2D Materials

PAPER

Purity of graphene oxide determines its antibacterial activity

I Barbolina[1], C R Woods[2], N Lozano[3,4], K Kostarelos[3,4], K S Novoselov[2,4] and I S Roberts[1]

[1] The Department of Life Sciences, University of Manchester, UK
[2] School of Physics and Astronomy, University of Manchester, UK
[3] Nanomedicine Laboratory, Faculty of Medical & Human Sciences, University of Manchester, UK
[4] National Graphene Institute, University of Manchester, Oxford Road, Manchester M139PL, UK

E-mail: i.s.roberts@manchester.ac.uk

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Abstract

Nanomaterials based on two-dimensional (2D) atomic crystals are considered to be very promising for various life-science and medical applications, from drug delivery to tissue modification. One of the most suitable materials for these purposes is graphene oxide (GO), thanks to a well-developed methods of production and water solubility. At the same time, its biological effect is still debated. Here we demonstrate that highly purified and thoroughly washed GO neither inhibited nor stimulated the growth of *E.coli*, ATCC25922; *E.coli* NCIMB11943 and *S.aureus* ATCC25923 at concentrations of up to 1 mg ml^{-1}. Moreover, transmission electron microscopy (TEM) of GO exposed bacteria did not reveal any differences between GO exposed and not exposed populations. In contrast, a suspension of insufficiently purified GO behaved as an antibacterial material due to the presence of soluble acidic impurities, that could be removed by extended purification or neutralisation by alkaline substrates. A standardised protocol is proposed for the generation of clean GO, so it becomes suitable for biological experiments. Our findings emphasise the importance of GO purification status when dealing with biological systems as the true effect of material can be masked by the impact of impurities.

1. Introduction

Research into graphene and graphene-based materials has expanded into medicine and life sciences focusing on imaging [1], biosensors [2, 3], drug delivery [4–6], and pathogen control [7, 8]. Graphene oxide (GO) is one of the materials of interest due to its aqueous colloidal stability, possibility to functionalise and vary its hydrophilicity by (partial) reduction into graphene [9, 10].

The effect of GO on microorganisms has been researched particularly intensively. However the results up to date have been extremely controversial, ranging from demonstration of potent antibacterial effects at minimum inhibitory concentration (MIC) similar to the clinically used antibiotics [11–15] to the evidence of enhanced microbial recovery and increased physiological activity [16–19]. This discrepancy in the published data is exemplified by the effect of GO on *Escherichia coli* where GO has been variously reported to be bactericidal with MICs in the region of 0.25 μg ml^{-1} [15] through to having no detectable effects at 200 μg ml^{-1} [20]. A number of hypotheses have been proposed to explain the possible antibacterial properties of GO, including cell wrapping [21], contact with sharp edges [12], oxidative stress [14], and destructive extraction of phospholipid [22]. However, to date there is not a single unifying model to explain possible toxicity of GO. Likewise the published data on the effect of the flake size of GO on bacterial viability is equally inconsistent [20, 23]. One possible explanation for this inconsistency in the reported data may be due to the source of the GO and the manner in which it is produced and prepared prior to experimentation [24–26].

In this paper we address these issues to establish the effect of GO on bacteria. We develop a standardised protocol for GO preparation prior to use and demonstrate that GO has no antibacterial properties against both Gram negative and positive bacteria and that reported inhibitory effects of GO are probably a result of the pH of the GO and it contamination with low molecular weight contaminants.

2. Experimental procedures

2.1. Synthesis of graphite oxide (GtO) for GO production

Graphite flakes (Graflake 9580) were obtained from Nacional Grafite Ltda. (Brazil). Nitric acid 70%, sodium nitrate, potassium permanganate, sulfuric acid 99.999% and hydrogen peroxide 30% were purchased from Sigma Aldrich. Water for injection was obtained from Fresenius Kabi. Graphite oxide (GtO) sheets have been synthesized using the modified Hummers' method previously described [24]. Briefly, 0.8 g of graphite flakes were mixed with 0.4 g of sodium nitrate in a round bottom flask, and then 18.4 ml of sulphuric acid 99.999% was added slowly to the mixture. After obtaining a homogenized mixture, 2.4 g of potassium permanganate was slowly added and the mixture was maintained for 30 min. 37 ml of water for injection was added drop-wise due to the violent exothermic reaction and the temperature was continuously monitored and kept at 98 °C for 30 min. The mixture was further diluted with 112 ml of water and 30% hydrogen peroxide was added for the reduction of the residual potassium permanganate, manganese dioxide and manganese heptoxide to soluble manganese sulphate salts.

2.2. Alternative method of GO production from commercial GtO samples

GtO for GO preparation was obtained from BGT Materials, UK, where it was prepared according to modified Hummers' method [27] from natural graphite of 99.95% purity and was purified with HCl and washed with deionised water by centrifugation. GtO was additionally washed in deionised water and separated by centrifugation at 11 872 g until pH of supernatant achieved pH-6-6.5, the same as pH of deionised water using Sorvall RC5B Plus centrifuge (USA). The exfoliation of GO was done by sonication for 30 min in the ultrasonic tank (Hilsonic, UK) and separated from remaining GtO by two times centrifugation at 11 872 g for 30 min. GO was additionally washed in deionised water by several steps of centrifugation at 34 310 g for 30 min until neutral pH of supernatant. For GO with small flakes the above prepared GO was additionally sonicated for 12 h. GO samples were sterilised by short exposure (10 min) to UV light (UviTec, UK).

2.3. Commercial samples of GO

Commercial samples of GO (CSA, CSB, CSC) were obtained as catalogue or research products from Graphenea (Spain), BGT Materials (UK), 2DTech (UK) and were used as reference materials for comparison with in-house produced GO.

