Electronic Supplementary Information for

Bacterial Physiology Is a Key Modulator of the Antibacterial Activity of Graphene Oxide

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1. A Brief Survey of Essential Reference Studies Regarding Antibacterial Nanocarbons

In the main text, due to space considerations, we preferred referring review articles whenever available instead of citing original studies. However, it is useful to compile prominent references herein for interested readers. In Table S1, we summarize essential information regarding antimicrobial activity of carbon-based nanomaterials (a.k.a. nanocarbons).

Nanocarbons used	Bacteria tested	Parameters of interest, major findings and mechanisms proposed, if any	Ref.
single-walled carbon nanotube (SWCNT)	E. coli	direct contact of SWCNTs to the bacteria damages the cellular membranes and causes death	[69]
SWCNT and multi- walled carbon nanotube (MWCNT)	E. coli	 the effect of nanotube size (diameter) direct contact causes membrane damage	[3]
MWCNT	E. coli	chemical and physical properties of MWCNTs affect their antibacterial efficacy	[70]
SWCNT	E. coli, P. aeruginosa, B. subtilis, S. aureus	 gram-negative bacteria are more resistant dispersion quality influences the killing efficacy mechanical puncture and oxidative stress involve in the inhibition of bacteria 	[7]
graphene oxide (GO), reduced GO (rGO)	E. coli	the observation of potent antibacterial activity of graphene materials	[71]
graphene (Gp), GO	E. coli, S. aureus	 gram-negative <i>E. coli</i> is more resistant damage caused through puncturing of membranes by sharp edges of nanosheets 	[72]
graphite (Gt), graphite oxide (GtO), GO, rGO	E. coli	 the order of antibacterial activity: GO > rGO > Gt > GtO transport inhibition by sheet wrapping, membrane puncturing and oxidative stress 	[4]
GO	E. coli	• larger GO sheets wrap cells more effectively and show higher antibacterial activity	[5]
GO	E. coli, S. iniae	gram-negative bacterium is more resistantreactive oxygen species involve in killing	[73]
Gp	E. coli	 simulation-based investigation of the interaction of Gp and cell membranes Gp extracts membrane phospholipids 	[74]
GO	P. syringae, X. campestris pv. undulosa	GO kills ~90% of cells through wrapping (intertwining) which eventually induces lysis	[9]

Table S1. A brief outline of selected studies on the antibacterial activity of nanocarbons.

2. Details of Cell Preparation and Antibacterial Studies

Details of Cell Preparation Routine: Bacterial cells stored at -80 °C were first resuscitated in 2 wt% Luria-Bertani broth (LBB), Lennox (Difco[™], BD Biosciences) at 37 °C under 200 rpm shaking by overnight culture (Fig. S1a). After ~12 hours, cells are directly diluted with isotonic saline (0.9 wt% aqueous NaCl) and spread on ~3.6 w/v% Luria-Bertani agar (LBA), Lennox (Difco[™], BD Biosciences) using the disposable L-shaped spreaders (Copan Diagnostics Inc.). Plates were incubated at ~36-37 °C for ~15-18 hours (Isotemp IncubatorTM, Fisher ScientificTM) and an arbitrary colony was selected to prepare a subculture (Fig. S1a). This subculture was used to prepare standardized inoculums (Fig. S1b) which are used in cell preparation for antibacterial tests (Fig. S1b). (Note that, the standard inoculums were refreshed using previously prepared standard inoculums approximately once in around 2-4 weeks.) Standard inoculums were used in the preparation of overnight (~12 hours) cultures and actual cell samples were gathered from the second subcultures using overnight-grown cells. In order to collect the cells having different physiological states, the growth kinetics of second subcultures were monitored with optical density measurements at 600 nm using a Cary 5000 spectrophotometer (Varian) with 2 mL of samples placed in disposable plastic cuvettes (BRAND UV-Cuvettes, BrandTech Scientific Inc.). Obtained data was used to construct standard growth curve (Fig. S1c) and used to check consistency throughout the work. Bacteria were harvested at OD₆₀₀ values of "0.60-0.65", "1.70-1.75", or "1.60-1.65" which correspond to exponential-, stationary-, and decline-phases, respectively. (Note that these values are valid for fast-growing *E. coli* DH5α strain. Since the appropriate optical density values and hence the suitable harvest times vary depending on the species studied.) Then, cellular products were cleaned by three cycles of centrifugation at 4000 rpm for 15 minutes and subsequent resuspension in isotonic saline solution to remove residual growth medium components and cellular waste. Resulting cellular suspensions were used in antibacterial assays. Accordingly, bacterial samples used for zeta potentiometry (ZPM), dynamic light scattering (DLS), X-ray photoelectron spectroscopy (XPS), and Microbial Adhesion to Solvents (MAtS) studies were prepared the same manner.



* 50 µL for Escherichia coli and Staphylococcus aureus; 100 µL for Pseudomonas aeruginosa.

