Electronic Supplementary Material (ESI) for Environmental Science: Nano.

Biotransformations and cytotoxicity of eleven graphene and inorganic two-dimensional nanomaterials using simulated digestions coupled with a triculture in vitro model of the human gastrointestinal epithelium

*Lila Bazina[1#], Dimitrios Bitounis[1#], Xiaoqiong Cao[1], Glen M DeLoid[1], Dorsa Parviz[2], Michael S Strano[2], Hao-Yu Greg Lin[3], David C Bell[3,4], Brian D Thrall[5], Philip Demokritou[1]**

1. Center for Nanotechnology and Nanotoxicology, HSPH-NIEHS Nanosafety Research Center, Department of Environmental Health, Harvard School T.H. Chan of Public Health, Boston, MA 02115, USA
2. Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
3. Center for Nanoscale Systems, Harvard University, Cambridge, Massachusetts 02138, USA
4. Harvard John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts 02138, USA
5. Biological Sciences Division, Pacific Northwest National Laboratory Richland, WA 99354, USA

Author e-mail addresses:
LB: lbazina@hsph.harvard.edu; DB: dbitounis@hsph.harvard.edu; GD: gdeloid@hsph.harvard.edu; XC: xicao@hsph.harvard.edu; DP: dparviz@mit.edu; MS: strano@mit.edu; HYGL: hlin@cns.fas.harvard.edu; DCB: dcb@seas.harvard.edu; BDT: Brian.Thrall@pnnl.gov; PD: pdemokri@hsph.harvard.edu

[#] Equally contributing first authors
*Corresponding author: Dr. Philip Demokritou
corresponding author email: pdemokri@hsph.harvard.edu

Supplementary information:

Synthesis and size-sorting of graphene-based and inorganic 2DNMs

Graphene oxides

Synthesis of graphene oxide samples was performed according to an improved Hummer's method developed by the authors and previously described by Parviz et al. [1]. Details on the size-sorting of GO-M and GO-L have been described in a previous work by Bitounis et al. [2]. For GO-S, endotoxin-free water was added to graphite oxide in a 200-mL beaker to obtain a 1 mg mL-1 solution. This sample was ultrasonicated using a probe sonicator (Q-sonica 700) at 40% amplitude for 1 hour. The dispersion was then centrifuged at 3220 g for 1 hour to remove large flakes and graphitic aggregates and the supernatant was collected and used as GO-S in further experiments.

Reduced graphene oxides

Graphene oxide suspensions of 1mg/ml concentration, prepared as described above for GO-S or GO-L were used to prepare the small- or large-size variants of reduced graphene oxide, rGO-S, and rGO-L, respectively. Specifically, ascorbic acid (L-AA) was added to either GO-S or GO-L to obtain a 1:10 mass ratio of graphene oxide: L-AA. The mixture was stirred until L-AA was completely dissolved. The solution was then placed on a hot plate and the graphitic plane of graphene oxide was reduced at 50°C for 24 hours. After the sample was cooled down to room temperature, it was centrifuged at 4000 rpm for 30 min to collect the precipitate. The supernatant was decanted, the precipitate was redispersed in endotoxin-free water, and centrifuged again. The washing process was repeated until pH values increased to 6. The final precipitate was collected and dried at room temperature. The powder was then re-dispersed in a Na-cholate solution of 4 mg/ml to obtain 1mg/ml reduced graphene oxide suspensions. The sample was further sonicated at 45% amplitude for 60 min with 10-second pulses separated by 2-second pauses. The dispersions obtained downstream of GO-S or GO-L treatment were centrifuged at 5500 rpm for 5 minutes to remove any remaining aggregates and their respective supernatants were used as rGO-S or rGO-L for further experiments.

Partially reduced graphene oxide

A graphene oxide dispersion of 1mg/ml concentration, prepared as described for GO-S, was used to prepare prGO. Ascorbic acid (L-AA) was added to the dispersion to obtain a 1:0.1 mass ratio of GO: L-AA. The mixture was stirred until the L-AA completely dissolved. The solution was then placed on a hot plate and the oxidized graphitic plane of graphene oxide was reduced at 50°C for 120 min. After the reaction was complete, the sample was cooled down to room temperature and was centrifuged at 4000 rpm for 30 min to collect the precipitate. The supernatant was decanted, the precipitate was redispersed in endotoxin-free water, and centrifuged again. The washing process was repeated until the pH values were increased to 6. The final precipitate was collected and dried at room temperature. The powder was then dispersed in a 4 mg/ml Na-cholate solution to obtain a 1mg/ml suspension of partially reduced graphene oxide. The sample was further sonicated at 45% amplitude for 60 min with 10-second pulses separated by 2-second pauses. The final dispersion was centrifuged at 5500 rpm for 5 minutes to remove any remaining aggregates and the supernatant was collected and used as prGO for further experiments.

Graphenes

To prepare graphene in Na-cholate, expanded graphite powder (Asbury Carbons) was added to a 5 mg/ml solution of Na-cholate at a final concentration of 10 mg/ml. The mixture was sonicated at 50% amplitude for 60 min. The resulting dispersion was then centrifuged at 5500 rpm for 4 hours and the supernatant was collected for further experiments as G-NaC-S.

To prepare graphene in PF108, expanded graphite powder (Asbury Carbons) was added to a 10 mg/ml solution of Pluronic F108 at a final concentration of 10 mg/ml. The mixture was sonicated at 50% amplitude for 60 min and centrifuged for 4 hours at 4000 rpm. The supernatant was collected and used as G-PF108-S in further experiments.

hBN 2DNM

Bulk hBN powder (Sigma-Aldrich) was added to a Na-cholate solution of 4 mg/ml at a final concentration of 10 mg/ml and sonicated at 50% amplitude for 60 min. The dispersion was centrifuged at 5500 rpm for 4 hours, the supernatant was decanted, and the precipitate was redispersed in a 4 mg/ml Na-cholate solution. The sample was sonicated at 50% amplitude for 3 hours, followed by 4 hours of centrifugation at 5500 rpm. The supernatant was collected and used in further experiments as hBN-S.

MoS_2 2DNM

Sodium cholate solution of 4 mg/ml was used for exfoliation. Bulk MoS_2 powder (Sigma-Aldrich) was added to a 4 mg/ml solution of Na-cholate at a final concentration of 10 mg/ml. The mixture was sonicated at 50% amplitude for 60 min. The resulting dispersion was centrifuged at 550 rpm for 4 hours, the supernatant was decanted, and the precipitate was redispersed in a Na-cholate solution of 4 mg/ml. The sample was sonicated at 50% amplitude for 2 hours and centrifuged at 550 rpm. The supernatant was collected, placed in a rotary evaporator at 35°C to increase its concentration and used as MoS_2 2DNm in further experiments.

WS_2 2DNM

Bulk WS_2 powder (Sigma-Aldrich) was added to a 10 mg/ml PF108 solution, and the mixture was sonicated for 60 min at 50% amplitude, followed by centrifugation at 4000 rpm for 100 min. The precipitate was collected in a 10mg/ml PF108 solution and re-sonicated at 50% amplitude for an additional 120 min. The dispersion was then centrifuged at 2000 rpm for 100 min and the supernatant was used as WS_2 2DNM in further experiments.

Physicochemical and biological characterization of as-synthesized graphene-based and inorganic 2DNMs

Atomic force microscopy (AFM)

As-synthesized 2DNM dispersions were diluted to 0.01 mg/mL by addition of deionized water (diw). A drop of the diluted sample was placed on an AFM-grade mica substrate. After 30 min, the sample was gently washed with diw for 5 second and dried using N_2 gas. The washing and drying cycle was repeated once more to assure the removal of aggregated particles and impurities. AFM images were obtained using a Veeco Multimode 8 atomic force microscope in tapping mode using 9 nm thick silicon tip. Images were taken at various spots on the sample using similar scanning rates (0.7 Hz). Features with heights larger than 5 nm appeared to be 2DNM aggregates or impurities and were not used in the image analysis. Gwyddion software was used to process the AFM images and evaluate the thickness of 2DNM nano-sheets. All obtained images were individually and manually processed.

Fourier-transform infrared spectroscopy (FTIR)

The attenuated total reflection (ATR) sampling technique is used to collect the FTIR spectra of 2DNM, as it is very simple and quick and does not require complicated sample preparation. As-synthesized 2DNM dispersions at 0.5 mg/mL were deposited on a SiO_2/Si substrate. Prior to deposition, the substrate was cleaned by rubbing it with three Q-tips soaked in acetone, isopropanol, or water, respectively. The substrate was then kept at 90° C for 30 min to remove all the solvent residuals from the surface. Three (3) drops of 2DNMs dispersion were then deposited on the surface and dried using Ar gas. A Thermo Scientific Nicolet 4700 FTIR was used to collect the FTIR spectra of 2DNMs. A cleaned SiO_2/Si substrate was used as blank to collect the background spectra of the substrate and subtract it from the spectra of the sample. For each sample, 5 scans were conducted in the range of 500 cm-1 to 4000 cm-1. The data were averaged, and the mean was used as the 2DNM representative spectra.

X-ray photoelectron spectroscopy (XPS) of pristine 2DNMs

As-synthesized 2DNM dispersions were dried at room temperature to obtain nanosheet powders which were then used for XPS characterization. About 20 mg of the nanosheets powder were mounted on a 0.5 cm^2 piece of double-sided carbon tape and fixed on the XPS sample holder substrate. Loose particles were removed from the carbon tape surface using Ar gas. PHI Versaprobe II XPS with an argon Ar ion X-Ray source was used for the XPS measurements. Samples were scanned over 0-1100 eV binding energy range to obtain the survey spectrum. The scanning was repeated 10 times for each element at three different points on the sample surface. XPS data were analysed using CasaXPS software. Data were calibrated using the carbon peak at 284.8 as the reference peak. Elemental percentage of the samples was determined using the ratio of the elemental peaks in the survey spectra.