2.4. X-ray photoelectron spectroscopy (XPS)

The composition of GO surfaces was studied by x-ray photoelectron spectroscopy (XPS) at NEXUS facility (the UK's National EPSRC XPS Users' Service, hosted by nanoLAB in Newcastle-upon-Tyne). GO samples were freeze-dried prior the analysis using Scan Vac Cool Safe 55-9 Pro freezer dryer, Labogene (Denmark). XPS was recorded using a Thermo Theta Probe XPS spectrometer with a monochromatic Al K-α source of 1486.68 eV. The survey XPS spectra were acquired with pass energy (PE) of 200 eV, 1 eV step size, 50 ms dwell time and averaged over 5 scans. The etching was 90 s. The high resolution C1s XPS spectra were acquired with PE of 40 eV, 0.1 eV step size, 100 ms dwell time and averaged over 20 scans. Spectra from insulating samples have been charge-corrected by shifting all peaks to the adventitious carbon C1s spectral component binding energy set to 284.6 eV. CasaXPS software was used to process the spectra acquired at NEXUS.

2.5. Atomic force microscopy (AFM)

AFM measurements were performed on a Bruker Dimension 3100V atomic force microscope, in Tapping (Dynamic) mode. The tips used have resonance of approximately 350 kHz.

2.6. Inductively coupled plasma atomic emission spectrometry (ICP-AES)

Metal content of GO suspensions was determined by ICP-AES measurements using Perkin-Elmer Optima 5300 (USA). Samples for ICP-AES analysis were chemically digested by drying 2.5 mg of GO dispersions followed by addition of 1 ml nitric acid (70%) at 70 °C–80 °C overnight. Then 1 ml of hydrogen peroxide ultrapure 30% was added and kept it at 70 °C–80 °C for 4 h. 5 ml of diluted nitric acid (2%) was added and the samples were filtered through 0.2 μm.

2.7. Bacteria strains and culture conditions

E.coli, ATCC25922 and *Staphylococcus aureus*, ATCC25923 were obtained from Thermo Scientific (UK); *E.coli*, NCIMB 11943 was obtained from National Collection of Industrial Food and Marine Bacteria (NCIMB, UK). Cultures were maintained as a frozen stock (−80 °C, RS Biotech freezer, UK) in growth media supplemented with 50% glycerol. Liquid cultures were grown in where appropriate Mueller-Hinton Broth (MHB), Tryptone Soy Broth (TSB) (Oxoid, UK) and M9 minimal media [28] supplemented with thiamine (0.01% w/v). Tryptone Soy Agar (TSA) (Oxoid UK) was used as solid media.

2.8. Effect of GO on cell growth monitored by measuring optical density (OD)

An inoculum of 10^5 coloniy-forming unit (cfu) ml^{-1} in the appropriate double strength growth media was prepared from overnight broth culture of test organism. GO water suspension dilutions in the range of 0–500 $\mu g\,ml^{-1}$ were prepared from GO stock solution of 1000 $\mu g\,ml^{-1}$. No GO was added to GO-free growth control. An aliquot of inoculum was mixed with the same

volume of GO dilutions or GO-free control to give a final concentration of GO in the range of 0–250 μg ml^{-1}. Double strength sterile growth medium mixed with GO dilutions was included as abiotic control. 200 μl of each mixture was dispensed into 6 wells of 96-well sterile microplate (Costar, Corning, USA). Cell growth was monitored by measuring the turbidity OD_{600} at 30 min intervals during 0–24 h incubation at 37 °C with fast shaking in Synergy™ Multi-Mode Microplate Reader (BioTek® Instruments, USA). The OD_{600} of bacteria culture was corrected for OD of abiotic control media with the same amount of GO at the same point of measurement.

2.9. Bacteria growth in the presence of GO determined by viable count

Test media was supplemented with required amount of GO to achieve concentration 0–250 μg ml^{-1}. Media without GO was used as a control. Media were inoculated with overnight culture of test organism to the final concentration 10^3–10^4 cfu ml^{-1} and the flasks were incubated at 37 °C and shaken at 200 rpm. The concentration of bacteria was determined at regular intervals during 24 h incubation by serial dilution of the sample and plating out the aliquot (20–100 μl) on the surface of TSA. The plates were incubated at 37 °C for 24 h and the total number of colonies was counted. Concentration of bacteria in the media at each sampling point was calculated taking into account dilution of the sample and the amount plated out on the solid media.

The resulted growth curves were analysed by fitting the logistic growth equation using formula:

$$N(t) = \frac{N_0 B}{N_0 + (B - N_0)e^{-rt}}$$

Where, N(t)—bacteria concentration (cfu ml^{-1}) at time point—t (hours); $N_{(0)}$—bacteria initial concentration (cfu ml^{-1}); B—maximum bacterial density (cfu ml^{-1}); r—growth rate constant (h^{-1}).

2.10. Quantitative suspension test

Quantitative suspension test was used to test effect of GO as following. An overnight culture of *E.coli* ATCC25922 grown in MHB was washed 2 times in deionised water and suspended into GO test solution at the concentration 1 mg ml^{-1}. The suspension was incubated for 4 h at 30 °C and 200 rpm shaking and the number of surviving organisms was evaluated after 2 and 4 h of incubation by plating out 20 μl from 10^0–10^{-2} dilutions onto surface of TSA plate and compared to the original inoculum. Recovery of bacteria from deionised water suspension after the same treatment was used as material-free control. Microbiocidal effect of test suspension (ME) is calculated as:

$$ME = \log(I) - \log(T)$$

Where, I—initial inoculum concentration, cfu ml^{-1}, and T—concentration of surviving microorganisms, cfu ml^{-1}.

2.11. Transmission electron microscopy (TEM)

Overnight culture of *E. coli*, ATCC25922 or *S. aureus*, ATCC25923 grown in MHB was washed in sterile saline solution and inoculated into MHB with added GO (100 μg ml^{-1}) to achieve approximate bacteria concentration of 10^6 cfu ml^{-1}. The cultures were grown at 37 °C with 200 rpm shaking until OD_{600} at ~1. The samples were fixed with 4% v/v formaldehyde +2.5% v/v glutaraldehyde in 0.1 M Hepes buffer (pH 7.2). They were post-fixed with 1% w/v osmiumtetroxide +1.5% w/v potassium ferrocynaide in 0.1 M cacodylate buffer (pH 7.2) for 1 h, then in 1% w/v tannic acid in 0.1 M cacodylate buffer (pH7.2) for 1 h and finally in 1% w/v uranyl acetate in water for 1 h. The samples were dehydrated in ethanol series infiltrated with TAAB 812 resin and polymerized for 24 h at 60 °C. Sections were cut with Reichert Ultracut ultramicrotome and observed with FEI Tecnai 12 Biotwin microscope (USA) at 100 kV accelerating voltage. Images were taken with GatanOrius SC1000 CCD camera.