Fig. S1. The cell preparation routine adopted. (a) Resuscitation of bacteria in growth media using main stock preserved at -80 °C. (b) Preparation of standardized stocks to keep at -20 °C. (c) Growth and harvest of cells of having different physiological states used for antibacterial assays. Note that steps shown in Fig.s S1b and S1c were repeated at least thrice before proceeding with the growth of cells used in the actual antibacterial assays. (Illustrated routine was developed largely based on the knowledge and tips acquired from [75]–[78].)

Spread- and Drop-Plate Colony Counting Assays: In all antibacterial assays, bacterial suspensions and GO solutions were mixed in equal volumes by adding GO solutions on bacterial suspensions slowly and the final concentration of bacteria was set to $\sim 2-8 \times 10^6$ CFU/mL. Reference bacterial suspensions and GO-bacteria mixtures were incubated for $\sim 30-180$ minutes at 37 °C under 200 rpm shaking similar to our group's previous reports [4], [5], [26]. For the spread-plate test, incubated samples were diluted five thousand times and were plated on LBA using $100-\mu$ L portions of diluted samples. For the drop-plate test, diluted samples used in the spread-plate test were further diluted to their two fifth and $25-\mu$ L drops were dropped on LBA without disturbing. Each experiment was repeated thrice on different

occasions to ensure repeatability using two-three replicate samples for spread-plate and foursix drops for drop-plate tests per sample at each run. All plates were incubated at \sim 36-37 °C for \sim 15-18 hours and colonies were counted manually. Results were reported in the form of normalized survival rates (*i.e.* percentage survival) by taking GO-free conditions as the reference for each case. Fig.s 2 and 6c seen in the main text and Fig. S2 seen below summarize the experimental results obtained by colony counting methods. In the main text, we have discussed the results shared in Fig.s 2 and 6c in thoroughly. Below, we provide further experimental evidence to support those discussions.

Based on the experiments performed by a period of two hours of incubation, we observed that the GO susceptibility of exponential-phase cells is much higher than that of stationary-phase cells. In our recent study,[26] using exponential-phase *E. coli* cells, we have shown that the antibacterial activity of GO is not a sudden effect, rather a time-dependent killing process. Thus, we wanted to compare the time-dependent survival rates of exponential-and stationary-phase cells as shown in Fig. S2a. As we observed before, a sharp drop takes place in the survival of exponential-phase cells in the first 30 minutes and overall trend resembles an exponential decay. However, although the drop of survival in the first 30 minutes is comparable with the upcoming 2.5 hours, the overall trend is rather linear-like in stationary-phase cells.

We have recently shown that the antibacterial activity of GO in dispersion phase depends on the environmental salinity particularly against gram-negative bacteria due to the osmotic sensitivity of cells [26]. Importantly, when the salinity of bacterial dispersion drops from 0.9 to 0.18 wt% NaCl suddenly, exponential-phase *E. coli* cells were being considerably more susceptible to GO. Therefore, in this study, we studied at 0.45 wt% NaCl final concentration and avoided the sudden mixing of cells inside GO solutions to suppress the possible effect of osmotic shock. To do so, we have changed the mixing order of bacteria and GO by adding GO solutions on bacterial dispersions slowly. Fig. S2b shows that there is just a very slight viability difference between 0.45 and 0.72 wt% conditions for both exponential-and stationary-phase *E. coli*. By the same change in salinity, on the other hand, *P. aeruginosa* shows more increase in viability: nearly two and three times increase for exponential- and stationary-phase cells, respectively. Therefore, regardless the level of salinity level, exponential-phase cells are more susceptible to GO for both gram-negative bacteria.



Fig. S2. (a) The time-dependent antibacterial activity of GO against *E. coli* cells harvested at exponential and stationary phases based on the drop-plate assay. (Dashed lines are guide-to-eye.) (b) The effect of osmotic conditions on the antibacterial activity of GO towards *E. coli* (*EC*) and *Pseudomonas aeruginosa* (*PA*) cells studied at 0.45 and 0.72 wt% saline. (Note that all experiments were performed using 100 μ g/mL of GO and the results were normalized to the colony counts of untreated samples as reference.)

Considerations Regarding the Selection of Complementary Antibacterial **Assay:** Despite the repetitive observation of the antibacterial activity of graphene-family materials by many groups (Table S1), there is an ongoing skepticism due to the appearance of a few conflicting reports [51], [52]. Moreover, the complexity of the interactions between biological systems and nanoparticles makes already hard assessment of cellular viability evaluations even trickier [79]–[82]. Thus, one may argue the comprehensiveness of colony counting methods to draw a solid conclusion. As complementary techniques, we have first considered Live-Dead assay and cytoplasm constituent detection method. However, we particularly hesitated to rely on Live-Dead assay for a two main reasons: First, as the membrane permeability of cells greatly influences the reliability of technique and physiological states of the bacteria dramatically impact the membrane permeability [83], [84], the comparison of growing and nongrowing cells seemed tricky to us. Second, we suspected that GO-wrapped and inhibited cells may remain intact and hamper the accuracy of the technique limiting the penetration of dyes [85]. Actually, we also questioned the reliability of cytoplasm constituent detection method for the current research due to the second concern we argue for Live-Dead assay. Putting all together, we decided to focus on growth monitoring method, which is a powerful alternative as it provides a kinetic information about the recovery of cells.