Single-particle tracking (SPT)

As-synthesized 2DNM dispersions were diluted to ~0.5 µg/mL by addition of diw. Hydrodynamic size distribution measurements were conducted in a NanoSight instrument. Approximately 1 ml of the diluted 2DNM dispersion was injected to the fluid channel of the NanoSight. After adjusting the camera focus and gain, the movement of the particles was captured over 30 second for 10 scans. Recorded videos were processed using NanoSight NTA 3.2 software. The software, using Stokes-Einstein equation, calculated the hydrodynamic radiuses of 2DNMs. Statistical moments such as minimum, maximum, mean, and standard deviation were also obtained from this video processing.

Ultraviolet-visible spectroscopy (UV-Vis)

As-synthesized 2DNM dispersions were diluted to appropriate concentration by addition of diw such that the maximum optical density was ~1. Measurements were carried out in a Shimadzu 1800 spectrometer. Na-cholate and PF108 solutions with concentrations similar to those used for the dispersion of hydrophobic 2DNMs (graphenes, reduced graphene oxide, partially reduced graphene oxide, and inorganic 2DNMs) were used as for background correction.

Inductively-coupled plasma mass spectrometry (ICPMS)

ICPMS was used to evaluate the elemental purity of as0synthesized 2DNM suspensions. following a protocol previously described by Herner et al. [3]. Exactly 5 ml of the each 2DNM suspension was placed in a Teflon pressure vessel with a mixture made from 1.5 mL of 16 N HNO_3, 0.5mL of 28 N HF, and 0.2 mL of 12 N HCl. The samples were then heated with a microwave oven to 180°C in 9 min, followed by a 10 min hold at that temperature, and 1 h of ventilation/cooling. After cooling, digests were diluted to 30 mL with high-purity water prior to ICPMS analysis. The digestion vessels were pre-cleaned using an acid solution consisting of 2.4N HCl for two days, then 3.2 N HNO_3 for two more days and, lastly, rinsed with high-purity water. In addition to the collected samples, sample spikes, sample duplicates, blanks, standards, and certified reference materials (NIST 2709, NIST 1648a, NIST 2556, NIST 2702) were used in the chemical testing.

Raman spectroscopy

As-synthesized 2DNM dispersions were used to prepare the samples for Raman spectroscopy. Twenty (20) ml of each dispersion were vacuum filtered over a PTFE membrane (0.2-micron pore size). Samples were dried in an oven at 80°C for 10 hours and then mounted on a glass slide using double sided tape to achieve a flat surface during the measurement. A Horiba Jobin-Yvon LabRam Raman microscope was used for the measurements with a laser source of 532 nm. The samples were scanned over appropriate range of Raman shift using 10 seconds acquisition times for 5 times on each point. Grating for these measurements was set at 1800 gr/mm.

Endotoxin quantification and microbiological sterility assessment - ESI

As-synthesized 2DNMs were tested for endotoxin using the EndoZyme® recombinant factor C (rFC) assay (Hyglos, Germany) following the manufacturer's instructions and as previously described by the authors [4]. In brief, 2DNM suspensions at 10 µg/ml, endotoxin standard dilutions, and 2DNM suspensions spiked with 0.5 EU/ml were prepared in endotoxin-free water. One hundred (100) µl of each sample were transferred in a pre-warmed (37°C) 96-well plate and mixed with 100 µl assay reagent at a final ratio of 8:1:1 of assay buffer, enzyme, and substrate, respectively. Fluorescence measurements were taken at t = 0 and at t = 90 min using excitation and emission wavelengths at 380 and 440 nm, respectively. Dilution of standard endotoxin samples were used to create a calibration curve which in turn was used to extrapolate the endotoxin concentrations in the 2DNM samples.

Microbiological sterility of as-synthesized 2DNM suspensions was assessed according to a protocol issued by the World Health Organization (WHO) available in the international pharmacopoeia and previously described by the authors [5]. In brief, 1 ml of as-synthesized 2DNM suspensions were mixed with 1ml of fluid thioglycolate medium at pH 6.9–7.3. The solutions were then incubated at 37°C for 14 days during which they were visually examined for indications of bacterial growth daily. Every third day, samples of broth were spread onto tryptic soy agar plates and pour plates were created by mixing with potato dextrose agar and plate count agar. Plates were incubated at 37°C for 3 days during which they were inspected for bacterial growth and fungal colonies daily.

Interference tests between 2DNMs and cytotoxicity assays

Pierce LDH assay kit:

This colorimetric assay measures the levels of LDH released in the cell supernatant because of cell membrane rupture. To test for interferences from 2DNMs, the assay was performed in the absence of cells but otherwise identically to how it is presented in the main text. Briefly, 100 µl of 2DNMs dispersed in DMEM + 10% FBS vol/vol were added to empty 96-well plates at a concentration of 250 µg/ml. Then, 50 µl were transferred to Protein LoBind Eppendorf tubes and centrifuged at 5000g for 5’. The supernatant was then aspirated and tested according to the manufacturer’s instructions and as described in detail by the authors in a previous publication [6]. **Figure S1-A** shows that the background signal from cell growth medium alone was comparable to that from the samples treated with 2DNMs, which led to some negative values. The test performed here excludes the possibility of false positive results. Therefore, the small LDH release caused by ingested 2DNMs as presented in the main manuscript can be safely attributed to real (albeit small) cellular membrane disruption by 2DNMs.

CellROX® green reagent:

Production of ROS was assessed using the CellROX® green reagent. Application of this assay leads to increased fluorescent signal in the presence of increased intracellular ROS species. To test for interferences, co-cultures of Caco-2 and HT29 cells were seeded in 96-well plates and exposed to 2DNMs at a concentration of 250 µg/ml for 1 hour. Such short timepoint (1 hour) and high 2DNM concentration (250 µg/ml) allowed to test for possible suppression of the fluorescent signal from residual 2DNMs without killing the cells. According to the manufacturer's instructions, the cell medium with 2DNMs was removed and the cells were washed with PBS before adding the assay reagents. The assay was then completed according to the manufacturer's instructions and as described in a previous publications by the authors [6]. **Figure S1-B** shows that the baseline cell production of ROS did not change as compared to untreated cells. This result suggests that the increased levels of oxidative stress caused by ingested 2DNMs as reported in the main text are not due to false positives.

PrestoBlue™ Cell Viability Reagent:

Viability (mitochondrial enzyme activity) was assessed using the PrestoBlue™ Cell Viability Reagent. The fluorescent signal produced by this assay is positively correlated with changes in the mitochondrial enzymatic activity (i.e., increased mitochondrial activity leads to increased fluorescent signal). To test for interferences, co-cultures of Caco-2 and HT29 cells were seeded in 96-well plates and exposed to 2DNMs at a concentration of 250 µg/ml. The manufacturer's instructions call for removal of the cell supernatant and washing the cells with PBS before adding the reagent. To test for interference from residual nanomaterials while the cells are still alive, exposure duration was kept to 1 hour. After washing the cells 3x with PBS, 100 µl of a 10% working solution of the PrestoBlue reagent (diluted in DMEM without phenol red) was added to each well. The assay was then completed according to the manufacturer's instructions and as described in a previous publications by the authors [6]. **Figure S1-C** shows a decrease in enzymatic activity which can be attributed to the extremely high 2DNM concentration. Results suggest that, if anything, residual 2DNMs may cause an underestimation of the enzymatic activity. In the main text, ingested 2DNMs at low concentrations do not suppress the metabolic activity of exposed cells. Based on the interreference tests, such negative results are not considered to mask the cytotoxicity of 2DNMs.

CellEvent Caspase-3/7 Green Detection kit:

Apoptosis was assessed using the CellEvent Caspase-3/7 Green Detection kit (ThermoFisher). This assay produces a green fluorescent signal that increases with increased nuclear production of caspases 3/7. To test for interference from 2DNMs, co-cultures of Caco-2 and HT29 cells were seeded in 96-well plates and exposed to 2DNMs at a concentration of 250 µg/ml for 1 hour. Such short timepoint (1 hour) and high 2DNM concentration (250 µg/ml) allowed to test for possible suppression of the fluorescent signal from residual 2DNMs without killing the cells. Importantly, the cell medium with

2DNMs was removed before applying the assay (as also described in the main text) to minimize potential interference from 2DNM with the assay reagents. Cells were then washed twice with pre-warmed PBS and 100 µl of an 8 µM working reagent solution was added to the wells. The 96-well plates were then incubated for 45 min at 37°C and 5% CO_2, washed once with warm PBS, and incubated with 100 µl of 4% formaldehyde at room temperature for 15 min. Formaldehyde was then replaced with 200 µl of room temperature PBS and fluorescence was measured at 502 nm (excitation)/530 nm (emission). Apoptosis activity was calculated by first subtracting the background values (measurements from untreated, unstained wells) from the values of 2DNMs-treated samples, then normalizing the background-corrected measurements to the stained negative control values and expressing the result in fold-change. **Figure S1-D** shows that unstained cells generated higher fluorescent signals from 2DNM-treated cells which excluded the possibility of false positive results."

