

## Antimicrobial activity of graphene oxide-metal hybrids

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### Abstract

With resistant bacteria on the increase, there is a need for new combinations of antimicrobials / biocidal agents to help control the transmission of such microorganisms. Particulate forms of graphite, graphene oxide (GO) and metal-hybrid compounds (silver-graphene oxide (AgGO) and zinc oxide graphene oxide (ZnOGO)) were fabricated and characterised. X-Ray diffraction and Diffuse Reflectance Infrared Fourier Transform Spectroscopy demonstrated the composition of the compounds. Scanning Electron Microscopy and Energy Dispersive X-Ray Spectroscopy determined the compounds were heterogeneous and irregular in shape and size and that the level of silver in the AgGO sample was 57.9 wt.% and the ZnOGO contained 72.65 wt. % zinc. The compounds were tested for their antimicrobial activity against four prominent bacteria; *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecium* and *Klebsiella*

26 *pneumoniae*. AgGO was the most effective antimicrobial (Minimum inhibitory concentration  
27 *E. coli* / *Enterococcus faecium* 0.125 mg mL<sup>-1</sup>; *S. aureus* / *K. pneumoniae* 0.25 mg mL<sup>-1</sup>). The  
28 addition of Ag enhanced the activity of GO against the bacteria tested, including the generally  
29 recalcitrant *K. pneumoniae* and *Enterococcus faecium*. These findings demonstrated that GO-  
30 metal hybrids have the potential to be utilised as novel antimicrobials or biocides in liquid  
31 formulations, biomaterials or coatings for use in the treatment of wounds where medically  
32 relevant bacteria are becoming increasingly resistant.

33 **Keyword:** Antimicrobials; graphene oxide, biocide; ESKAPE; nano / micro particles;  
34 pathogens

### 35 **1. Introduction**

36 Concerns about bacterial resistance from community-acquired and food-borne pathogens has  
37 been growing for a number of years at both national and international levels. Several Gram-  
38 positive and Gram-negative bacteria including *Escherichia coli*, *Klebsiella pneumoniae*,  
39 *Enterococcus faecium* and *Staphylococcus aureus* are currently considered as emergent global  
40 pathogens, which pose a huge global health problem (Boucher et al., 2009).

41 Metals have been used for decades to treat various infectious diseases, and their antimicrobial  
42 efficacies are now being re-evaluated owing to the emergence of resilient pathogens. A  
43 particular interest has emerged particularly in the use of these compounds for topical /  
44 therapeutic use as well as for disinfection to prevent the adhesion and transmission of bacterial  
45 species. Silver is one of the most widely investigated metals for antimicrobial applications, and  
46 is being used in a number of medical purposes including catheters, biomaterials and wound  
47 dressings. Zinc oxide (ZnO) is used in such applications as food packaging (Tayel et al., 2011),  
48 textiles (Velmurugan et al., 2016), as antimicrobials (Deokar et al., 2016), and in wound  
49 dressings (Chaturvedi et al., 2016). Nanoparticles are interesting in that they can be synthesized  
50 with a high surface area to volume ratio and with unusual morphologies that contain sharp

51 edges and corners. Graphite and the graphene derivatives have traditionally been used in  
52 electrochemistry, from applications in energy technologies, such as batteries and fuel cells and  
53 they have also been used in an array of functional composites (Unwin, et al., 2016). Work has  
54 recently suggested that the graphene family of compounds also possess antimicrobial properties  
55 (Liu et al., 2011; Wang et al., 2012). By combining the antimicrobial activity of metals together  
56 with the physical effect of GO on the bacterial cell walls, it may be hypothesised that the  
57 antimicrobial activity of graphene products may be increased.

58 A number of disinfectants and antiseptics have been reported to be showing signs of becoming  
59 less effective so there is a need for the development of novel microbicides due to the current  
60 limitations (Russel and Chopra, 1990; Jennings et al 2015). Transmission and infection  
61 problems due to bacterial adhesion to surfaces can be mitigated in part by the development of  
62 alternative antimicrobial sources / biocides. The aim of this work was to determine if metal-  
63 GO hybrid compounds demonstrated increased antimicrobial efficacy compared to graphite  
64 and GO, against a range of bacteria. The development of such alternative antimicrobial actives  
65 may prove beneficial for use in such formulations such as biocidal, disinfecting or topical  
66 antimicrobials or cleaning agents or for incorporation into biomaterial coatings.