3. Results

3.1. Preparation and characterisation of GO suspensions

In-house produced samples for our study (marked UMT—stands for the University of Manchester) were prepared by exfoliation of graphite oxide (GtO). Before exfoliation, GtO was washed in deionised water to remove impurities from oxidation process. The efficiency of purification process and the number of washing—centrifugation cycles was controlled by pH of supernatant with the purification stopped when the pH of supernatant was close to pH of deionised water used for washing (pH 6-6.5). GO prepared from purified GtO was also washed in deionised water and separated by centrifugation to complete the purification process. Figure 1 shows AFM image of UM samples prepared by this method. The sample mainly consists of single-layer flakes, which is confirmed by the height measurement (figure 1(B)). Raman spectra obtained from our samples (see supplementary information) are characteristics for GO.

To demonstrate the role of purification process on the effect of GO towards bacteria, we applied different number of washes to four subsamples of freshly synthesized GtO as shown (figure 2(A)) and then prepared corresponding GO suspensions: UMT-0; UMT-1; UMT-2; UMT-3. These resultant GO suspensions were adjusted to the same concentration of 1 mg ml^{-1}.

XPS analysis of samples of GO at different points in the washing protocol is shown in table 1. The C:O ratio of the GO samples ranged from 2.2 to 2.4 without correlation to the number of GtO washes before exfoliation. The purity of GO increased with the number of washes from 97.7% to 99.8% in UMT-0 and UMT-3 samples respectively. The sulphur content reduced from 1.68% in the UMT-0 sample (2 washes) to 0.05%

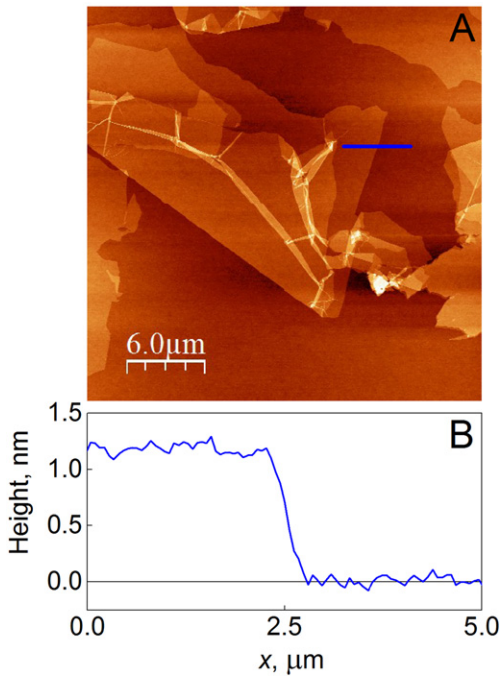
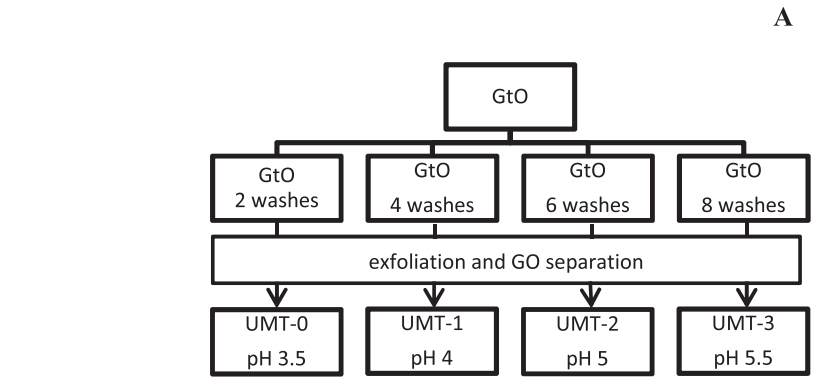


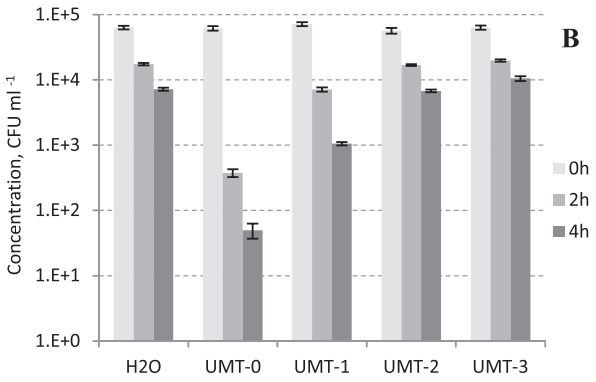
Figure 1. AFM image of GO UMT-3 sample on SiO wafer (A) and the corresponding height profile (B). The height profile diagram of the blue line outlined on the AFM image. The height of ~1.2 nm is typical for monolayer GO as measured by AFM [30].

in the UMT-1 sample (4 washes) and was not detectable in the UMT-2 (6 washes) and UMT-3(8 washes) samples. Likewise boron was present only in the first UMT-0 sample (table 1). At the same time, the presence of nitrogen was shown only in UMT-1, UMT-2 and UMT-3 samples. We believe this nitrogen in a contaminant of the GO preparation procedures as reported elsewhere [29]. The impurities of GO samples after different purification was further analysed by quantification of metals known to be present in GO. Concentrations of Al, Cr, Mn, Fe, Co, Ni, Cu, Zn, Mo in UMT-0 and UMT-3 samples were measured by ICP-AES (table 2). The concentrations of Al, Cr, Ni and Mo were similar in both samples tested. The concentration of Fe, Cu and Mn decreased with the number of washing steps and the most reduction was observed for Mn, where GO obtained from 2 times washed GtO contained 7871.50 ± 0.27 ppm and was reduced 4.5 times to 1734.83 ± 1.14 ppm in GO produced from 8 times washed GtO. The concentration of two metals: Co and Zn showed small increase in UMT-3 sample in comparison to UMT-0.