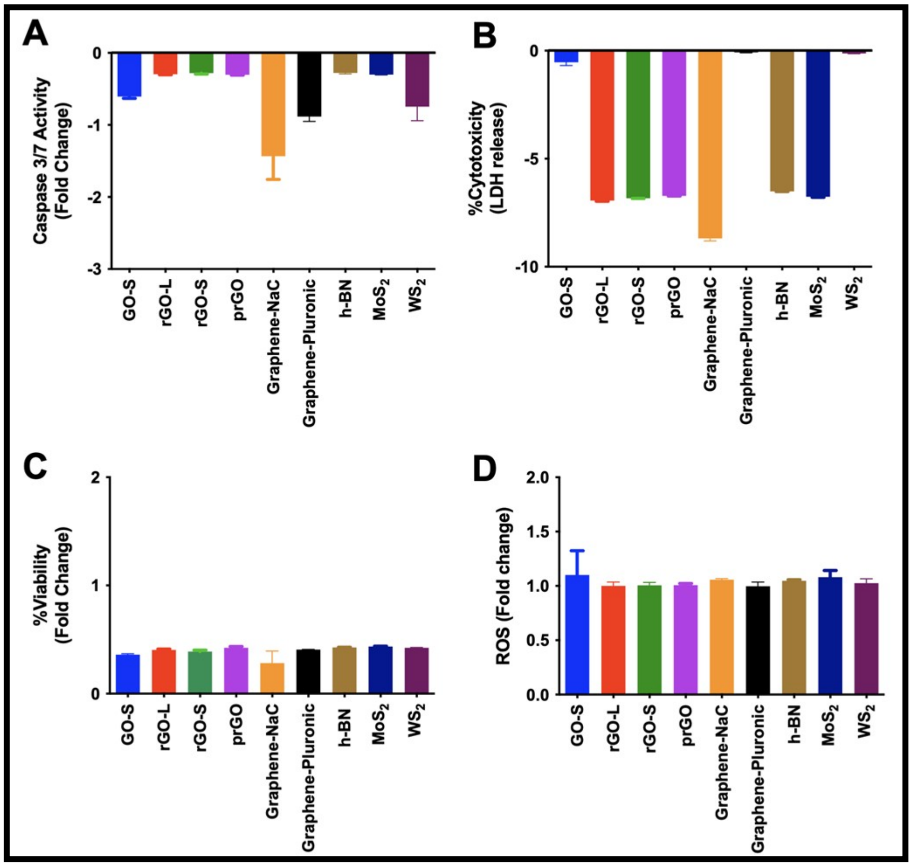
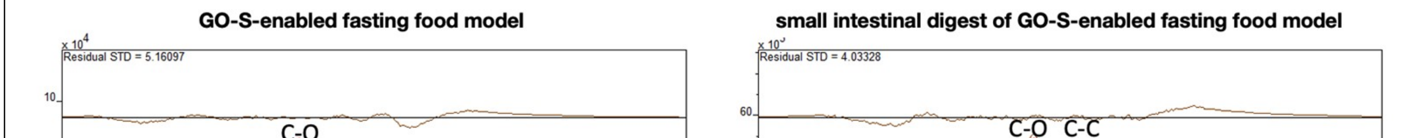
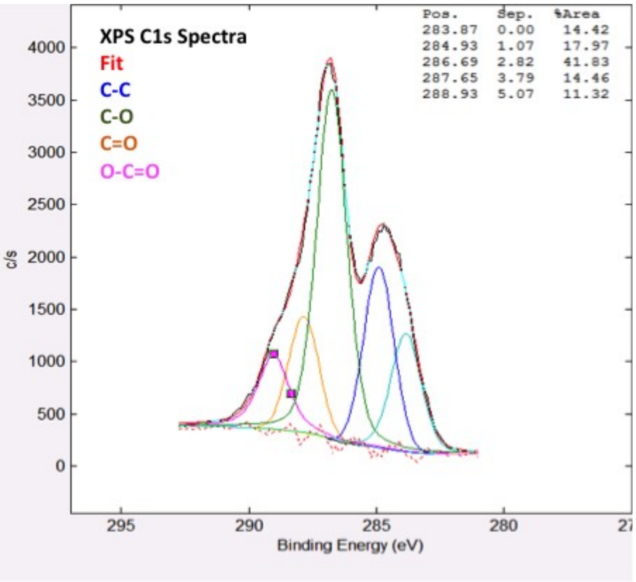
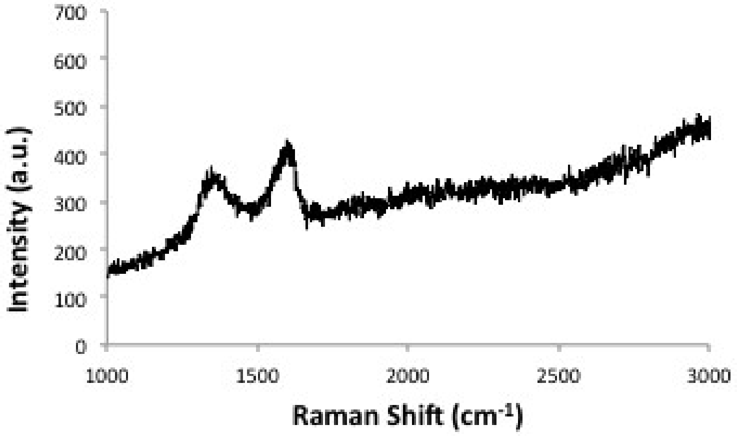


Figure S1. Assessment of 2DNMs interference across toxicological assays. Interference results of toxicological assessments of GO-S, rGO-S, rGO-L, prGO, graphene-Na-cholate, and graphene-PF108, h-BN, MoS_2 and WS_2 nanomaterials. **A.** Caspase 3/7 activity (apoptosis) after 2DNM exposure expressed as fold change relative to that in cells exposed to cell culture media (24-hour exposure). **B.** Cytotoxicity (LDH release) calculated as % of LDH in lysed control (24-hour exposure). **C.** Cell viability (mitochondrial enzymatic activity) expressed as % of activity (fluorescence) measured in cells treated with negative control wells with cell culture media (24-hour exposure). **D.** ROS generation expressed as fold change relative to that in cells exposed to cell culture media (measured after 6-hour exposure). All exposures were performed in triplicate. Error bars represent mean ± standard deviation. * p < 0.05; *** p < 0.001.



Supplementary Tables:

Table S1: Complete physicochemical, morphological, and biological characterization of GO-S

Chemical properties	**X-Ray Photoelectron Spectroscopy** **(XPS)**	**GO Atomic Ratio** Carbon (C1s peak): 62.26% Oxygen (O1s peak): 37.17% Sulfur (S1s): <0.57% 	Analysis Software: Multipak Instrument: PHI Versaprobe II
Chemical properties	**Raman Spectroscopy**	 **Peak (cm-1)** **Correspondence** 1350 D band 1580 G band	Instrument: Horiba Jobin-Yvon LabRam Raman Microscope

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<td>Fourier Transformed Infrared Spectroscopy (FTIR)</td>
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<tr><td>1726</td><td>C=O Stretching</td></tr>
<tr><td>1620</td><td>C=C Stretching</td></tr>
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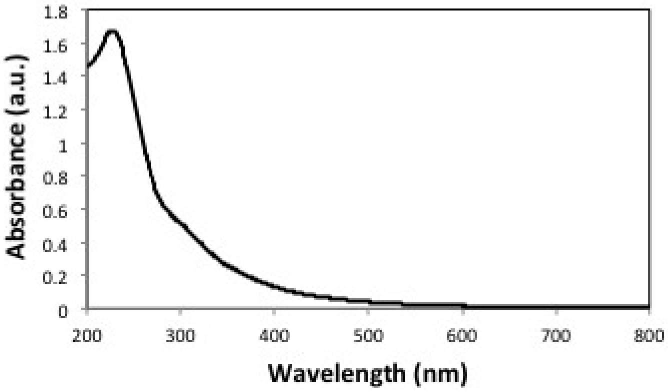