## 67 **2. Materials and Methods**

### 68 *2.1 Synthesis of compounds and characterisation*

69 For the synthesis of the compounds, all chemicals (analytical grade or higher) were used as  
70 received from Sigma-Aldrich (UK) without any further purification and all solutions were  
71 prepared with deionised water of resistivity not less than 18.2 MΩ cm. Synthetic graphite  
72 powder was commercially obtained from Gwent Group (Pontypool, UK).

73 Graphene oxide (GO) was synthesized by the Hummers method *via* the oxidation of synthetic  
74 graphite (Hummers Jr and Offeman, 1958). Graphite flakes (5 g) and NaNO<sub>3</sub> (2.5 g) were  
75 combined in 115 mL of H<sub>2</sub>SO<sub>4</sub> (conc.) and stirred for 30 min. Whilst kept in an ice bath (<5

76 °C),  $\text{KMnO}_4$  (15.0 g) was gradually added to the suspension and the rate of addition was  
77 controlled to keep the reaction temperature below 15 °C. The mixture was heated to 35 °C for  
78 a 30 min period and underwent continuous stirring producing a brown paste. A further dilution  
79 was made by adding 250 ml of water to the mixture and the temperature was increased to 70  
80 °C for 15 min. The resultant mixture was diluted by adding  $\text{H}_2\text{O}$  until a final volume of 1 L  
81 was obtained. Finally, the solution was treated with 15 mL of  $\text{H}_2\text{O}_2$  (30 % w/w) to terminate  
82 the reaction, at which stage the solution became yellow in appearance. For purification, the  
83 mixture was filtrated and the obtained solid was washed thoroughly with Milli Q water several  
84 times in order to avoid sulphate contamination. After purification, the powder was dried at 60  
85 °C during 48 h.

86 In the preparation of the AgGO, a sonochemical reduction method was utilised (Anandan and  
87 Muthukumaran, 2015). Following preparation of the GO, 0.5 g was added to 150 mL of  
88 ethylene glycol and sonicated for 30 min. In a separate vesicle, 1.0 g of silver nitrate was added  
89 to 20 mL of ethylene glycol and sonicated for 30 min. The silver nitrate dispersion was added  
90 drop-wise to the GO solution whilst undergoing sonication for 30 min to produce a  
91 homogeneous mixture. Finally, 50 mL of 0.1 M  $\text{NaBH}_4$  was added to the resultant AgGO  
92 mixture and a further 30 min of sonication was performed. The product was purified with  
93 repeated steps of  $\text{H}_2\text{O}$  and ethanol washing, after which the solution was dried at 50 °C.

94 The ZnOGO was fabricated by dissolving 5.0 g GO in 200 mL of N, N,-dimethylformamide  
95 (DMF), along with 20 ml of 1 M zinc acetate dihydrate (pH of 6.5). The homogeneous solution  
96 was heated to 60 °C and was stirred continuously for 120 min, after which the solution was  
97 heated to 250 °C. Following solvent evaporation, partial ZnO / ZnOHGO was produced. The  
98 resulting dried product was collected and ground in an agate mortar prior to being annealed at  
99 450 °C for 120 min within atmospheric conditions to obtain the final ZnOGO product (Liu et  
100 al., 2012).

101 *2.1.1 Preparation of compounds for testing*

102 For the analysis of the fabricated compounds, 20 mg of each test compound was added to 20  
103 mL of sterile distilled water. The samples were vortexed for 10 s and immediately 10  $\mu$ L of  
104 prepared sample was pipetted onto a 10 mm x 10 mm polished silicon wafer (Montco Silicon  
105 Technologies, USA) and air dried for 30 min. The samples were stored at room temperature,  
106 in desiccators until use.

107 *2.1.2 X-Ray Diffraction (XRD)*

108 In order to identify the crystal phase of the compounds, X-Ray Diffraction (XRD) was  
109 performed using a PANalytical X'pert powder diffraction platform. Nickel filtered copper  $K_{\alpha}$   
110 radiation ( $\lambda = 1.54 \text{ \AA}$ ) was used, with an anode voltage of 40 kV an anode current of 30 mA. A  
111 reflection transmission spinner stage (15 rpm) was implemented to hold the powder samples.  
112 The XRD parameters were step size: 0.13; sample: powder; slit (antiscatter) size:  $1/4^{\circ}$ . The  $2\theta$   
113 range was set between  $10^{\circ}$  and  $100^{\circ}$ , in correspondence with literature ranges associated with  
114 the characterised samples (Li et al., 2007; Zhou et al., 2007; Kumar et al., 2013; Chowdhuri et  
115 al., 2015; Liu et al., 2016). Additionally, to ensure well-defined peaks, an exposure of 50 s per  
116  $2\theta$  step was implemented.