3. Physicochemical Characterization of the E. coli Cells

Zeta Potentiometry: All ZPM measurements were performed on a Brookhaven ZetaPALS (Brookhaven Inc.) \sim 36-38 °C using cells washed and dispersed in isotonic saline to have OD₆₀₀ of \sim 0.2. Statistics were made out of eight data points, each of which is an average of five cycles, per sample. Results are available in Table 1 of the main text. In brief, exponential-phase cells were found to be more negatively-charged than stationary- and decline-phase cells. We discussed this observation in length within the main text and below with the help of DLS observations.

Dynamic Light Scattering: We performed DLS studies to better treat ZPM results. All DLS measurements were taken on a Zetasizer Nano-ZS (Malvern Instruments) device equipped with a detector positioned at 173° and a 633 nm He-Ne laser (4.0 mW) source. For all measurements, "automatic measurement position optimization" and "attenuation selection" modes were selected and minimum ten minutes of temperature equilibration time was given.

Eight data points were collected for each measurement by setting data accumulation time as 15 seconds. To avoid aggregation issues, all measurements were performed using fresh samples (<2 hours). Data was analyzed with Zetasizer Software (Version 7.11) provided by the manufacturer. The refractive index value of bacteria was taken as "1.39" [86], [87].

Due to the ion-penetrable, "soft", nature of bacterial outer membranes, there might be an overestimation in zeta potential results and the deviation is known to be physiologydependent as a result of the variations in surface structures of cells [41], [88], [42], [89]. Previously, pH-based changes detected in hydrodynamic size of bacteria has been linked to the collapse of bacterial surface structures which correlates with electrokinetic "softness" of bacteria [90], [91]. In our preliminary studies, we observed that hydrodynamic behavior of bacteria is also influenced by the temperature. Performing DLS measurements at 4 °C and 37 °C. we think that it is possible to compare the "softness" of exponential-, stationary-, and decline-phase cells based on the changes takes place in the average hydrodynamic diameter values (Fig. S3). Temperature dependence of the size difference is almost 100 nm for exponential-phase cells, practically no change occurs in stationary-phase cells, and declinephase cells show a drop of \sim 240 nm. These observations suggest that surface polymers (*i.e.* lipopolysaccharides and lipoproteins) of exponential-phase cells are less densely packed than those of stationary-phase cells and may collapse on the cell surfaces. The highest drop observed in decline-phase cells may indicate the presence of unevenly and highly extended surface polymers on cell surface presumably including extracellular polymeric substances. (Note that broadening in the cross-sectional profile of decline-phase cells seen in Fig. 4g of main text may also be implying the production and release of extracellular substances in decline-phase cells.) So these observations suggest that, exponential-phase cells are "softer" than stationary-phase cells, but decline-phase cells are the "softest". As a result, the drop of zeta potential values cannot be completely attributed to electrokinetic "softness" and hence the ranking of zeta potential values seems largely reliable. Important to note that, XPS studies further confirm this conclusion.



Fig. S3. DLS measurements of **(a)** exponential-, **(b)** stationary- and **(c)** decline-phase cells at 4 °C and 37 °C. Note that, data was constructed based on the results of experiments performed on two different occasions. (Dashed vertical lines mark "1" micrometer to help to compare the size shifts.)

X-ray Photoelectron Spectroscopy: In order to compare the changes in total and surface elemental compositions, we measured sonolyzed and intact cells, respectively. Sonolysis was performed using Vibra-Cell VCX-130 ultrasonic processor (Sonics & Materials, Inc.) equipped with titanium alloy microtips (tip penetration depth was kept ~3-4 cm due to foaming). At each experiment, ~20 mL of dense cellular samples cooled by ice-water bath was subjected to sonication at 20% amplitude for 15 minutes. Sonolyzed cells were vacuumdried for days at room temperature to obtain powders of cellular homogenates. To obtain intact and dried cells, we first tried freeze-drying in our preliminary experiments on exponential-phase cells. However, we have detected a considerable level of K signal which suggests cellular burst due to freeze-drying. Therefore, we have switched to vacuum-drying at low-temperature which apparently preserves the cellular integrity better with no or less K signal (Fig. S4). For this, washed cellular suspensions were first centrifuged and redispersed with 5-to-10 mL of isotonic saline yielding highly concentrated cellular suspensions. Concentrated suspensions were subjected to vacuum filtration onto Teflon filters resulting in cellular lawns. Obtained cellular lawns were then further oven-dried at ~40-45 $^\circ C$ for <3 hours and were subjected to vacuum-drying at 40 °C ~24 hours.



Fig. S4. XPS spectra of exponential-phase cells obtained using samples prepared by (a) freeze-drying and (b) vacuum-drying. (Note that K signals disappears in Fig. S4b indicated the intactness of bacteria.)