3.2. The Effect of GO purity on bacterial viability

We used a modified quantitative suspension test recommended for disinfectants [31] using reference culture of





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Sample	Microbiocidal effect (ME) log10 reduction in CFU ml^{-1}	
	2h	4h
H_2O	0.56	0.94
UMT-0	2.22	3.09
UMT-1	1.00	1.83
UMT-2	0.53	0.92
UMT-3	0.50	0.78

Figure 2. Preparation of purified GO suspensions. (A) Effect of GO suspensions (1 mg ml^{-1}) on recovery of *E.coli*, ATCC25922 in suspension test (incubation at 30 °C for 2 and 4 h) (B) Microbiocidal effect calculated for different GO suspension and for control (C).

Table 1. XPS survey of GO suspensions different by the purification process. All GO suspension were prepared from the same graphite oxide sample (GtO): UMT-0—graphite oxide was washed 2 times in the same volume of deionised water before sonication and GO separation by centrifugation; UMT-1—graphite oxide was washed 4 times before exfoliation and separation; UMT-2—graphite oxide was washed 6 times before exfoliation and separation; UMT-3—graphite oxide was washed 8 times before exfoliation and separation.

	Element content, %						
Sample	C 1s	O 1s	N 1s	B 1s	S 2p	Purity, %	C:O
UMT-0	67.32 ± 0.98	30.39 ± 0.84	<0.01	0.61 ± 0.18	1.68 ± 0.07	97.7 ± 0.2	2.2 ± 0.1
UMT-1	69.95 ± 0.09	29.64 ± 0.04	0.36 ± 0.03	<0.01	0.05 ± 0.07	99.6 ± 0.1	2.4 ± 0.0
UMT-2	70.08 ± 0.13	29.83 ± 0.18	0.09 ± 0.13	<0.01	<0.01	99.9 ± 0.1	2.3 ± 0.0
UMT-3	70.86	28.95	0.19	<0.01	<0.01	99.8	2.4

Table 2. ICP-AES analysis of GO samples before and after purification showing the concentration contaminating metals.

	Elemental concentration (ppm by mass)	
Element	UMT-0	UMT-3
Al	1405.36 ± 1.38	1413.97 ± 1.49
Cr	15.51 ± 1.89	14.71 ± 2.33
Mn	7871.50 ± 0.27	1734.83 ± 1.14
Fe	240.41 ± 0.45	137.05 ± 1.20
Co	0.99 ± 1.33	1.63 ± 1.92
Ni	3.57 ± 1.41	3.76 ± 5.90
Cu	47.86 ± 0.83	38.97 ± 1.26
Zn	54.06 ± 0.54	113.22 ± 0.38
Mo	0.81 ± 7.50	0.76 ± 11.19

E.coli ATCC25922. Here the number of viable bacteria was determined after exposure to GO aqueous suspension (1 mg ml^{-1}). The contact time was extended from 5–30 min recommended for disinfectants to 2–4 h and contact temperature was increased from room temperature to 30 °C. As a material-free control, bacteria were incubated for the same period of time in deionised water. Although there is some loss of viability in deionised water since there are no nutrients present and the bacteria will experience an osmotic shock [32] any relative changes in bacteria recovery from the GO-water suspension would clearly indicate the effect of suspended material. The levels of bacteria recovery from different GO suspensions and corresponding calculated microbiocidal effects (ME) are presented (figures 2(B) and (C) respectively).

As predicted the GtO purification process had significant impact on bacteria viability from corresponding GO suspensions. The lowest recovery of *E. coli* inoculum was observed from the list purified UMT-0 sample with 2.22 log reduction after 2 h of incubation and 3.09 log reduction after 4 h. GO samples from further purified GtO (UMT-3) showed increased recovery of bacteria cells with UMT-2 suspension giving the same recovery as from GO-free water.

Therefore these data confirm that bactericidal properties of GO are critically dependent on the purification and preparation method and probably are as a result of pH and contaminating small molecules.

All GO suspensions were prepared from the same graphite oxide sample (GtO): UMT-0—graphite oxide was washed 2 times in the same volume of deionised water before sonication and GO separation by centrifugation; UMT-1—graphite oxide was washed 4 times before exfoliation and separation; UMT-2—graphite oxide was washed 6 times before exfoliation and separation; UMT-3—graphite oxide was washed 8 times before exfoliation and separation. H_2O—deionised water used as GO-free control.

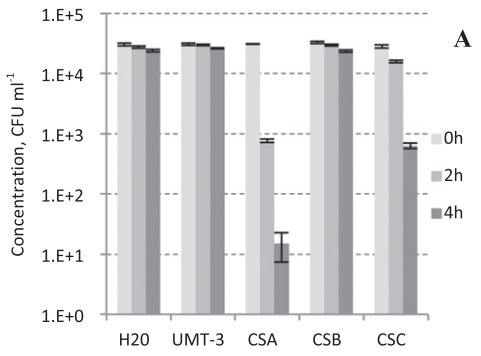
3.3. The mechanism of GO toxicity towards bacteria

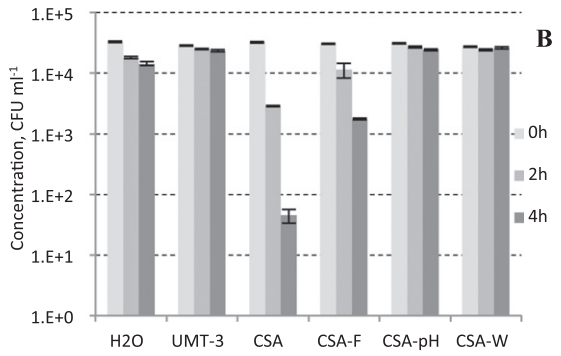
To study the effects of commercial GO in comparison to GO produced and purified at Manchester University three samples from commercial sources (CSA, CSB and CSC—this notation stands for as supplied commercial samples) were purchased. Comparative elemental composition of these samples is shown (table 3). The highest purity of 99.4% was measured for UM and CSB samples, with lower purity for CSA and CSC measured 97.6% and 98.2% respectively. Furthermore, we prepared washed sample CSA-W using the protocol described in this paper, where we made sure that the pH of the suspension is close to that of deionised water (6–6.5). An alternative sample with neutral pH was achieved by neutralising GO suspension with ammonium hydroxide to pH 6.8 to generate CSA-pH. Finally, for control purpose, we also produced filtrate from the commercial sample through the removal of GO material by centrifugation and filtration through 0.22 μm membrane filter to generate the filtrate CSA-F. We then assayed these samples in the modified quantitative suspension test using *E. coli* ATCC25922 (figure 3).