</td>
<td>Analysis Software: UVProbe
Instrument: Shimadzu 1800</td>
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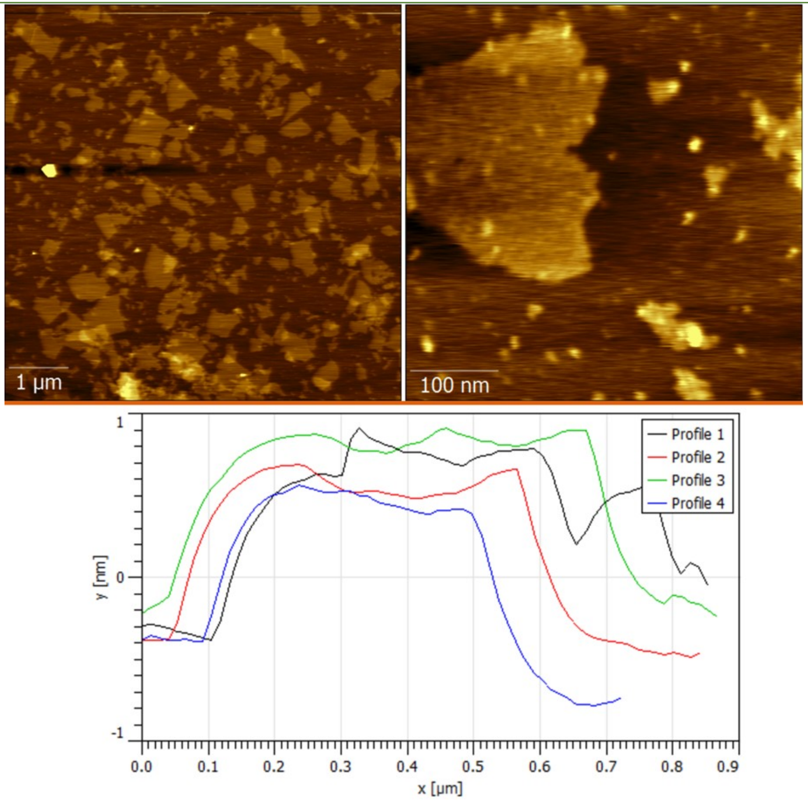
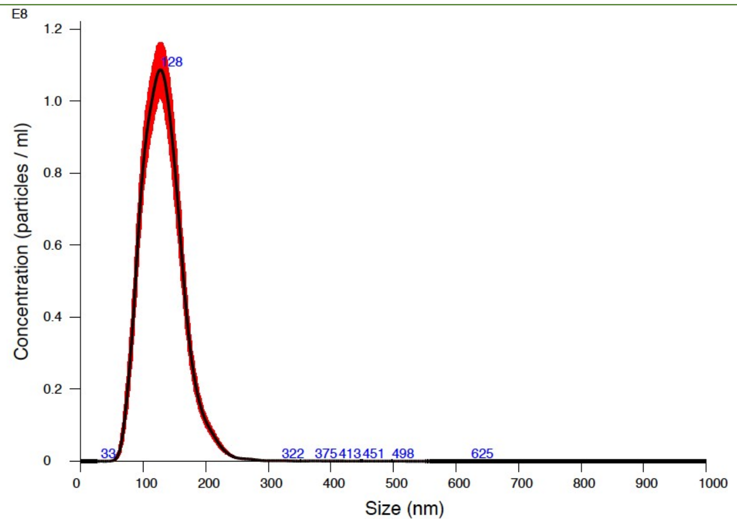
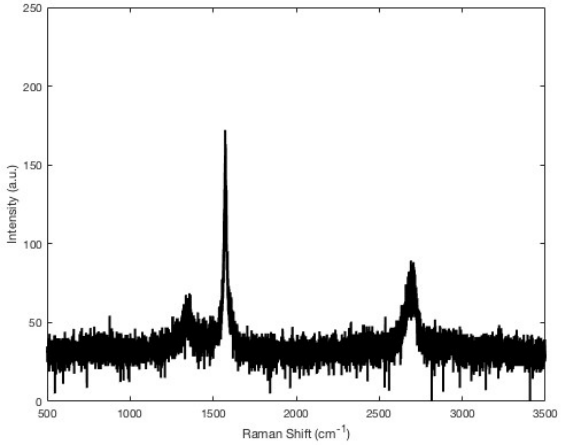
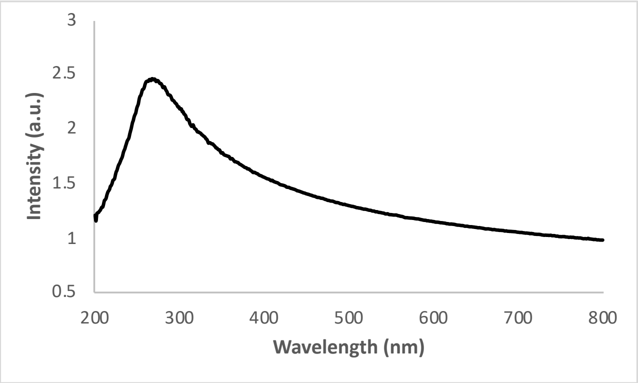
Physical properties	**Inductively Coupled Plasma Mass Spectrometry (ICP-MS)**	**Trace Metals Puirty** Per dry mass: 98.697 ± 0.097 % Per suspenssion 99.999 ± 0.000 % **Chemical Analysis (wt% of the three most abundntant metals):** Sulfure: 1.120 ± 0.021 % Boron: 0.046 ± 0.011 % Sodium: 0.046 ± 0.002 % **Measured elements:** Metals: Li, Be, Na, Mg, Al, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Y, Zr, Nb, Mo, Rh, Pd, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Dy, Yb, W, Pt, Hg, Pb ,Th, U Nonmetals: B, P, As	
	Atomic Force Microscopy (AFM)	 **Average Nanosheet Height:** 1.2 nm **Single Sheet > 95%**	Instrument: Research Asylum AFM Analysis software: Gwyddion

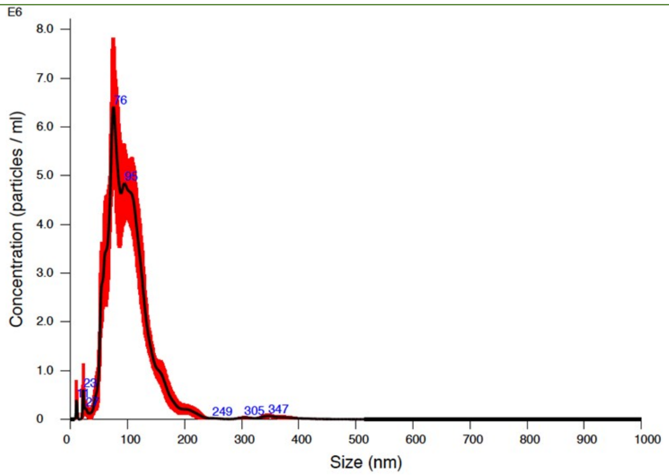
Physical properties	**Single Particle Tracking**	 **R_H Distribution** Mean (nm): 131.5 Mode (nm): 127.5 St. Dev. (nm): 33.3	Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10
	Lateral Size Estimated from Hydrodynamic Radius (R_H) according to [7]	**Lateral Size Moments (nm)** Mean: 313.5 Mode: 297.9 D_{10}: 172.6 D_{50}: 298.6 D_{90}: 483.5	Calculated
Concentration	**Calculated using Beer-Lamberts' Law**	Concentration = 0.5 mg/mL *	Measured using vacuum filtration
Endotoxins	**Recombinant Factor C** [8]	<0.005 EU/ml ** (below measurement threshold)	Suspension tested at 10 µg/ml
Microbiological sterility	**Pharmacopeia Protocol for Sterility WHO Document QAS/11.413 FINAL**	0 bacteria/mg	Suspension tested at 100 µg/ml

* UV-Vis Spectroscopy was performed on an aqueous graphene oxide dispersion of 0.03 mg/mL concentration. The aggregation of nanosheets over time may be monitored by the change in absorption at 260 nm wavelength.

** For more information on the endotoxin concentration measurement please refer to APPENDIX C: Endotoxin Characterization.

Table S2: Complete physicochemical, morphological, and biological characterization of G-PF108-S

Crystalline Structure	**Raman Spectroscopy**	 D peak: 1360 cm^{-1} G Peak: 1572 cm^{-1} 2D peak: 2690 cm-^{1}	Horiba Jobin-Yvon LabRAM Raman Microscope
UV Absorption	**UV-Vis Spectroscopy**[*]		Analysis Software: UVProbe Instrument: Shimadzu 1800
Concentration	**Calculated using Beer-Lambert Law**	graphene concentration = 0.32 mg/mL Pluronic F108 concentration = 10 mg/mL	Calculated

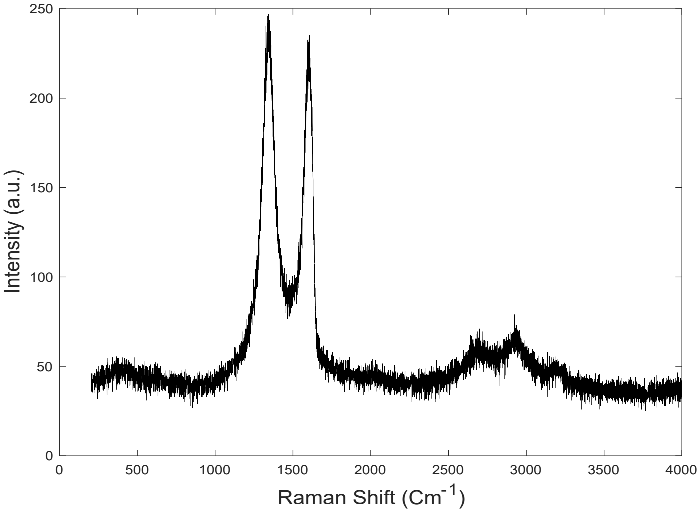
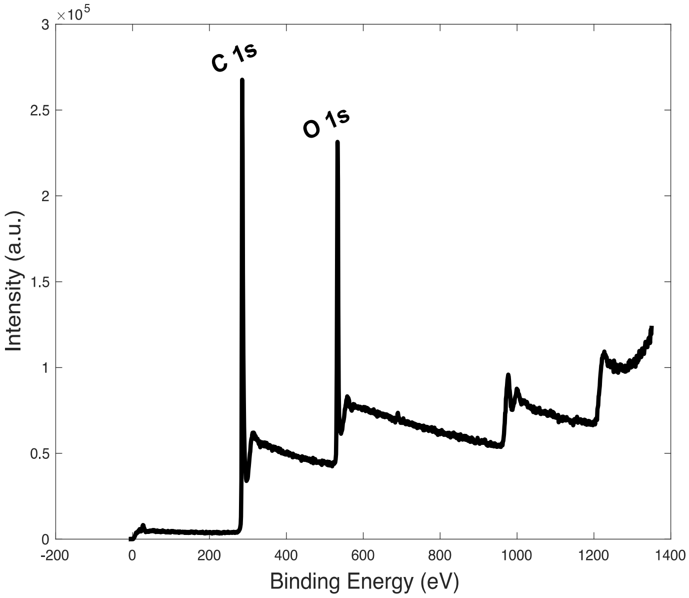
Size Distribution	**Hydrodynamic Radius (R_H) Measurements** **by Nanoparticle Tracking Analysis** **		Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10
Size Distribution	**Hydrodynamic Radius (R_H) Measurements** **by Nanoparticle Tracking Analysis** **	***R_H* Distribution Statistics** Mean (nm): 102.1 Mode (nm): 75.3 St. Dev. (nm): 43.5	Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10
Lateral Size	**Estimated from** **Hydrodynamic Radius (R_H)** (APPENDIX A)	**Distribution Statistics** Mean (nm): 206 Mode (nm): 125 Min (nm): 27 Max (nm): 900 D10 (nm): 87 D50 (nm): 181 D90 (nm): 368	Calculated