Results of sonolyzed cells are summarized in Table S2. As expected, there is a gradual and significant reduction in C content of cells by the switch from growing-to-nongrowing physiology. Accordingly, both O and N levels show an increase. It is hard to interpret the reason behind the increase in O level. However, the increase of N might be due to the increase of wastes. P level of exponential- and stationary-phase cells are quite similar. The main causes of increase detected in P content of decline-phase cells may be sustain or increase of DNA content and the accumulation of polyphosphates due to elevated nutritional stress [92], [93], [29], [94].

Table S2. Percentage elemental compositions of sonolyzed cellular homogenates of bacteria harvested at different growth stages as obtained by XPS analysis. (Note that all numbers show atomic percentages (at%) and calculations are done just based on four major elements using C1s, N1s, P2p, and O1s signal regions.)

Growth phase	%C	%O	%N	%P
Exponential	65.6	21.7	11.1	1.6
Stationary	62.0	22.4	14.1	1.5
Decline	56.4	26.2	14.3	3.1

No matter how different the detailed surface compositions of the cells having different physiologies are, as they are all covered with certain types of biopolymers. So, unlike as observed for sonolyzed cells (Table S2), C content of intact cells remains relatively unchanged with minor decreases by exponential-to-stationary and stationary-to-decline phase

transitions (Table S3). O contents are also quite close to each other with a slight decrease from growing to nongrowing physiologies. However, there is a marked increase in N content of nongrowing cells which supports the decrease of surface charge of nongrowing cells. P levels are relatively interesting. Exponential-phase cells reveal the highest content supposedly due to lower surface coverage density of surface LPSs and proteins in comparison to stationary-phase cells. The accumulation of polyphosphates due to nutritional stress [94] may supposedly explain the observation of higher P signal in decline-phase cells than that of in stationary-phase cells. Below in Fig. S5, XPS C1s, O1s, N1s, and P2p spectra of cells harvested at different growth phases are also given to support the discussions held in the main text.

Table S3. Surface elemental compositions of dried bacteria harvested at different growth stages as measured by XPS. (Note that all numbers show atomic percentages (at%) and calculations are done just based on four major elements using C1s, O1s, N1s, and P2p signal regions.)

Growth phase	%C	%O	%N	%P
Exponential	65.6	26.7	4.8	2.9
Stationary	64.2	25.3	8.7	1.8
Decline	62.9	25.3	9.3	2.5



Fig. S5. XPS C1s (a-c), O1s (d-f), N1s (g-i) and P2p (j-l) spectra with standard mathematical deconvolution for bacteria harvested at (a,d,g,j) exponential, (b,e,h,k) stationary and (c,f,i,l) decline phases.

Above in Fig. S5, we present C1s, O1s, N1s, and P2p spectra of bacteria harvested different growth phases with standard mathematical deconvolution process that fits purely Gaussian fits. We have analyzed C1s spectra (Fig.s S5a-to-S5c) with an advanced peak resolution algorithm called Compfit as well. Compfit makes peak decomposition using a model C1s spectrum (*i.e.* CompfitComponents.mat) created by multivariate analysis of a large set of data including standard samples and various bacterial strains [28]. We have fixed some minor bugs of this useful algorithm to be able to use it in newer versions of MATLAB® (R2013a and above) and also extended the capability of it by enabling the analysis of multiple data sets at one time. Thus, we named this updated algorithm as CompfitV2 which includes CompfitV2Analysis and CompfitV2Extension scripts. (Those codes and associated data set to run the codes are available in the Appendix section below.) Using CompfitV2, we are able to resolve our C1s spectra to its lipid, polysaccharide, and protein components with a typical fit error of $\sim 10\%$ which is good enough to interpret chemical composition of bacterial surfaces (Fig.s 5b-to-5d of the main text). The errors observed presumably originate from instrumental factors and natural dissimilarities between our samples and the samples used by Ramstedt et al. [28] in generating the model C1s spectrum used in the fitting procedure.

Surface Analysis of Bacteria by a Two-phase Partitioning Assay: For MAtS tests, chloroform was obtained from Sigma while the origin of hexadecane, diethyl ether or hexane were purchased from Fischer and all solvents were used as received from the vendor without further purification. As confirmed below in Fig. S6, the optical density of bacterial suspension is quite linearly dependent on the actual concentration of bacteria below "OD₄₀₀ \approx 1". So, the actual fraction of the cells partitioned into the solvent phase can be simply calculated by the relation given by Bellon-Fontaine *et al.* [32] as follows:

Adhesion to Solvents (%) =
$$\left(\frac{OD_{before} - OD_{after}}{OD_{before}}\right) \times 100$$

where " OD_{before} " is the initial optical density of bacterial suspension before mixing with solvent and " OD_{after} " is the optical density measured after vortex mixing of the two-phase system and subsequent separation of solvent and water phases.



Fig. S6. The correlation of optical density measurements with actual cellular concentration starting with a concentrated suspension of exponential-phase cells. (Experiment was carried out by gradually replacing equal volume of cellular suspension with isotonic saline.)