The levels of bacteria recovery from different GO suspensions and corresponding calculated microbiocidal effects (ME) are presented (figure 4 panels A and C respectively). Based on the data here none of the GO samples can be considered as disinfectants at tested concentration 1 mg ml^{-1} (figure 3(C)) as a ME of $\geqslant 5$ is required for a substrate to be classified as disinfectant [31]. The results of bacteria recovery (figure 3(A)) showed noticeable variations between tested GO suspensions. The ME of UM and CSB samples were found to be similar to ME of water for both exposure times 2 h and 4 h indicating that presence of GO materials did not cause any additional inhibition or stimulation in comparison to water, which itself resulted in 10% (0.05 log)

Table 3. XPS survey of GO samples. UMT-3—GO sample prepared at the University of Manchester according to the method described in this paper; CSA, CSB, CSC—commercial as supplied samples of GO.

	Element content, %						
Sample	C 1s	O 1s	N 1s	B 1s	S 2p	Purity, %	C:O
UMT-3	71.09 ± 0.19	28.34 ± 0.18	0.04 ± 0.06	<0.01	0.53 ± 0.05	99.4 ± 0.1	2.5 ± 0.0
CSA	68.78 ± 0.04	28.80 ± 0.31	0.09 ± 0.13	<0.01	1.74 ± 0.05	97.6 ± 0.3	2.4 ± 0.0
CSB	68.44 ± 0.19	30.98 ± 0.05	0.18 ± 0.13	<0.01	0.40 ± 0.03	99.4 ± 0.2	2.2 ± 0.0
CSC	66.42 ± 0.75	31.82 ± 0.79	0.01	0.17 ± 0.05	1.60 ± 0.04	98.2 ± 0.1	2.1 ± 0.1





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Sample	Microbiocidal effect (ME) log10 reduction in CFU ml^{-1}	
	2h	4h
H_2O	0.05	0.10
UMT-3	0.01	0.06
CSA	1.60	3.31
CSB	0.04	0.14
CSC	0.25	1.65

D

Sample	Microbiocidal effect (ME) log10 reduction in CFU ml^{-1}	
	2h	4h
H_2O	0.26	0.36
UMT-3	0.06	0.09
CSA	1.05	2.86
CSA-F	0.43	1.24
CSA-pH	0.06	0.11
CSA-W	0.05	0.02

Figure 3. (A), (B) Effect of GO suspensions (1 mg ml^{-1}) of different preparations on recovery of *E.coli*, ATCC25922 in suspension test (incubation at 30 °C for 2 and 4 h). (C), (D) Microbiocidal effect calculated for experiment A—(C) and B (D). H_2O—deionised water used as GO-free control; UMT-3—graphene oxide suspension in deionised water; CSA, CSB, CSC—commercial graphene oxide samples adjusted to concentration 1 mg ml^{-1}; CSA-F—filtrate of CSA suspension obtained by centrifugation and filtration; CSA-pH—suspension of CSA adjusted to pH 6.85 by addition of ammonium hydroxide; CSA-W—suspension of CSA additionally purified by 5 times washing in deionised water.

and 20% (0.10 log) reduction in *E.coli* concentration after 2 h and 4 h respectively. However, a significant drop in viability was detected with two of the commercial GO preparations CSA and CSC with ME of 3.31 and 1.65 respectively after 4 h of incubation. Comparison of elemental content of GO samples used in the experiment (table 3) shows higher concentration of sulphur and the presence of boron in both CSA and CSC samples in contrast to other tested GO suspensions. This would indicate the presence of impurities in these preparations.

These data suggest that the antibacterial effect observed in CSA and CSC is not due to GO flakes, but rather from impurities present in the suspension. To verify this assumption we tested the washed and neutralised versions of CSA (CSA-W and CSA-pH) as well as its filtrate (CSA-F). All the preparations were tested for recovery of *E.coli* ATCC25922 cells in the suspension test (figures 3(B) and (D)). Recovery of *E.coli* from the CSA-F after 4 h of incubation was reduced by over 1 log and was lower than recovery from deionised water after 4 h of incubation. The washed (CSA-W) and neutralised (CSA-pH) subsamples adjusted to the same concentration—1 mg ml^{-1} supported bacteria recovery similar or slightly higher than recovery from deionised water. These results indicate that the toxicity of CSA sample can be explained by presence of soluble acidic impurities which can be removed by additional washing or neutralised by base compounds.

3.4. Effect of GO on bacteria growth in planktonic culture

The growth of *E.coli* NCIMB11943, *E.coli* ATCC25922 and *S. aureus* ATCC25923 in the presence of GO (UM