Elemental Composition	**Inductively Coupled Plasma Mass Spectrometry** **(ICP-MS)**	**Graphene suspension total purity (wt%):** 97.0177 ± 0.1571 **Graphene suspension total trace elements concentration (ppm):** 9.5434 ± 0.5027 **Chemical analysis** **(wt% of the three most abundntant metals):** Calcium : 0.8113 ± 0.0925 Potasium : 0.7837 ± 0.0900 Magnesium : 0.6858 ± 0.0782 *Metals: Li, Na, Mg, Al, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Y, Nb, Mo, Rh, Pd, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Dy, Ho, Yb, Lu, Hf, W, Pt, Hg, Tl, Pb ,Th, U Nonmetals: B, P, As, Se	Microwave digested hBN suspension in H_2SO_4, HCl, and HF
Endotoxins	**Recombinant Factor C** (APPENDIX A)	< 0.005 EU/ml (Below the limit of detection)	Suspension tested at 10 μg/ml
Sterility	**Pharmacopeia Protocol for Sterility WHO Document** **QAS/11.413 FINAL**	0 bacteria/mg	Suspension tested at 1 mg/ml

* UV-Vis Spectroscopy was performed on aqueous graphene/Pluronic F108 with a dilution factor of 5. The concentration of Pluronic F108 in the dispersion is 10 mg/mL. The absorption at 660 nm wavelength was used to calculate the graphene concentration in the dispersion.

** NanoSight measurements were performed on aqueous graphene/Pluronic F108 dispersions with graphene concentration of 5 μg/mL.

Table S3: Complete physicochemical, morphological, and biological characterization of rGO-S

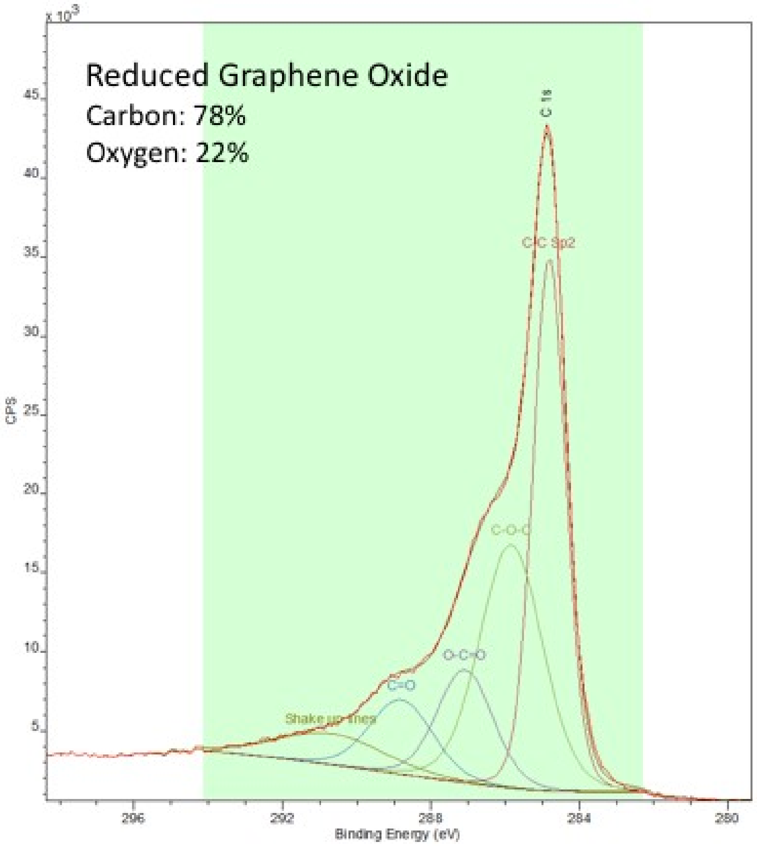
Crystalline Structure	**Raman Spectroscopy**	 Graphitic hexagonal structure	Perkin Elmer Raman Station 400
Elemental Analysis	**X-Ray Photoelectron Spectroscopy** **(XPS)**	 **Atomic Ratio** Carbon: 78% Oxygen: 22%	Thermo Scientific K-Alpha XPS

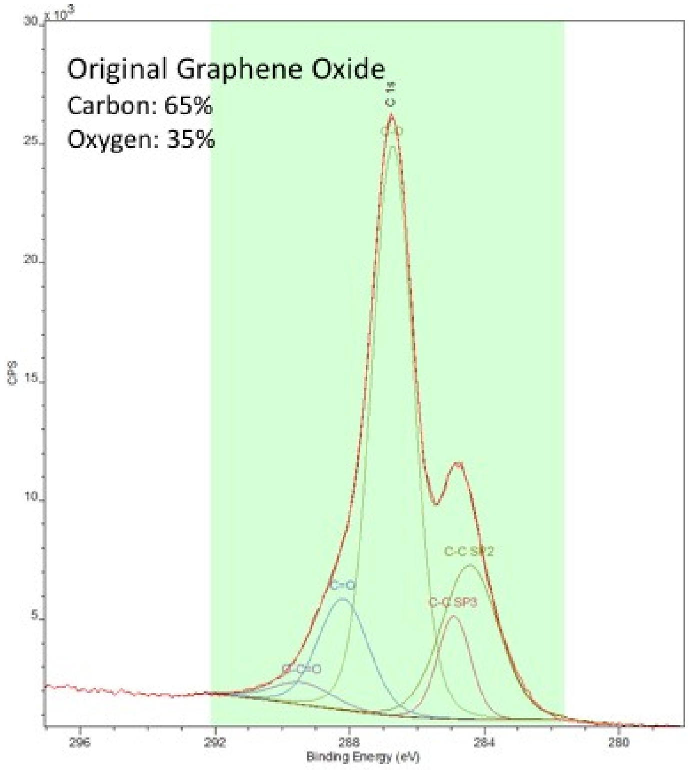
Elemental Analysis

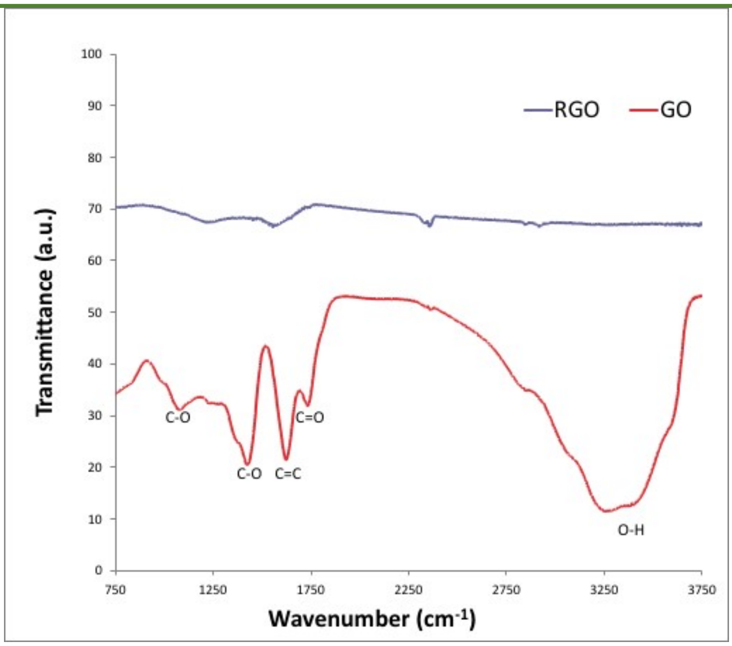
X-Ray Photoelectron Spectroscopy

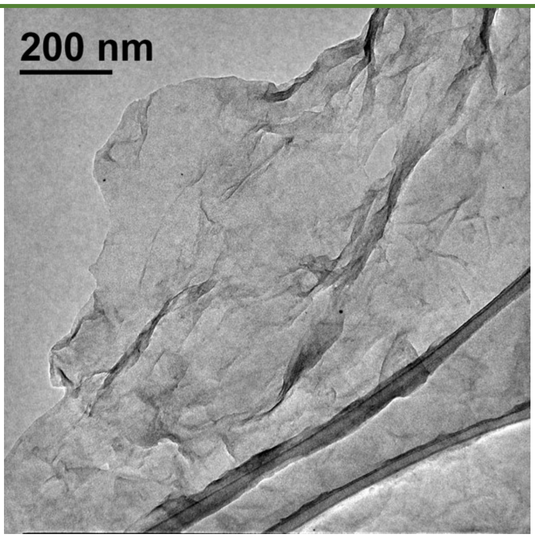
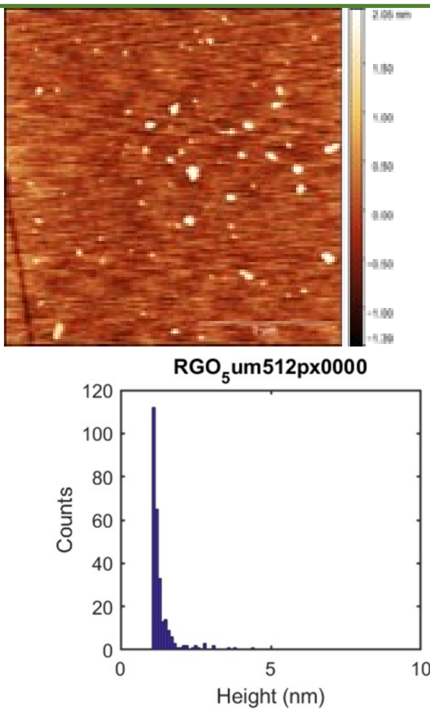
(XPS)