117 *2.1.3 Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS)*

118 Diffuse Reflectance Fourier Transform Infrared Spectroscopy (DRIFTS) was carried out using  
119 a Spectra-Tech DRIFTS cell fitted in a Thermo – Nicolet Nexus FTIR spectrophotometer. The  
120 instrument was thoroughly purged (30 L / min) with  $\text{CO}_2$  and water-free air, produced using a  
121 Balston purge gas generator. All samples were diluted to ca. 5 % wt. in finely ground KBr  
122 (Sigma, UK). The samples were used as received, with no further grinding. The sample was  
123 folded into the pre-ground KBr using a micro-spatula. The micro-sampling cup was over-filled  
124 slightly and the cup dropped from a height of 1 cm onto the bench in order to shake off the  
125 excess mixture whilst at the same time, produce a slightly domed and naturally randomised,

126 surface of KBr diluted sample. The same batch of ground KBr was used as the background.  
127 The background and sample spectra were made up of 164 scans with resolution set to 4 cm<sup>-1</sup>.  
128 As the sample was diluted with KBr there were no specular reflection components so a blocker  
129 was not used. Spectra were plotted in absorbance (Liauw, 2003).

#### 130 *2.1.4 Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray Spectroscopy (EDX)*

131 In order to determine the shape, size and atomic elemental weight of the compounds, the  
132 samples were fixed to stubs using carbon tabs (Agar, UK). Scanning Electron Microscopy (Carl  
133 Zeiss Ltd.) was carried out using a Supra 40VP SEM with SmartSEM software. Energy  
134 Dispersive X-Ray (EDAX Inc.) was carried out using an Apollo 40 SDD system with Genesis  
135 software.

## 136 **2.2 Microbiology and antibacterial testing**

### 137 *2.2.1 Stock cultures of bacteria*

138 In preparation for the antimicrobial assays, stock cultures of *S. aureus* NCTC 4137, *K.*  
139 *pneumoniae* NCTC 9633 or *E. coli* NCTC 10418 were inoculated onto nutrient agar (NA) or  
140 nutrient broth (NB) and incubated at 37 °C for 24 h. Stock cultures of *Enterococcus faecium*  
141 NCTC 7171 were cultured onto Columbia blood agar with horse blood in a 5 %, Brain heart  
142 infusion agar (BHIA) (Oxoid, UK) or brain heart infusion broth (BIHB) and incubated in 5 %  
143 CO<sub>2</sub> for 24 h at 37 °C. All medias were obtained from Oxoid (UK).

### 144 *2.2.2 Preparation of microbiological cultures*

145 Ten millilitres of appropriate broth was inoculated with a single colony of bacteria and  
146 incubated overnight according to the above conditions. Following incubation, cells were  
147 harvested at 567 × g for 10 min and washed once, re-suspended in sterile distilled water,  
148 vortexed for 30 s, and then centrifuged again at 567 × g for 10 min. The inocula were examined  
149 in a spectrophotometer at 540 nm and compared against a blank of sterile distilled water to  
150 determine their optical density. They were then diluted accordingly and quantified using serial

151 dilutions. The cell concentrations corresponded to; *E. coli*  $4.20 \times 10^8$ , *S. aureus*  $1.30 \times 10^8$ ,  
152 *Enterococcus faecium*  $3.95 \times 10^8$  and *K. pneumoniae*  $2.82 \times 10^8$  colony forming units per mL  
153 (CFU / mL).

#### 154 2.2.3 Zones of inhibition

155 The zones of inhibition assays were performed to test the antimicrobial efficacy of each  
156 individual compound (n = 24). One hundred microliters of prepared cell suspension was  
157 pipetted and spread across the surface of the agar. Three equal wells (8 mm diameter) were cut  
158 out of the each agar plates. To each of the wells, 100  $\mu$ L of suspended compound was added.  
159 The plates were incubated in the appropriate air conditions and temperature for 24 h. Following  
160 incubation, the zones of inhibition was measured in mm from four sides of each well to  
161 determine an average mean value (n = 24).