In order to evaluate electron donor / electron acceptor (a.k.a. Lewis acid-base) property of the cells, we studied MAtS test with two pairs of solvents having similar Lifshitz - van der Waals surface tension components: i) chloroform, an electron acceptor solvent, and hexadecane, a nonpolar solvent, ii) diethyl ether, an electron donor solvent, and hexane, a nonpolar solvent (Fig. S7). Thus, the difference between "Adhesion to Solvents" values obtained within the first pair of solvents (*i.e.* chloroform and hexadecane) indicates electron donor property of the bacterial surfaces while the same operation for the other pair (*i.e.* diethyl ether and hexane) gives the electron acceptor property. Results show that all growth phases have higher affinity to polar solvents (*i.e.* chloroform) and basic (*i.e.* diethyl ether) solvents [32], [95]. It should be also noted that "Adhesion to Solvents" values obtained for hexadecane also provide a measure of cell surface hydrophobicity particularly useful in a relative manner. (See Table 2 of the main text.) Two-phase partitioning tests have been vastly employed to investigate the correlations between microbial surface hydrophobicity and cellular physiology or even antibiotics susceptibility [45], [96], [97].



Fig. S7. (a) Adhesion of exponential-, stationary-, and decline-phase cells to four different solvents namely chloroform (CF), hexadecane (HD), diethyl ether (DEE), and hexane (HEX). (Dashed vertical line separates two pairs of solvents having similar surface energy components: i) CF-HD and ii) DEE-HEX.) **(b-c)** Representative images of a set of actual CF test samples before **(b)** and after **(c)** vortex mixing. (Note that different growth phases are denoted by their initials in Fig.s S3b and S3c and around 16-to-20 minutes were given for phase separation before photo shootings in Fig. S7c.)

4. Preparation and Characterization of Graphene Oxide Nanosheets

Synthesis: GO was synthesized according to a modified-Hummers method [25] with small changes. Unless otherwise stated, all chemicals purchased from Sigma and used without further purification. For pre-oxidation and cleaning, 2.7 g of natural graphite powder (Bay Carbon Inc.) was added into a mixture of H₂SO₄ (98 vol%, 21.6 mL), K₂S₂O₈ (4.5 g), and P_2O_5 (4.5 g) previously mixed in a round-bottom glass flask. Once all graphite chunks get wet, the mixture was heated to ~80 °C. After ~4.5 h, the system was cooled down to room temperature and transferred into ~1 L of water. After waiting a few hours for solid materials to deposit at the bottom, the supernatant was decanted and solid materials were filtered on a nitrocellulose membrane (pore size: ~0.45 µm, Merck Millipore). Obtained filter cake was thoroughly washed with deionized water until the pH of filtrate reached above pH 5 and then cleaned cake was oven-dried at 60 °C overnight. All dried solid materials were transferred into a dry container before adding H₂SO₄ (69 mL), NaNO₃ (1.5 g) and KMnO₄ (9 g) one-byone and slowly on an ice bath. A few minutes later, the ice bath was removed, and the mixture was set to ~35 °C under vigorous stirring. After giving ~2.5 hours for the reaction, ~260 mL of water and 15 mL of H₂O₂ were added slowly one after the other. (CAUTION: All these steps were performed in a fume hood and flasks were kept unsealed to let the release of outlet gas.)

Purification and X-ray Photoelectron Spectroscopy Analysis: To reach purity level desirable for biological work, graphite oxide product was washed with an excessive amount of deionized water with the help of first vacuum filtration on Teflon filters and then several cycles of centrifugation (at 12000 rpm). When pH of supernatant reaches to pH 6, the product was also subjected to dialysis for a week by refreshing the surrounding water bath once a day using cellulose tube membrane (D9652-100FT, M_w cut-off: 14k, Sigma-Aldrich). The final product (*i.e.* graphite oxide slurry) was kept in a well-sealed glass jar and was protected from light exposure by covering the jar with aluminum foil. The same stock was used throughout the studies for GO preparation. To check the purity level of stock graphite oxide samples, we have performed XPS survey scan (Fig. S8a). This analysis confirmed that the sample is highly pure with only a minor level of impurities (*i.e.* S, N, and P). We have also focused on C1s and O1s spectral regions which show that O incorporates in C in a variety of forms like carboxyl (O–C=O), epoxide (C–O–C), and carbonyl (C=O) functions (Fig. S8b and S8c).



Fig. S8. XPS analysis of purified Hummer's product used for the preparation of GO: (a) XPS survey spectrum and high-resolution (H-R) spectra of (b) C1s and (c) O1s regions with standard mathematical deconvolution. (Note that the elemental compositions obtained by both survey and H-R scans are given in Fig. S8a).