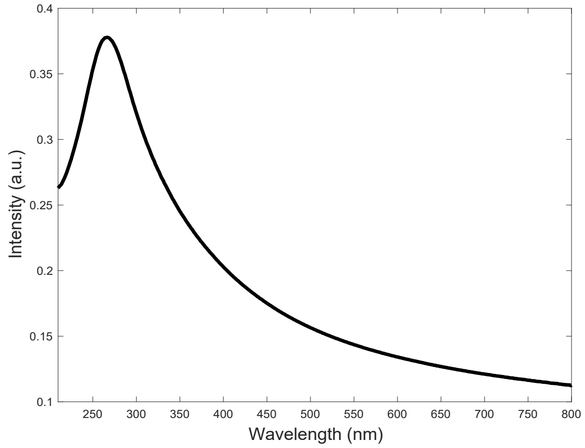
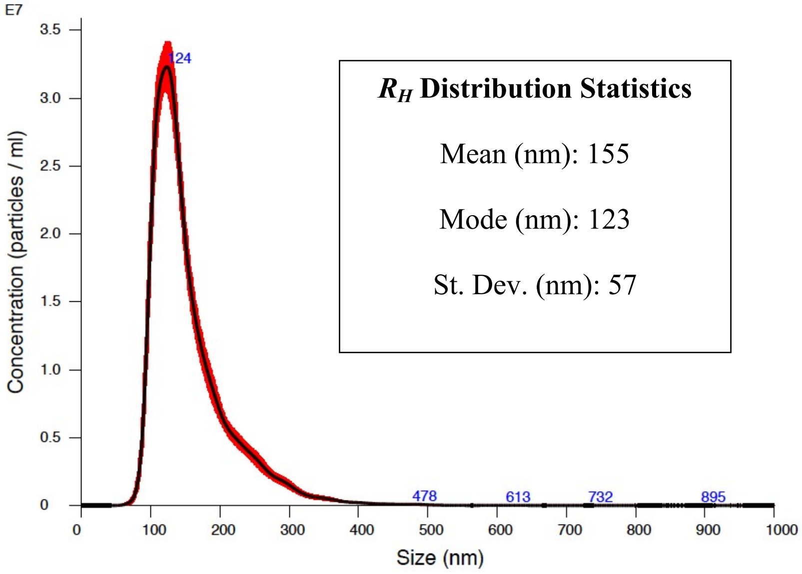
C S1 Peak Deconvolution





Elemental Composition	**Inductively-coupled Plasma Mass Spectrometry** **(ICP-MS)**	**rGO-S total* trace purity (wt%):** 99.7284 ± 0.0353 **rGO-S suspension total* trace concentration (ppm):** 1.3578 ± 0.1765 **Chemical analysis** **(wt% of the three most abundntant metals):** Aluminum : 0.0615 ± 0.0064 Calcium : 0.0462 ± 0.0106 Titanium : 0.0444 ± 0.0052 *Metals: Li, Mg, Al, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Y, Nb, Mo, Rh, Pd, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Dy, Ho, Yb, Lu, W, Pt, Hg, Tl, Pb ,Th, U Nonmetals: P, S, Se	Microwave digestion of rGO-S in H_2SO_4, HCl, and HF
Chemical Bonds	**Fourier Transform** **Infrared** **Spectroscopy** **(FTIR)** **C S1 Peak Deconvolution**		

Imaging	**Transmission Electron Microscopy** **(TEM)**	 Representative TEM image of rGO-S	TEM: Zeiss 2010
Thickness	**Atomic Force Microscopy** **(AFM)**	 <3nm: 1-3 layers	

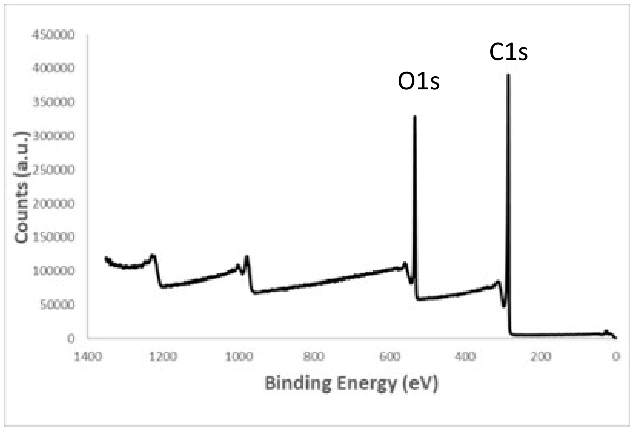
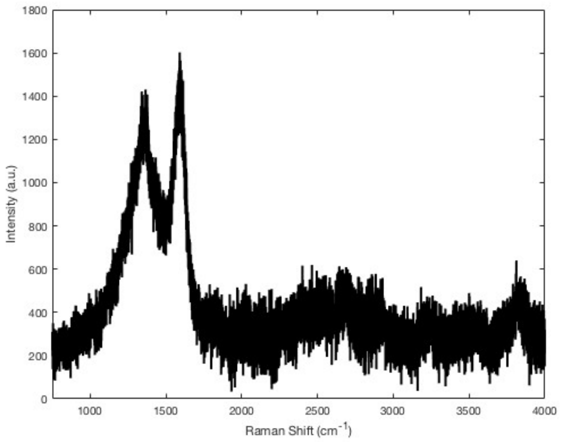
UV Absorption	**UV-Vis Spectroscopy***	 Peak absorption at 280 nm	Analysis Software: UVProbe Instrument: Shimadzu 1800
Concentration	**Calculated using Beer-Lambert Law**	Concentration = 500 µg/mL	Measured using vacuum filtration
Size Distribution	**Hydrodynamic Radius (R_H) Measurements** **by Nanoparticle Tracking Analysis****[**]		Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10

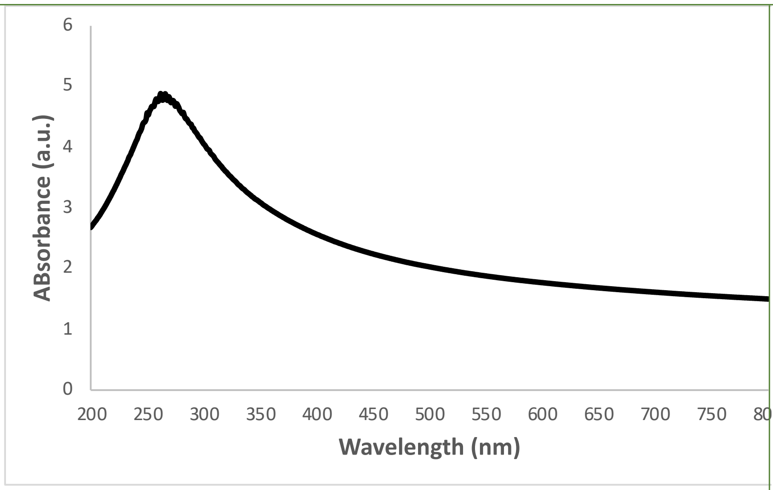
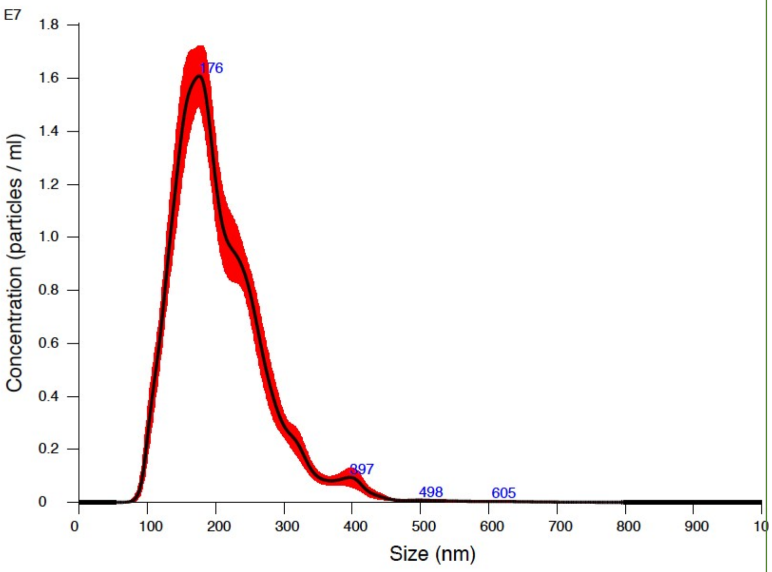
Lateral Size	**Estimated from Hydrodynamic Radius (R_H)**	Mean (nm): 411 Mode (nm): 280 Min (nm): 138 Max (nm): 2386 D10 (nm): 206 D50 (nm): 335 D90 (nm): 794	Calculated [1]
Endotoxins	**Recombinant Factor C**	**Concentration on Suspension** C_{End} < 0.005 EU/ml (Below the limit of detection) Endotoxins in PBS C_{End} = 76 EU/ml	Suspension Tested at 10 µg/ml [2]
Sterility	**Pharmacopeia Protocol for Sterility WHO Document** **QAS/11.413 FINAL**	0 bacteria/mg	Suspension tested at 10 µg/ml
Disp. Preparation	**Appendix C**	Dispersion preparation for 2D anisotropic ENMs	

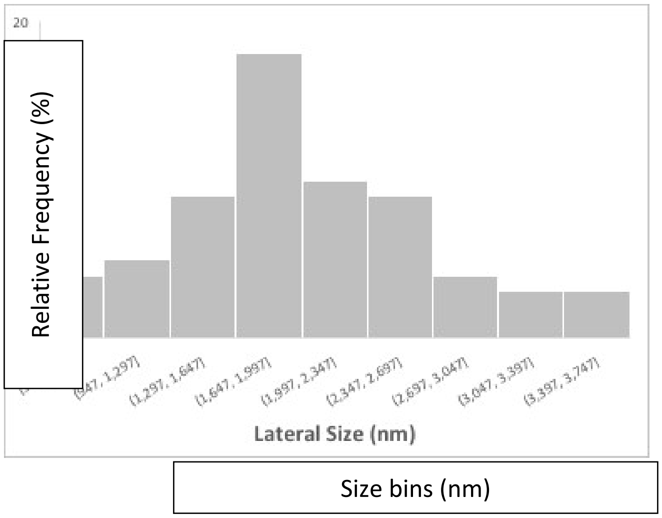
* UV-Vis Spectroscopy was performed on an aqueous reduced graphene oxide dispersion of 0.005 mg/mL concentration. The concentration of sodium cholate in the dispersion is 4 mg/mL. The aggregation of nanosheets over time may be monitored by the change in absorption at 260 nm wavelength.