#### 162 2.2.4 Minimum inhibitory concentrations (MIC)

163 The minimal inhibitory concentration (MIC) is defined as the lowest concentration of  
164 antimicrobial to prevent bacterial growth (Russel and Chopra, 1990). One millilitre of triphenyl  
165 tetrazolium chloride (TTC) blue metabolic dye (Sigma-Aldrich, UK), was added into 9 mL of  
166 the cell suspension so that the working concentration of the dye was 0.15 % w/v. To determine  
167 the MIC, the samples and bacteria were added to a 96 well flat-bottomed microtiter plate (MTP)  
168 and a serial dilution method used across the plate. A bacterial suspension without any  
169 compound (positive control) and un-inoculated broth (negative control) was included. After  
170 incubation, the MIC was taken as lowest concentration that inhibited the visible growth of the  
171 bacteria by comparison with the controls. Growth was indicated by a change of colour in the  
172 well to dark blue / purple.

#### 173 2.3.5 Minimum Bactericidal Concentration (MBC)

174 The MBC is defined as the lowest concentration required to completely inactivate the inoculum  
175 at a given time (Humphreys et al., 2011). To perform the MBC assays, 25  $\mu$ L was sampled and

176 pipetted onto agar plates from the MIC well that showed no growth and also from the first well  
177 that showed growth and incubated overnight in appropriate conditions. After incubation, the  
178 lowest concentration well sample that showed no growth on the agar plate was determined to  
179 be the MBC for that test sample.

#### 180 *2.3.6 Statistical analysis*

181 Statistical tests were carried out using a two tailed distribution *t*-test with two sample  
182 homoscedastic variance. Results were reported as mean  $\pm$  standard error or percentage and any  
183 observed differences were considered significant at a  $p < 0.05$ .

### 184 **3. Results**

#### 185 *3.1 Particle characterisation*

186 In order to assess the antibacterial activity of four compounds, graphite, GO, AgGO and  
187 ZnOGO, the compounds and hybrid molecules were firstly obtained or synthesized, then  
188 characterised and then tested using well-established antibacterial assays. The XRD patterns  
189 relating to the graphite powder produced the expected characteristic diffraction peaks at  $2\theta =$   
190  $26.6^\circ$ ,  $44.7^\circ$  and  $54.6^\circ$ , corresponding to the (002), (101) and (004) diffraction peaks of graphite  
191 powder respectively (Fig. 1a and a'). XRD (Fig.1 a') plotted over a narrower  $2\theta$  range and with  
192 a finer counts scale, showed some disordered material as evidenced by the wide peak between  
193 ca.  $7^\circ$  and  $17^\circ$  and by a characteristic 'sharp' peak was evident at  $2\theta = 11.8^\circ$  (Fig. 1b). The  
194 composition of the GO sample was confirmed as corresponding to the (002) diffraction peak  
195 of disordered GO. Application of the Bragg equation to the reflection peak angles, revealed  
196 that the interplanar distance increased from 0.35 nm in graphite to 0.75 nm in graphene oxide.  
197 For the latter, EDX gave 54.6 wt.% C and 45.3 wt.% O (O/C ratio = 0.83), whilst the former  
198 (graphite) had an oxygen content of only 8.9 wt.%, with all of the remainder being carbon  
199 (Table 1).



200 The ZnOGO was confirmed by XRD to have a high concentration of ZnO (Fig. 1c). Diffraction  
201 peaks were evident at  $2\theta = 32.2^\circ, 34.8^\circ, 36.7^\circ, 48.0^\circ, 57.0^\circ, 63.3^\circ, 66.8^\circ, 69.5^\circ$  and  $72.9^\circ$  which  
202 corresponded to the (100), (002), (101), (102), (110), (103), (112), (201) and (004) crystalline  
203 planes of ZnO, respectively (Liu et al., 2012). EDX revealed that the ZnOGO contained C (8.60  
204 wt.%), O (18.75 wt.%) and Zn (72.65 wt.%). Due to the low level of carbon in ZnOGO, the  
205 (002) reflection for GO (centred at ca.  $10^\circ$ ) (Fig. 1c') was very weak. The ZnOGO was light  
206 grey in colour thus confirming the presence of carbon in the sample.