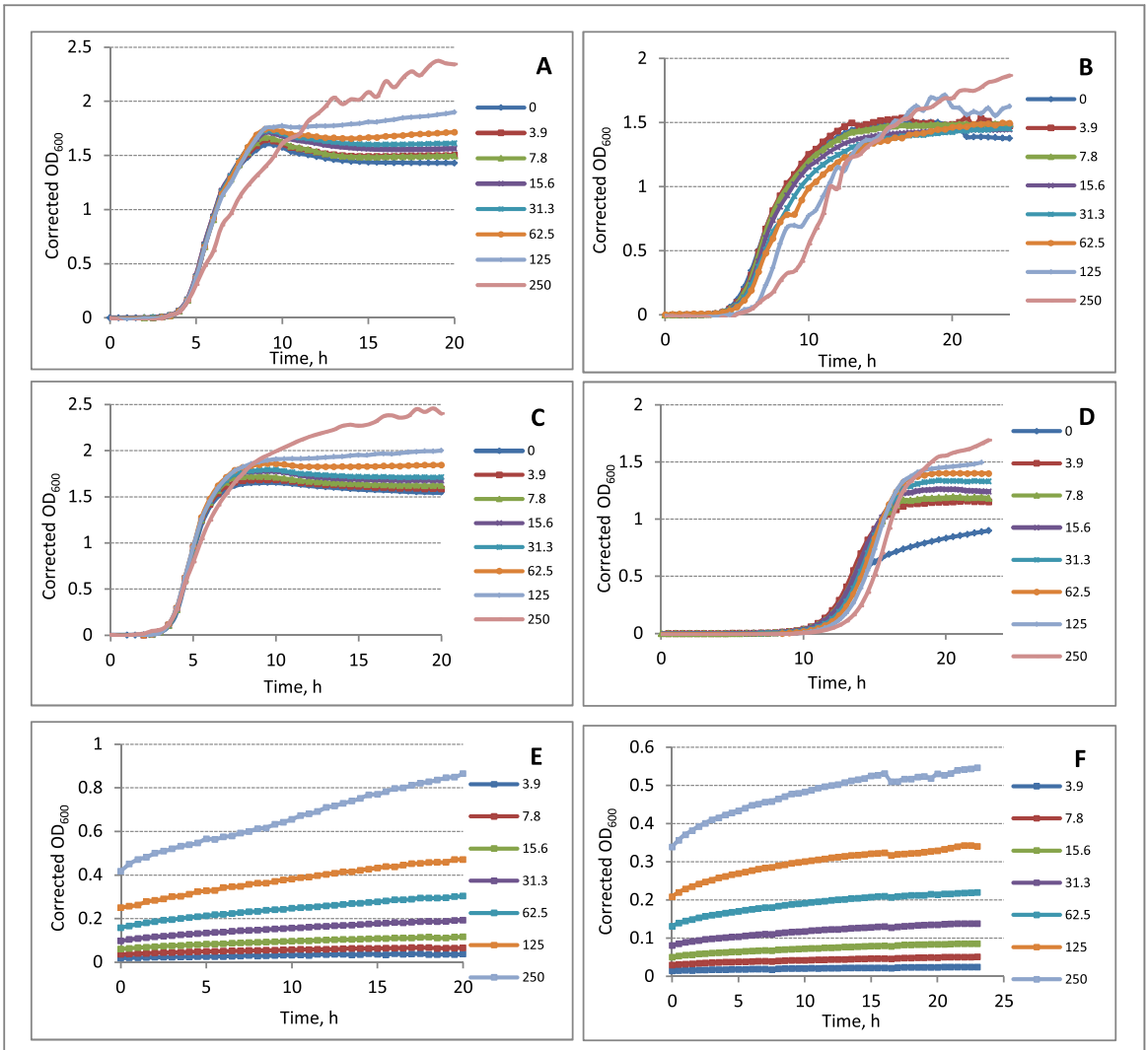

Figure 4. Optical density of test cultures in the media supplemented with GO at the concentration 0–250 μg ml^{-1}. A—*E.coli*, ATCC25922 in MHB media; B—*S.aureus*, ATCC25923 in MHB media; C—*E.coli*, NCIMB11943 in MHB media; D—*E.coli*, NCIMB11943 in M9 minimum media supplemented with thiamine; E—OD of MHB bacteria-free media with GO; F—OD of M9 bacteria-free media with GO.

preparation, 0–250 μg ml^{-1}) was evaluated by measuring OD_{600} of growth media during 24 h incubation (figures 4(A)–(D)) The results show that both *E.coli* strains and *S. aureus* were able to grow in MHB the presence of GO at the concentrations up to maximum tested—250 μg ml^{-1}. These results are in contrast with the previous reports where complete inhibition of bacteria growth was found with GO - 0.25 μg ml^{-1} for *E.coli* [15]. In the case of *S. aureus* concentrations of 125 μg ml^{-1} or above induced an extended lag phase but even at these higher GO concentrations *S. aureus* was capable of growing to the same final OD_{600} (figure 4(B)).

Although measurement of OD_{600} is widely used to measure bacterial growth, in the case of media supplemented with GO the interpretation of data is complicated by the presence of the material. As shown (figures 4(E) and (F)), the incubation of GO in growth media without bacteria resulted in continuous increase of optical density probably through GO reduction. Therefore the OD_{600} of the bacteria cultures were corrected for OD_{600} of sterile media with GO at each time point. Material transformation was also observed in the inoculated media containing GO with a change in the colour of the medium from light brown to dark brown and material precipitation by the end of incubation. GO instability and reactivity in the biological solutions was reported previously [33, 34], where aggregation of material was observed. Also bacterial reduction of GO was shown in the number of investigations in which reduction of GO to graphene resulted of blackening of the media and precipitation of graphene [35–37]. Increased maximum optical density of bacteria grown in the media supplemented with GO was described previously [16] but was assigned to stimulatory effect of material on microbial population. We would question this interpretation.

Taking into account the limitations of OD_{600} method and to establish what if any growth stimulatory effects GO had, an alternative viable count approach was utilised to investigate the growth response of bacteria to the presence of GO. Figure 5 shows growth curve of *E. coli* NCIMB11943 in M9 media supplemented with GO,

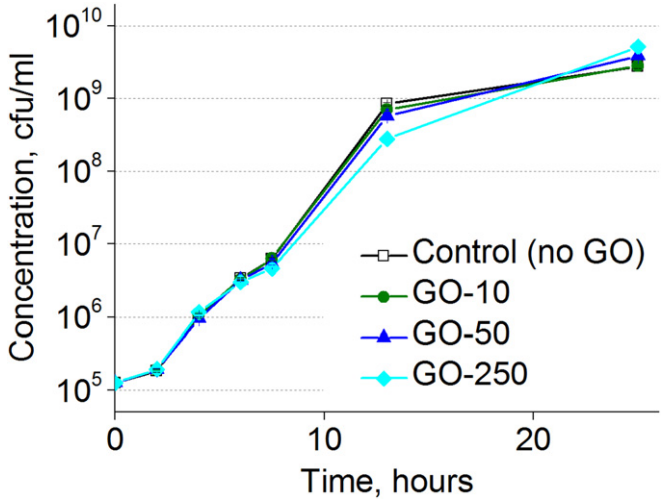

Figure 5. Growth of *E.coli*, NCIMB11943 in M9 media supplemented with GO UMT-3 preparation. Control—deionised water; GO-10—minimum M9 media supplemented with GO 10 μg ml^{-1}; GO-50—media with GO 50 μg ml^{-1}; GO-250—media with GO 250 μg ml^{-1}.