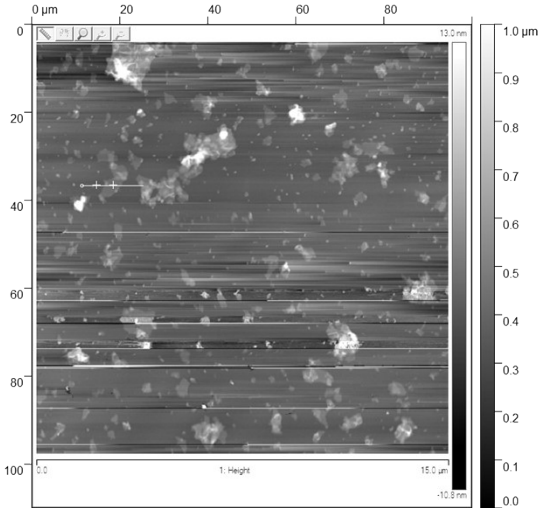
** NanoSight measurements were performed on aqueous rGO-S/sodium cholate dispersions with rGO-S concentration of 1 µg/mL.

Table S4: Complete physicochemical, morphological, and biological characterization of rGO-L

Elemental Analysis	**X-Ray Photoelectron Spectroscopy** **(XPS)**	 **rGO-L Atomic Ratio** Carbon: 77.7% Oxygen: 22.3%	Thermo Scientific K-Alpha XPS
Crystalline Structure	**Raman Spectroscopy**	 D peak: 1360 cm^{-1} G Peak: 1590 cm^{-1}	Horiba Jobin-Yvon LabRAM Raman Microscope

UV Absorption*	**UV-Vis Spectroscopy**	 Peak: 260 nm	Analysis Software: UVProbe Instrument: Shimadzu 1800
Concentration	**Calculated using Beer-Lambert Law**	rGO-L concentration = 0.40 mg/mL Sodium cholate concentration = 2.5 mg/mL	Measured using vacuum filtration
Size Distribution	**Hydrodynamic Radius (*R_H*) Measurements** **by Nanoparticle Tracking Analysis**		Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10

Size Distribution	**Hydrodynamic Radius (R_H) Measurements** **by Nanoparticle Tracking Analysis**	Mean (nm): 204.4 Mode (nm): 175.8 St. Dev. (nm): 68.4	Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10
Lateral Size Distribution	**Atomic Force Microscopy** **(AFM)**	**Distribution Statistics**  Mean (nm): 2015 St. Dev. (nm): 674	Analysis Software: Gwyddion Instrument: Veeco Multimode 8 AFM

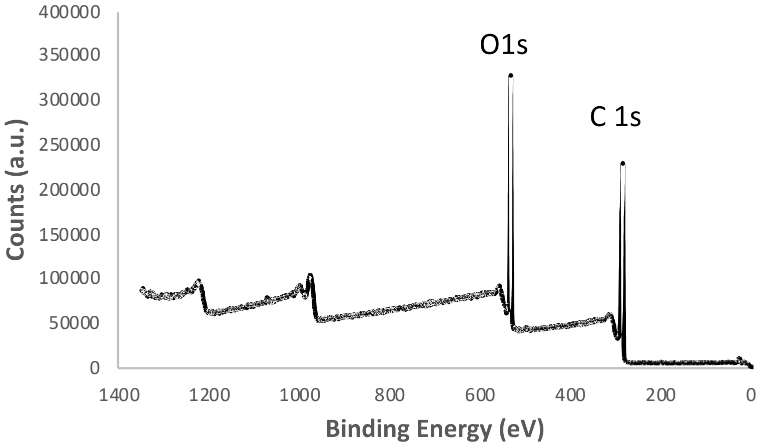
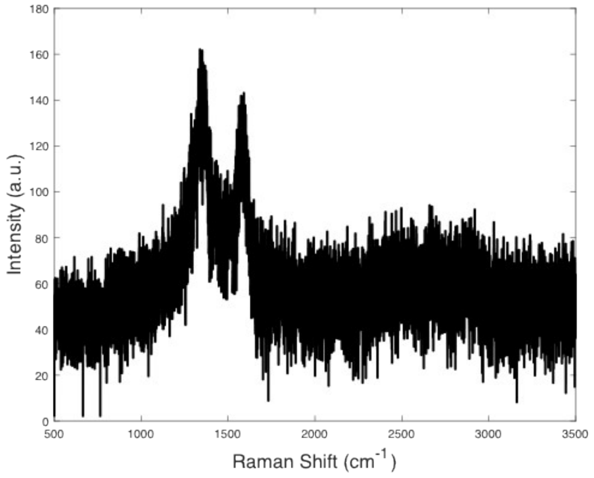
Thickness	**Atomic Force Microscopy** **(AFM)**	 Mean thickness: 62 nm (multiple layers: 2-60 layers)	Veeco Multimode 8 AFM Images analysed in Gwyddion 2.52
Elemental Composition	**Inductively Coupled Plasma Mass Spectrometry** **(ICP-MS)**	**rGO-L suspension total purity (wt%):** 98.6575 ± 0.6145 **rGO-L suspension total trace concentration (ppm):** 5.3698 ± 2.4578 **Chemical analysis** **(wt% of the three most abundntant metals):** Zinc : 0.2383 ± 0.5697 Manganese : 0.1075 ± 0.0060 Potasium : 0.0220 ± 0.0129 *Metals: Li, Na, Mg, Al, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Y, Nb, Mo, Rh, Pd, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Dy, Ho, Yb, Lu, Hf, W, Pt, Hg, Tl, Pb ,Th, U Nonmetals: B, P, As, Se	Microwave digested hBN suspension in H_2SO_4, HCl, and HF

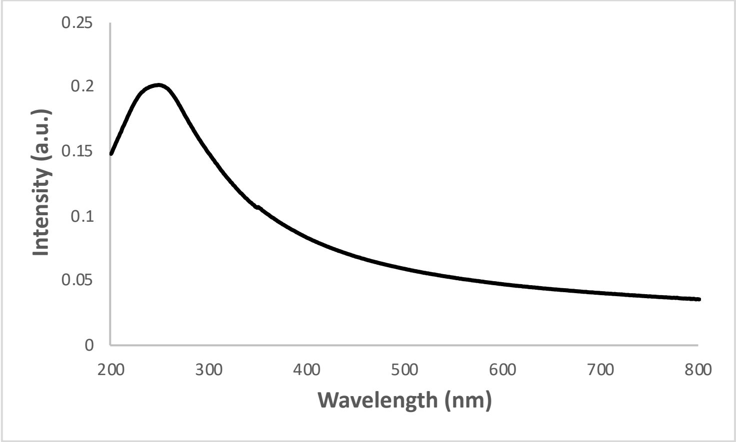
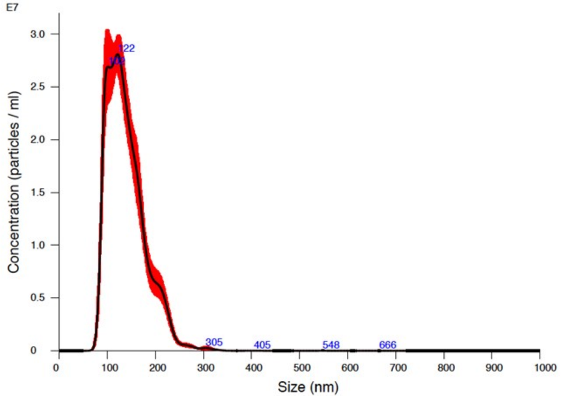
Endotoxins	**Recombinant Factor C** [8]	< 0.005 EU/ml (Below measurement threshold)	Suspension Tested at 10 µg/ml
Sterility	**Pharmacopeia Protocol for Sterility WHO Document** **QAS/11.413 FINAL**	0 bacteria/mg	

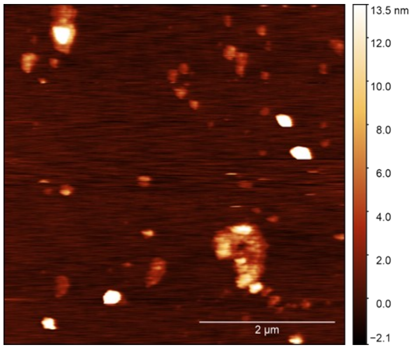
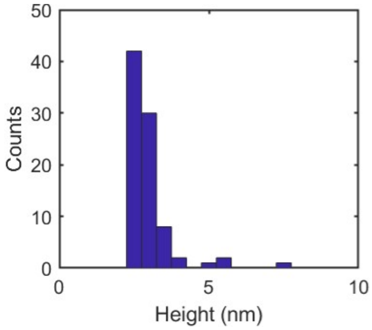
* UV-Vis Spectroscopy was performed on an aqueous reduced graphene oxide dispersion of 0.1 mg/mL concentration. The aggregation of nanosheets over time may be monitored by the change in absorption at 260 nm wavelength.