Exfoliation and Atomic Force Microscopy Characterization: Purified stock dispersion was vigorously stirred with a magnetic bar for a few days to yield a homogenous dispersion. After homogenization, the concentration of the product was measured by weighing a number of vacuum-dried (60 °C, ~24 hours) samples with an ultra-microbalance (XSE105, Mettler-Toledo). GO nanosheets were prepared by exfoliating and comminuting graphite/graphene oxide dispersions with a two-step sonication process. In a typical experiment, first, ~20 mL of as-prepared graphite oxide slurry (~6.7 mg/mL) was placed in a glass tube and was bathsonicated for 3 hours. In the second step, pre-exfoliated graphite/graphene oxide dispersion was diluted to ~0.5 mg/mL using deionized water and was tip-sonicated maximum 2-3 days before the antibacterial tests. Tip-sonication was performed exposing ~20 mL of diluted sample to 50% amplitude for 30 minutes with tip penetration depth of ~1 cm. Both in the bath- and tip-sonications, in order to prevent overheating and minimize temperature-induced reduction, samples were cooled using an ice-water mixture. After exfoliation processes, resulting GO solutions were kept overnight in a dark place and then were centrifuged at 4000 rpm for 10 minutes (Kubota, Model 2420) to eliminate titanium alloy contamination comes from the unavoidable corrosion of sonic tip during irradiation.

For atomic force microscopy (AFM) measurements, ~25 μ L of 5 μ g/mL GO solution was deposited on freshly cleaved mica surfaces (ca. 0.5×0.5 inch²) by drop casting and drying in ambient conditions. Measurements were performed in ambient conditions. (See above for the details of AFM device used.) AFM studies showed that GO is well exfoliated as monolayers and is comminuted as small particles typically below than a few hundred nanometers in lateral dimension (Fig. S9).



Fig. S9. Typical AFM images of GO nanosheets prepared with two-step sonication procedure: (a) large view, (b) closer view with (c) cross-sections.

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APPENDIX – CompfitV2 Algorithm

The script of CompfitV2 is available below as two separate MATLAB® codes with the associated data stands for component fitting: i) analysis, ii) extension, and iii) components. (It is recommended to use the file names as given below preferably in MATLAB® R2013a or above.)

i) CompfitV2Analysis.m

```
%% Definition: XPS C1s Peak Resolution - Analysis
% This script determines optimal fractions of protein/peptidoglycan
% (Component#1), lipid (Component#2), and polysaccharide (Component#3).
2
%% User Manual:
% Steps:
% 1. Place MATLAB files "CompfitV2Function.m" & "CompfitV2Extension.m"
00
   & data file "CompfitComponents.mat" in a working directory.
% 2. Store all your data (C1s spectra) inside the same directory
  in separate text files (".txt") by keeping binding energies (eV)
2
8
   in the first columns & intensities (cps) in the second columns.
% 2. Open "CompfitV2Extension.m" and run. (Click F5)
% 3. Step2 will return the following:
8
  modelC (sum of components)
8
  model (individual components)
8
          (fractions of components)
  Х
8
  BE
          (Binding energy = Components(:,1))
% Notes:
% 1. Please do not place any extra ".txt" files into working directory!
% 2. Please do not keep non-numeric items in ".txt" files like titles!
% 3. Make sure the range of your data is wider than "291-280.5 (eV)"!
%% Defining function:
% Function command:
function [model, modelC, x, BE, data] = CompfitV2Analysis(R, C, Count i)
%% Data interpolation to BE, normalization, and offset to "0" intensity:
% Rearrangement of data in components file (CompfitComponents.m):
BE = C(:, 1);
A = interp1(R(:,1), R(:,2), C(:,1));
data = Count i; % To be used for saving outputs
% Elimination of 'nan' terms from A in case experimental BE does not cover
% those of seen in CompfitComponents data:
[i] = isnan(A(:,1));
for n = 1:size(A, 2)
    if i(n) == 1
        A(1,n) = 0;
    end
end
2
% Taking intensity to "0" at A(end,2);
for n = 1:size(A, 2)
    AA = A - A(end, :);
```

```
end
% Normalization of AA(:,2):
A = AA . / sum(AA);
%% Optimization of x:
options = optimset('Diagnostics','off','Display','off','MaxPCGIter',200,...
    'TolPCG',1e-16,'Tolfun',1e-12,...
    'MaxIter',200, 'MaxFunEvals',500);
lb = [0 \ 0 \ 0]; ub = [1 \ 1 \ 1];
x0 = [.1 .1 .1 ];
x = lsqnonlin(@(x)(A - [C(:,2:4)*x']), x0, lb, ub, options);
model = [C(:,2:4)*x'];
modelC = [C(:,2).*x(1) C(:,3).*x(2) C(:,4).*x(3)];
number = num2str(data,'%03d'); % "001", "002", ..., "NoDD"
C1 = modelC(:,1); % Component#1
C2 = modelC(:,2); % Component#2
C3 = modelC(:,3); % Component#3
%% Plotting & printing results:
figure('position', [50, 50, 500, 450]); % Create new figure with given size
plot(BE,A,'--',BE,model,'Color',[0.5 0 0.5],'LineWidth',2.5); hold on;
plot(C(:,1),C1,'Color',[0 0 0.8],'LineWidth',2.5); hold on; % Component#1
plot(C(:,1),C2,'Color',[1.0 0 0],'LineWidth',2.5); hold on; % Component#2
plot(C(:,1),C3,'Color',[0 0.5 0],'LineWidth',2.5); hold on; % Component#3
legend('experimental','fitting','peptide','lipid','polysaccharide',...
    'Location', 'NorthWest');
legend boxoff;
xlabel('Binding Energy (eV)', 'Color', 'b', 'FontSize', 22, ...
    'FontWeight', 'Demi');
ylabel('Intensity (normalized cps)','Color','b','FontSize',22,...
    'FontWeight', 'Demi');
xMin = 281; xMax = 291; yMin = -0.001; yMax = 0.08;
axis([xMin, xMax, yMin, yMax]); % Axis scales
set(gca,'xdir','reverse','LineWidth',2);
set(gca, 'FontSize', 17); % Setting fontsize of axis scales and legend
set(gca,'XMinorTick','off'); % Minor X-ticks
set(gca,'YMinorTick','off'); % Minor Y-ticks
set(gcf,'Color',[1 1 1]); % Background color ("white": [1 1 1])
fprintf('Peptide
                          = %3f\n', x(1)); % Component#1
fprintf('Lipid
                          = %3f\n', x(2)); % Component#2
fprintf('Polysaccharide
                          = %3f\n\n', x(3)); % Component#3
output1 = [BE A];
output2 = [BE model modelC];
output3 = x;
eval(['dlmwrite(''Data' number '-Experimental.asci'',output1,''\t'');']);
eval(['dlmwrite(''Data' number '-Components.asci'',output2,''\t'');']);
eval(['dlmwrite(''Data' number '-Fractions.asci'',output3,''\t'');']);
%% Code History:
% Version01: The main version.
% --> by Ramstedt et al., J. Biol. Chem. (2011), 286(14), 12389-12396.
% Version02: Bug-Fix + extension for enabling multiple data analysis.
% --> by H. Enis Karahan @R2013a (23.02.2016).
```