207 Following analysis of the AgGO, the diffraction peaks occurred at  $2\theta = 38.7^\circ, 44.9^\circ, 65.0^\circ$  and  
208  $77.9^\circ$  (Fig. 1d). These peaks corresponded to the (111), (200), (220) and (311) crystallographic  
209 planes of face-centred cubic silver. A small amount of  $\text{Ag}_2\text{O}$  was present as evidenced by the  
210 corresponding (110) and (111) reflections at  $28.4^\circ$  and  $33.8^\circ$  respectively. The (002)  
211 reflection of the GO was significantly attenuated (Fig. 1d) and shifted from  $11.8^\circ$  to  $10^\circ$  ( $d_{(002)}$   
212  $= 0.86$  nm). There was also a broad reflection peak over the range  $12^\circ$  and  $18^\circ$ , with two small  
213 peaks centred at  $15^\circ$  and  $20^\circ$  (Fig. 1d') whereby corresponding  $d$  values were 0.60 nm and 0.44  
214 nm, respectively, indicating the presence of disordered structures.

215 Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) was used to further  
216 characterise the compounds. Overlaid DRIFTS spectra of the  $4000\text{ cm}^{-1} - 2000\text{ cm}^{-1}$  region for  
217 the graphite, GO and AgGO demonstrated that the DRIFTS spectrum of graphite (Fig. 2a) was  
218 largely featureless as expected, though there was a small and negative hydrogen bonded OH  
219 stretching peak which was due to there being slightly more moisture in the background than  
220 the sample. The size and position of this band did not hinder interpretation of this spectral  
221 region for GO or AgGO. Graphene oxide (Fig. 2b) showed the expected broad envelope of  
222 hydrogen bonded OH stretching vibrations from  $3700\text{ cm}^{-1}$  to  $2500\text{ cm}^{-1}$ , together with some  
223 OH bands at  $3650\text{ cm}^{-1}$  that appeared to be much less involved in hydrogen bonding. Interaction  
224 of the GO with the silver (Fig. 2c) appeared to remove the latter OH stretching band and

225 generally attenuated the hydrogen bonded OH stretching within the region above 3350  $\text{cm}^{-1}$ .  
226 There were some small aliphatic C-H stretching vibrations at 2946  $\text{cm}^{-1}$  (asymmetric) and 2877  
227  $\text{cm}^{-1}$  (symmetric). The 2000  $\text{cm}^{-1}$  to 400  $\text{cm}^{-1}$  region of the same three samples demonstrated  
228 that the graphite spectrum (Fig. 3a) was again featureless apart from a small negative peak at  
229 1650  $\text{cm}^{-1}$  which could be assigned to an O-H bend of water, indicating again that there was  
230 slightly more moisture in the background than the sample; this peak did not interfere with  
231 interpretation. The GO featured all the expected peaks (Fig. 3b); carbonyl stretching (1738  $\text{cm}^{-1}$ );  
232 skeletal aromatic C=C vibrations (1615  $\text{cm}^{-1}$ ); C-OH stretching (1356  $\text{cm}^{-1}$ ); C-O-C  
233 stretching (1225  $\text{cm}^{-1}$ ); C-O stretching (1056  $\text{cm}^{-1}$ ); aromatic C-H bending (849  $\text{cm}^{-1}$ ). The  
234 AgGO (Fig. 3c) also featured the same absorption bands but with the following significant  
235 differences: carbonyl stretching, skeletal aromatic C=C vibrations and C-O-C stretching  
236 vibrations were all red-shifted by 10  $\text{cm}^{-1}$ , 29  $\text{cm}^{-1}$  and 5  $\text{cm}^{-1}$ , respectively. Furthermore, the  
237 C-O vibration was split and consisted of a blue shifted component (1078  $\text{cm}^{-1}$ ) and a red shifted  
238 component (1037  $\text{cm}^{-1}$ ) (Table 2).

239 Overlaid spectra of the synthesised ZnO and ZnOGO demonstrated in both spectra, carbon  
240 dioxide absorption at 2350  $\text{cm}^{-1}$  (Fig. 4) and carbonate absorptions at ca. 1580  $\text{cm}^{-1}$  and 1380  
241  $\text{cm}^{-1}$  (Fig. 5). SEM showed that the compounds were heterogeneous and irregular in size (Table  
242 3) and shape (Fig. 6). Graphite (Fig. 6a) had a flattened, irregular, random orientation,  
243 fractured, sheet like morphology with sharp, cleaved edges (0.10  $\mu\text{m}$  – 25.7  $\mu\text{m}$ ). Graphene  
244 oxide (Fig. 6b) was composed of aggregated creased platelets (0.20  $\mu\text{m}$  – 20.0  $\mu\text{m}$ ). The  
245 ZnOGO (Fig. 6c) consisted of numerous aggregated nanoparticles and / or nanoparticles  
246 covering micron-sized particles (0.05  $\mu\text{m}$  – 30.0  $\mu\text{m}$ ). AgGO (Fig. 6d) was similar to GO in  
247 appearance; creased aggregated platelets with a random scattering of nanoparticles (possibly  
248 silver and / or silver oxide) (0.01  $\mu\text{m}$ - 13.0  $\mu\text{m}$ ).