** High-accuracy total carbon concentration will be measured using complementary analytical techniques as part of Tier 2 Characterization.

Table S5: Complete physicochemical, morphological, and biological characterization of prGO

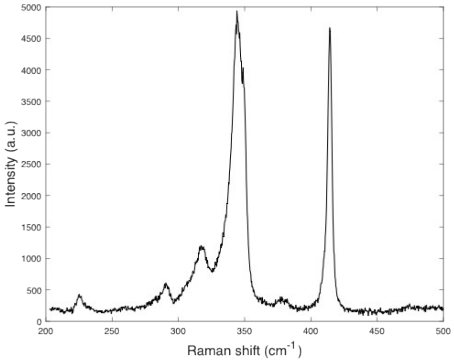
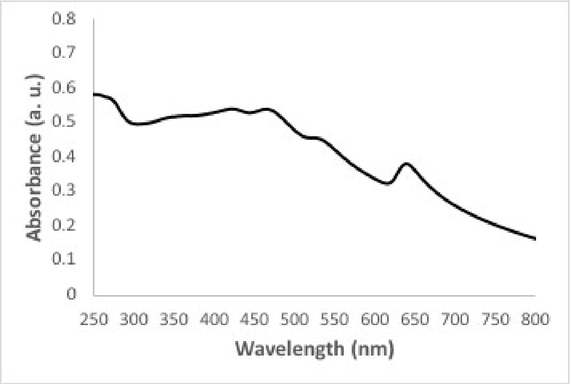
Elemental Analysis	**X-Ray Photoelectron Spectroscopy** **(XPS)**	 **RGO Atomic Ratio** Carbon: 72% Oxygen: 27.7% Impurities (Na): <0.3%	Thermo Scientific K-Alpha XPS
Crystalline Structure	**Raman Spectroscopy**	 D peak: 1360 cm^{-1} G Peak: 1590 cm^{-1}	Horiba Jobin-Yvon LabRAM Raman Microscope

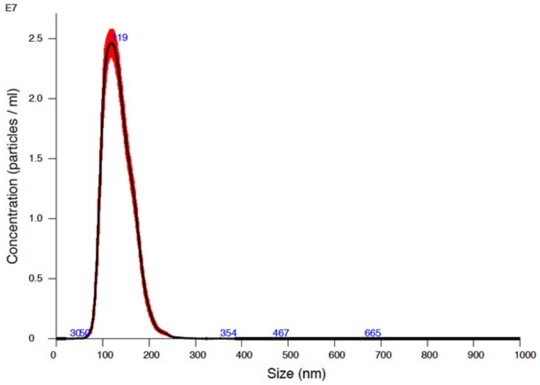
UV-Vis Absorption	**UV-Vis Spectroscopy*** 	 Peak: 260 nm	Analysis Software: UVProbe Instrument: Shimadzu 1800
Concentration	**Calculated using Beer-Lambert Law**	prGO Concentration = 0.5 mg/mL Na-cholate concnetration = 4.0 mg/mL	Measured using vacuum filtration
Size Distribution	**Hydrodynamic Radius (R_H) Measurements** **by Nanoparticle Tracking Analysis****		Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10
Size Distribution	**Hydrodynamic Radius (R_H) Measurements** **by Nanoparticle Tracking Analysis****	**R_H Distribution Statistics** Mean (nm): 139.4 Mode (nm): 121.5 St. Dev. (nm): 39.4	Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10

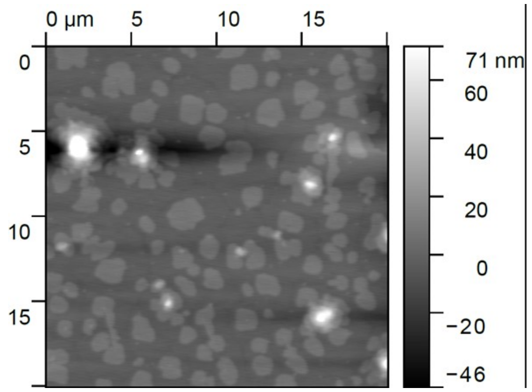
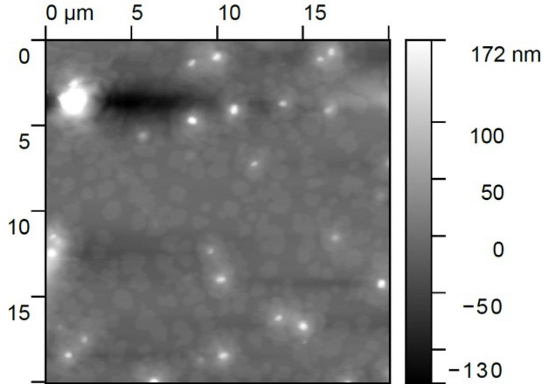
Lateral Size Distribution	**Estimated from** **Hydrodynamic Radius (*R_H*)** (APPENDIX A)	**Distribution Statistics** Mean (nm)**:** 357 Mode (nm): 273 Min (nm): 138 Max (nm): 2005 D10 (nm): 183 D50 (nm): 311 D90 (nm): 695	Calculated [1]
Nanosheet Thickness	**Atomic Force Microscopy** **(AFM)**	  **Average Nanosheet Height:** ~ 3 nm (1-3 layers)	Instrument: Research Asylum AFM Analysis software: Gwyddion

Elemental Composition	**Inductively Coupled Plasma Mass Spectrometry** **(ICP-MS)**	**prGO suspension total purity (wt%):** 99.7748 ± 0.0169 **prGO suspension total trace elements concentration (ppm):** 1.1262 ± 0.0843 **Chemical analysis** **(wt% of the three most abundntant metals):** Tatanium : 0.0800 ± 0.0068 Calcium : 0.0396 ± 0.0034 Potasium : 0.0099 ± 0.0041 *Metals: Li, Na, Mg, Al, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Y, Nb, Mo, Rh, Pd, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Dy, Ho, Yb, Lu, Hf, W, Pt, Hg, Tl, Pb ,Th, U Nonmetals: B, P, As, Se	Microwave digested hBN suspension in H_2SO_4, HCl, and HF
Endotoxins	**Recombinant Factor C** (APPENDIX A)	< 0.005 EU/ml (0.002 EU/ml)	Suspension Tested at 10 µg/ml [2]
Sterility	**Pharmacopeia Protocol for Sterility WHO Document** **QAS/11.413 FINAL**	0 bacteria/mg	Suspension tested at 1 mg/ml

Table S6: Complete physicochemical, morphological, and biological characterization of WS_2

	Method	Results	Notes
Crystalline structure	**Raman Spectroscopy**	 E^1_{2g} peak: 345 nm A_{1g} peak: 414 nm	Horiba Jobin-Yvon LabRAM Raman Microscope
UV Absorption‡	**UV-Vis Spectroscopy**		Analysis Software: UVProbe Instrument: Cary 5000
Concentration ‡	**Calculated using Beer-Lambert law ***	WS_2 concentration = 0.31 mg/mL Pluronic F108 concentration = 10 mg/mL	Calculated

Size distribution ‡	**Hydrodynamic radius (R_H) measurements** **by nanoparticle tracking analysis** **		Analysis Software: NanoSight 3.1 Instrument: NanoSight LM10
		***R_H* distribution statistics:** Mean (nm): 134.0 Mode (nm): 118.8 St. Dev. (nm): 30.2	
Lateral size ‡	**Estimated from** **hydrodynamic radius (R_H)**	Mean (nm): 323.38 Mode (nm): 265.12 D10 (nm): 192.33 D50 (nm): 303.33 D90 (nm): 495.27	Calculated

Thickness	**Atomic Force Microscopy** **(AFM)**	  Mean thickness: 6nm (includign ~1nm layer of PF108) WS_2 layers per particle: 2-5	Veeco Multimode 8 AFM Images analysed in Gwyddion 2.52

Elemental Composition	**Inductively Coupled Plasma Mass Spectrometry** **(ICP-MS)**	**WS_2 suspension total purity (wt%):** 96.8998 ± 0.5002 **WS_2 suspension total trace elements concentration (ppm):** 5.0262 ± 0.8110 **Chemical analysis** **(wt% of the three most abundntant metals):** Potasium : 1.0632 ± 0.2684 Calcium : 0.0107 ± 0.1057 Titanium : 0.2534 ± 0.0778 *Metals: Li, Na, Mg, Al, K, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Y, Nb, Mo, Rh, Pd, Ag, Cd, Sn, Sb, Cs, Ba, La, Ce, Pr, Nd, Sm, Eu, Dy, Ho, Yb, Lu, Hf, W, Pt, Hg, Tl, Pb ,Th, U Nonmetals: B, P, As, Se	Microwave digested hBN suspension in H_2SO_4, HCl, and HF
Endotoxins †	**Recombinant factor C**	< 0.005 EU/mL (below measurement threshold)	Suspension Tested at 10 µg/mL
Sterility	**Pharmacopeia protocol for sterility WHO document** **QAS/11.413 FINAL**	0 bacteria/mg	Suspension Tested at 1 mg/mL

* UV-Vis Spectroscopy was performed on aqueous WS_2/PF108. The concentration of PF108 in the dispersion is 10 mg/mL. The absorption at 345 nm wavelength was used to calculate the WS_2 concentration in the dispersion.

** NanoSight measurements were performed on aqueous WS_2/PF108 dispersions with WS_2 concentration of 1 μg/mL.

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