ii) CompfitV2Extension.m

%% Definition: XPS C1s Peak Resolution - Extension

```
% This script determines optimal fractions of protein/peptidoglycan
% (Component#1), lipid (Component#2), and polysaccharide (Component#3).
2
%% User Manual:
% Steps:
% 1. Place MATLAB files "CompfitV2Analysis.m" & "CompfitV2Extension.m"
   & data file "CompfitComponents.mat" in a working directory.
9
% 2. Store all your data (C1s spectra) inside the same directory
   in separate text files (".txt") by keeping binding energies (eV)
8
   in the first columns & intensities (cps) in the second columns.
8
% 2. Open this file and run. (Click F5)
% 3. Step2 will return the following:
   modelC (sum of components)
8
8
   model (individual components)
           (fractions of components)
8
   х
           (Binding energy = Components(:,1))
8
   BE
% Notes:
% 1. Please do not place any extra ".txt" files into working directory!
% 2. Please do not keep non-numeric items in ".txt" files like titles!
% 3. Make sure the range of your data is wider than "291-280.5 (eV)"!
%% General Arrangements:
clc; clear; % Cleanup
format short; % Output scaling into 5 digits
beep off; % Turn off "beep" sound in case of (the majority of) errors
%% Importing and arrangement of the data into MATLAB environment:
files = dir('*.txt'); % Capturing the names of ".txt" documents
NoDD = length(files); % # of data documents
Count_i = 0; % Counter
for i = 1:1:NoDD; % Loading of data documents into the memory
    Count i = Count_i + 1;
    % Converting # character to string character:
    V = num2str(i,'%03d'); % "001", "002", ..., "NoDD"
    2
    % Loading & arrangement of the data [eV, cps]:
    eval(['DataXPS' V ' = load(files(i).name);']); % Data to be analyzed
    eval(['R = DataXPS' V ';']); % To keep original letters... --> R & C
    load -ASCII CompfitComponents.mat; % Data taken from Ramstedt et al.
    C = CompfitComponents; % To keep original letters... --> R & C
    fprintf('The fractions are as follows for data set #%1d:\n', i);
    % Calling the main analysis script:
    [modelC, model, x, BE, data] = CompfitV2Analysis(R, C, Count i);
    pause (0.5); % Waiting time between successive analysis
end
8
%% Code History:
% Version01: The main version.
% --> by Ramstedt et al., J. Biol. Chem. (2011), 286(14), 12389-12396.
% Version02: Bug-fix + extension for enabling multiple data analysis.
% --> by H. Enis Karahan @R2013a (23.02.2016).
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iii) CompfitComponents.mat (as obtained from the supporting information of the reference [28])

291.01	0.00128	3.681E-4	3.226E-4
290.91	0.00119	3.851E-4	3.252E-4
290.81	0.00117	3.582E-4	3.406E-4
290.71	0.00117	4.151E-4	4.283E-4
290.61	0.00110	4.510E-4	4.936E-4
290.51	0.00111	4.716E-4	5.257E-4
290.41	0.00111	5.179E-4	6.602E-4
290.31	0.00117	5.947E-4	7.584E-4
290.21	0.00120	7.199E-4	7.657E-4
290.11	0.00128	8.839E-4	8.099E-4