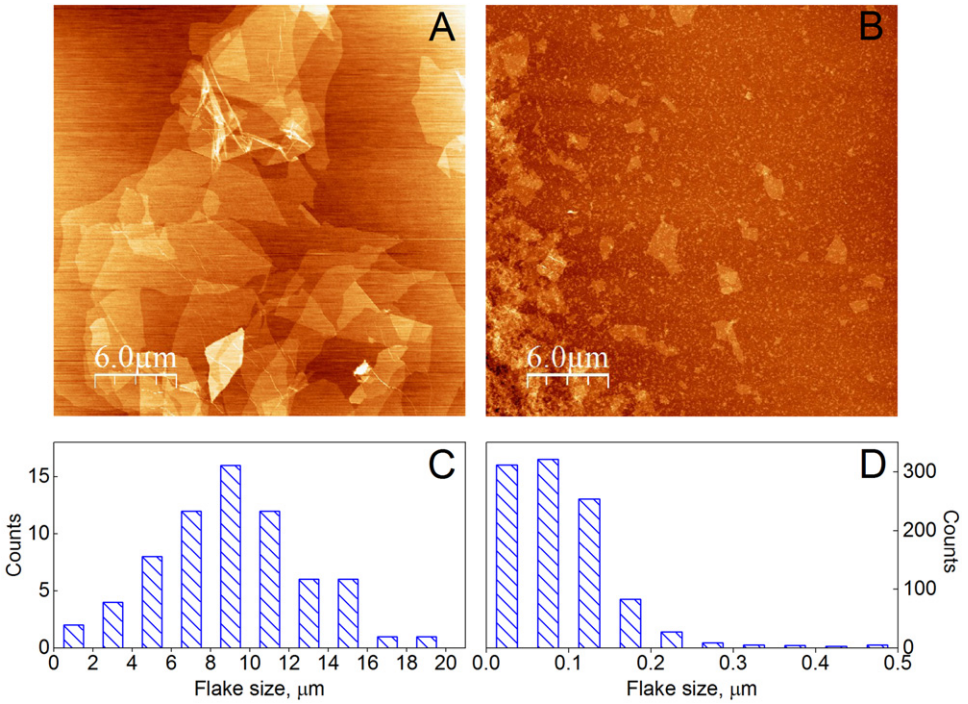

Figure 6. AFM images of GO with large flakes (A) and small flakes (B). (C) and (D) are the flake size distributions for samples similar to (A) and (B) respectively.

UM preparation at the concentrations 0–250 μg ml^{-1}. GO had no significant effect on bacterial growth indicating that it was neither inhibitory nor stimulatory for growth under the conditions used here.

3.5. Bacteria growth in the presence of GO of different lateral size

Following the number of reports where effect of GO was dependant on the lateral size of material flakes [20, 23, 38, 39] we have tested growth of *E.coli* ATCC25922 in the presence of GO prepared by the same method but varied by flake size: GO with large flakes (5–20 μm) and GO with small flakes (up to sub 100 nm). In both cases the GO was purified by the process described above and GO with small flakes was produced by prolonged sonication (figure 6).

E. coli ATCC25922 was grown in MHB in the presence of the two different flake sizes (figure 7). We also fitted the concentration of bacteria as a function of time with the exponential growth law: N(t) = N0Ns/(N0 + (Ns − N0)e-rt), where N0 is the initial concentration of bacteria, Ns—the saturation concentration (maximum

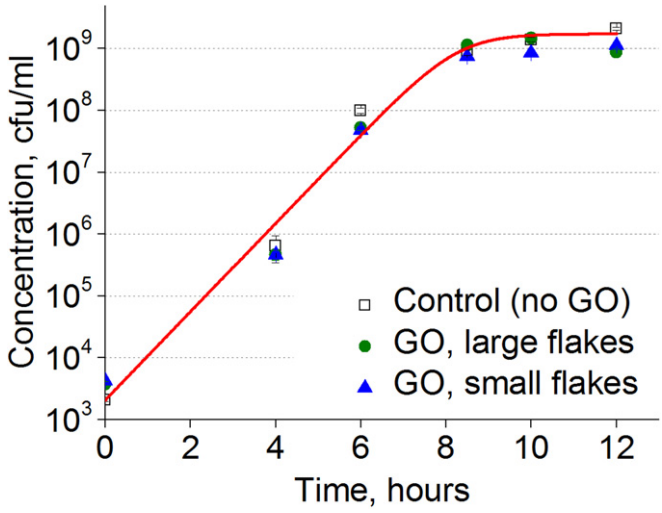

Figure 7. Growth curve of *E.coli* ATCC25922 in MHB media. No GO added (open black squares), large GO flakes added (solid green circles) and small GO flakes added (solid blue triangles). Red solid curves—theoretical fit to the experimental data in case of large GO flakes added. Orange dashed curve—theoretical fit to the experimental data in case of no GO added. GO concentration in the media—50 μg ml^{-1}.

population density), r—the growth rate. It is clear, that in the case of MHB media (figure 7) the growth rate and the maximum density concentration are not sensitive to the addition of GO—the data for the control sample (with no GO added) and for samples with large and small flakes are indistinguishable within the accuracy of the experiment.

3.6. Morphology of bacterial cells grown in the presence of GO

Effect of GO, (UM preparation) on the morphological status of bacteria was assessed by TEM after growth of test organisms (*E.coli* ATCC25922 and *S.aureus* ATCC25923) in the media with GO (100 μg ml^{-1}). TEM analysis of the same bacteria grown in the GO-free media was carried out for comparison. TEM images of bacteria with and without exposure to GO are shown (figure 8). As it followed from TEM results the presence of GO in the growth media did not have any visual effect on cell morphology and did not affect cell membranes. It is clear that although GO flakes (arrows in figures 8(B) and (D)) can surround bacteria cells and come in close contact with a cell membrane there is no permanent attachment and no uptake of GO by bacteria.

