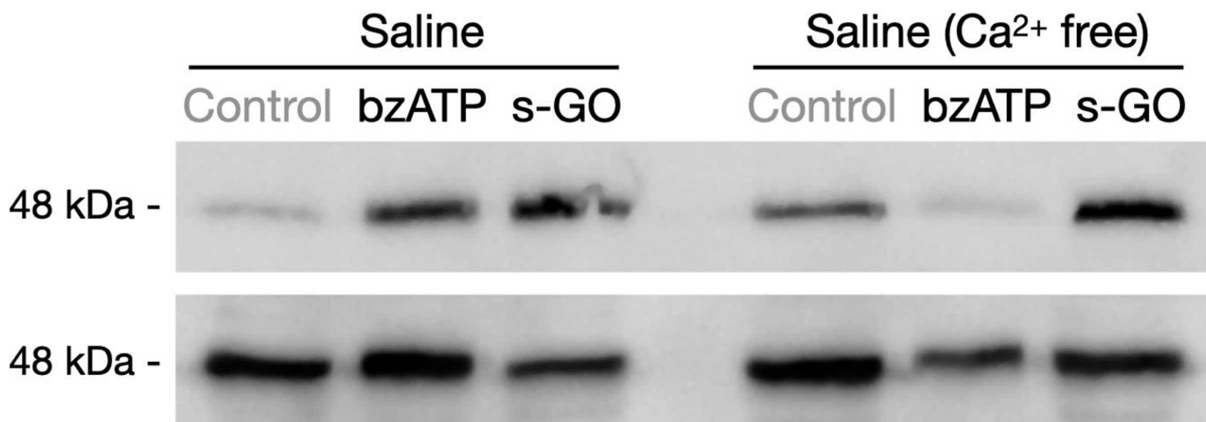


**Fig. S3.** Western blot analysis of pellets (top row) and cell lysates (bottom row) using the MV marker flotillin-1. Glial cultures were treated with bzATP and s-GO (6 days) under two conditions, the traditional saline solution and the saline $Ca^{2+}$ free solution. The saline $Ca^{2+}$ free solution was applied 45 minutes before the MVs harvest in order to allow the $Ca^{2+}$ intracellular storage depletion (s-$GO_{saline}$ is quantified as 150% more than $Control_{saline}$; $bzATP_{saline}$ is quantified as 220% more than $Control_{saline}$ while s-$GO_{ca2+\ free}$ is quantified as 90% more than $Control_{ca2+\ free}$; $bzATP_{ca2+\ free}$ is quantified as 10% less than $Control_{saline}$. Calculated over three independent experiments).

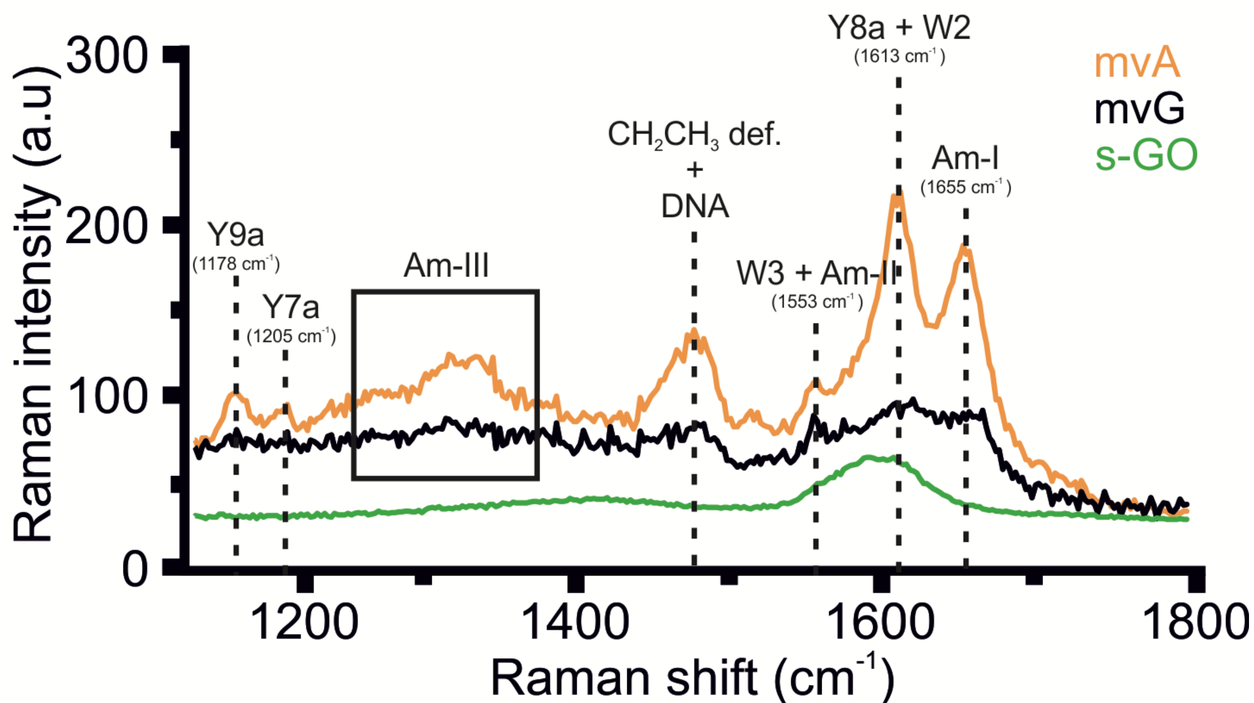


**Fig. S4.** UVRR spectra of extracellular vesicles mvA (in orange), mvG (in black) and graphene oxide (in green). Dashed black lines and the black box are used to highlight the presence of secondary structure and side chains contribution, i.e. amide bands (I, II and III) and the peaks associated to the vibrational modes of tryptophan (W), tyrosine (Y) residues. Due to the complexity of the samples, overlapping bands arisen from diverse vibrational modes characterizing protein secondary and tertiary structures as well as those of nucleic acids are clearly visible in the UVRR spectral region reported here.