290 01	0 00134	0 00110	9.627E-4
289 91	0 00153	0 00133	0 00110
289.81	0.00183	0 00154	0.00116
289.71	0.00103	0.00194	0.00116
200.71	0.00204	0.00105	0.00150
209.01	0.00234	0.00213	0.00107
209.JI	0.00270	0.00231	0.00101
209.41	0.00329	0.00201	0.00102
209.31	0.00396	0.00313	0.00207
289.21	0.00464	0.00342	0.00247
289.11	0.00557	0.00355	0.00268
289.01	0.0066/	0.00358	0.00302
288.91	0.00795	0.00352	0.00391
288.81	0.00935	0.00341	0.00463
288.71	0.01114	0.00321	0.00528
288.61	0.01282	0.00300	0.00653
288.51	0.01425	0.00259	0.00838
288.41	0.01554	0.00242	0.01001
288.31	0.01633	0.00210	0.01197
288.21	0.01657	0.00184	0.01379
288.11	0.01628	0.00169	0.01510
288.01	0.01533	0.00158	0.01656
287.91	0.01386	0.00156	0.01794
287.81	0.01222	0.00162	0.01865
287.71	0.01036	0.00204	0.01965
287.61	0.00882	0.00246	0.02093
287.51	0.00744	0.00298	0.02284
287.41	0.00637	0.00333	0.02639
287.31	0.00583	0.00364	0.03077
287 21	0 00559	0 00411	0 03588
287 11	0.00583	0 00412	0 04242
287 01	0.00651	0 00426	0.01212
286 91	0 00764	0 00441	0.05567
286 81	0.00704	0.00441	0.05507
286 71	0.000000	0.00457	0.06431
286 61	0.01071	0.00457	0.06431
200.01	0.01202	0.00495	0.00430
200.01	0.01494	0.00554	0.06196
286.41	0.01066	0.00617	0.05691
286.31	0.01857	0.00734	0.04933
286.21	0.01983	0.00896	0.04020
286.11	0.02060	0.0111/	0.03140
286.01	0.02125	0.01360	0.02341
285.91	0.02154	0.01633	0.01688
285.81	0.02199	0.01970	0.01185
285.71	0.02258	0.02387	0.00870
285.61	0.02328	0.02896	0.00648
285.51	0.02473	0.03444	0.00489
285.41	0.02627	0.03994	0.00419
285.31	0.02760	0.04555	0.00358
285.21	0.02881	0.05017	0.00347
285.11	0.02965	0.05351	0.00326
285.01	0.02962	0.05465	0.00313

284.91	0.02913	0.05369	0.00303
284.81	0.02761	0.05032	0.00298
284.71	0.02544	0.04488	0.00282
284.61	0.02307	0.03808	0.00240
284.51	0.02003	0.03101	0.00209
284.41	0.01692	0.02423	0.00175
284.31	0.01391	0.01802	0.00139
284.21	0.01090	0.01325	0.00103
284.11	0.00854	0.00953	6.556E-4
284.01	0.00642	0.00675	7.008E-4
283.91	0.00476	0.00487	6.137E-4
283.81	0.00348	0.00361	4.246E-4
283.71	0.00253	0.00261	3.645E-4
283.61	0.00188	0.00188	3.230E-4
283.51	0.00131	0.00140	3.193E-4
283.41	9.574E-4	0.00107	3.652E-4
283.31	7.509E-4	8.284E-4	2.549E-4
283.21	5.920E-4	7.489E-4	1.672E-4
283.11	5.174E-4	5.697E-4	1.670E-4
283.01	4.659E-4	4.857E-4	1.040E-4
282.91	3.927E-4	4.296E-4	6.967E-5
282.81	3.261E-4	3.952E-4	1.523E-4
282.71	2.626E-4	3.766E-4	9.695E-5
282.61	2.377E-4	2.901E-4	9.302E-5
282.51	2.529E-4	2.574E-4	5.181E-5
282.41	1.927E-4	2.534E-4	8.165E-5
282.31	1.956E-4	2.079E-4	6.265E-5
282.21	1.767E-4	1.743E-4	3.992E-5
282.11	1.769E-4	1.665E-4	3.086E-5
282.01	1.463E-4	1.981E-4	8.347E-5
281.91	1.096E-4	1.577E-4	1.689E-4
281.81	8.729E-5	1.109E-4	1.363E-4
281.71	7.871E-5	1.531E-4	0
281.61	8.501E-5	1.365E-4	0
281.51	3.019E-5	1.378E-4	1.313E-4
281.41	5.907E-5	1.025E-4	3.344E-5
281.31	9.057E-5	1.230E-4	0
281.21	9.220E-5	8.202E-5	0
281.11	4.753E-5	4.433E-5	6.888E-5
281.01	4.686E-5	4.482E-5	7.775E-5