4. Discussion

There has been considerable research studying possible interactions between GO and bacteria [11–19]. However the published data are conflicting with no clear definitive role for GO with claims that GO can either inhibit or promote bacterial growth. This prompted us to answer the question as to why the previous activities between GO and bacteria had been observed. One possibility was that the effects were due to chemical contaminants present in the GO preparations as a consequence of the generation of the GO [40]. To determine the presence of different impurities XPS and ICP-AES analysis was performed on GO samples used in our study. The detection of boron is probably originated from initial graphite where it was detected previously [41]. The fact that the boron concentration detected in GO samples (0.17%–0.61%) (tables 1 and 3) was below the inhibitory levels necessary to kill bacteria when the GO suspension was at concentration of 1 mg ml^{-1} would argue that this not responsible for the observed antibacterial properties in unwashed GO [42]. The presence of sulphur impurities can be expected because of the use of sulphuric acid in the oxidation process. The highest level of sulphur (1.7%) was detected in commercial samples or our own GO suspension with 2 washes (tables 1 and 3). Assuming that all sulphur is present in a form of sulphuric acid, then in a GO suspension (1 mg ml^{-1}) the predicted H_2SO_4 concentration will be 0.5 mM and a pH of 3, similar to pH measured value in low purity samples (UMT-0, CSA) with shown inhibitory properties (figures 3, 4). The appearance of nitrogen in GO purified and some commercial samples is likely caused by samples preparation and freeze-drying. The metallic elements detected in our GO samples are the contaminants known to be present in GO produced by Hummers' method [40]. We believe that they are not responsible for the antibacterial properties of unwashed UMT-0 GO sample since the concentration of each metal was well below the known inhibitory level of such metals [43–45]. The presence of sodium and potassium was reported previously and is most likely as a result of the use of sodium nitrate and potassium permanganate in the Hummers' method [40]. The levels of sodium and potassium were

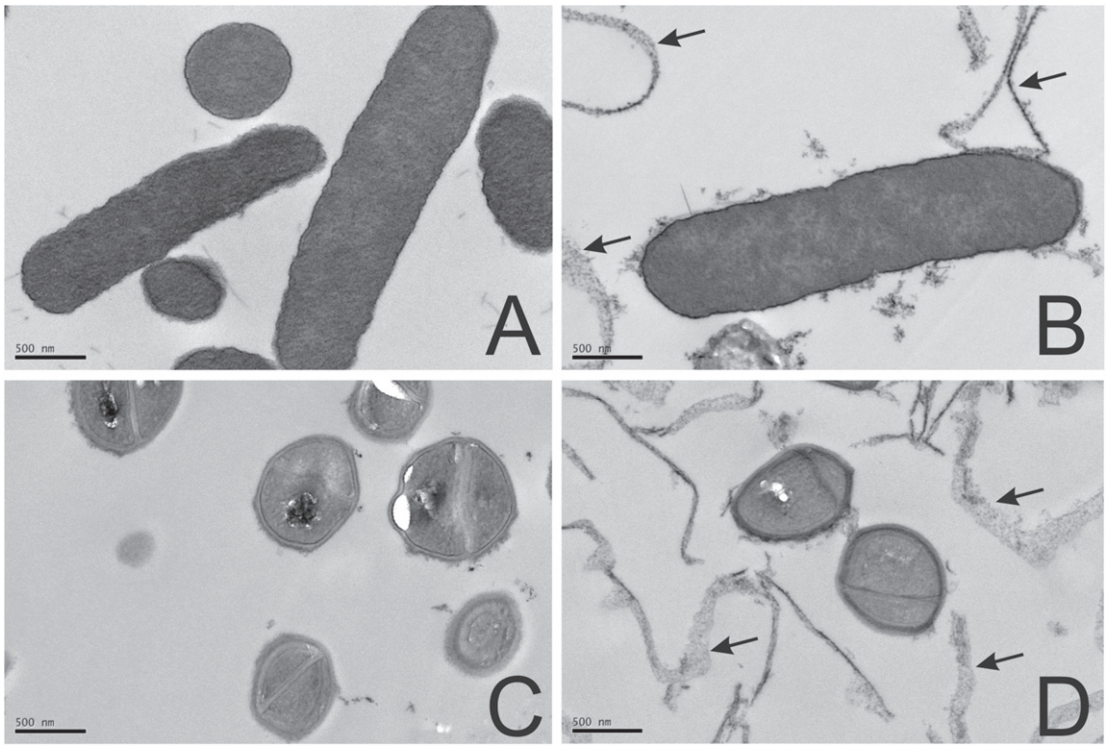

Figure 8. TEM images of *E.coli* ATCC25922 (A, B) and *S.aureus* ATCC25923 (C, D) after incubation in MHB media (A, C) and MHB media with GO (UM preparation) at 100 μg ml^{-1} (B, D).

detected there at the ppm and are also well below the toxicity levels for these metals in bacteria [46].

To test the effect of such impurities we developed an extensive washing protocol (see Experimental Procedures) in such that the initial acidic pH of the GO was removed. XPS analysis of this showed that following this clean up process the GO was highly pure with no detectable boron contamination and low levels of sulphur (tables 1 and 2). By using GO samples at different points in the clean up process we could demonstrate that as the pH increased during the purification process the bactericidal effects of GO disappeared (figure 3). We would argue that likely biological properties of GO in particular its antibacterial effects are most likely explained by using poorly purified GO which has a low pH. This was confirmed by assaying the biological properties of three commercial GO preparations. One of the preparations CSA had significant antimicrobial effects (figure 4). However by subjecting this sample to our purification procedure or by neutralising the acidic pH directly the antimicrobial properties of this GO preparation were no longer detectable. Taken as a whole we believe that this confirms our hypothesis that the reported antibacterial properties of GO are most likely explained by the acidic pH.

In conclusion the data in this study has for the first time generated definitive data that clearly demonstrates that under the *in vitro* conditions used here no antibacterial properties could be assigned to highly purified GO. It was neither bactericidal nor bacteriostatic over a broad concentration range against planktonic cultures of either *E. coli* or *S. aureus* in a number of assays. In addition, it had no detectable growth enhancing effects in the assays that were used in this study. Moreover, although previous publications had reported that the effect was dependant on the lateral size of the GO flakes [20, 23, 38, 39] we found that varying the GO flake size had no effect on the growth of *E.coli*. The overriding conclusion from our data is that GO itself is inert in its interactions with planktonic bacteria. However this does not preclude that GO might interact with biofilm bacteria in a different way and these studies are underway in our laboratory. However would strongly argue that any interactions between GO and biofilms are highly unlikely to be either bactericidal or bacteriostatic. We would suggest that the preparation protocol used in this study is used for all future experiments studying the biological properties of GO to avoid erroneous results due to chemical contamination.

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