### 249 *3.2 Microbiological analysis*

250 Zones of inhibition assays were carried out against Gram-negative *E. coli* and *K. pneumoniae*  
251 and Gram-positive *S. aureus* and *Enterococcus faecium* (Fig. 7). Following the zone of  
252 inhibition assays, all the compounds demonstrated antimicrobial activity against *E. coli* and all  
253 the GO-containing compounds demonstrated antimicrobial activity against *S. aureus*. Graphite  
254 was only effective against *E. coli* and thus demonstrated a significantly greater antimicrobial  
255 efficacy than GO or ZnOGO against this bacteria ( $p > 0.05$ ). The most effective antimicrobial  
256 overall against the bacteria using zones of inhibition was AgGO which provided the greatest  
257 zones of inhibition against *E. coli* (4.48 mm) and *S. aureus* (4.50 mm).

258 The MIC results demonstrated that against *E. coli* all the compounds were effective at  
259 concentrations of 0.125 mg mL<sup>-1</sup>. Against *S. aureus*, graphene oxide was the most effective  
260 (0.125 mg mL<sup>-1</sup>) whilst AgGO was the most effective against *Enterococcus faecium* (0.125 mg  
261 mL<sup>-1</sup>). *K. pneumoniae* was again the most difficult bacteria to inhibit. However, the ZnOGO  
262 and AgGO compounds demonstrated statistically significant inhibitory effects compared to the  
263 graphite and GO compounds against *K. pneumoniae* at concentrations of 0.25 mg mL<sup>-1</sup> ( $p >$   
264 0.05). MBCs demonstrated that against *E. coli*, ZnOGO and AgGO were the most effective at  
265 0.125 mg mL<sup>-1</sup>. GO, ZnOGO and AgGO were all effective against *S. aureus* at a concentration  
266 of 0.25 mg mL<sup>-1</sup> whilst AgGO was the most effective against *Enterococcus faecium* (0.125 mg  
267 mL<sup>-1</sup>) (Fig. 8b). It was demonstrated that as with the other assays, *K. pneumoniae* was the most  
268 difficult bacteria to eradicate, demonstrating the greatest MBC values. However, AgGO was  
269 the most effective hybrid compound against this bacteria at a concentration of 0.25 mg mL<sup>-1</sup>.

## 270 **4. Discussion**

### 271 *4.1 Characterisation of compounds*

272 The XRD patterns relating to the graphite powder produced the expected characteristic  
273 diffraction peaks (Peng et al., 2013). The composition of the GO sample was confirmed  
274 (Chowdhuri et al., 2015) and it was evident that the (002) reflection had shifted to a lower

275 angle and was of much lower intensity, relative to the same reflection in graphite. These  
276 observations are well established and indicate the formation of pendent oxygen containing  
277 functional groups on the top and bottom surfaces of the basal planes that increase the  
278 interplanar distance; this resulted in the shift of the (002) reflection to a lower angle  
279 demonstrating significantly decreased stacking uniformity (resulting in reduced reflection  
280 intensity). This is consistent with the DRIFTS data that indicated prolific functionalisation. The  
281 XRD also demonstrated disruption of the relatively ordered stacking of GO platelets due to  
282 non-uniform intercalation by the ZnO nanoparticles and / or coverage of the ZnO particles by  
283 GO, which may have contributed to a reduced intensity of the reflection (Chowdhuri et al.,  
284 2015). The AgGO peaks corresponded to crystallographic planes of face-centred cubic silver  
285 (Zhou et al., 2007). A small amount of Ag<sub>2</sub>O was present but as the atomic radius of silver is  
286 0.17 nm, it is conceivable that individual silver atoms may have intercalated the platelets  
287 (Dhoondia and Chakraborty, 2012). The attenuation of the (002) reflection indicated that the  
288 otherwise relatively regular stacking of GO had probably been disrupted by non-uniformly  
289 sized Ag nanoparticles between the GO platelets. It is also plausible that the GO may form a  
290 coating on the Ag nanoparticles (Oo, 2007; Das et al., 2011; Ma et al., 2011). The level of  
291 silver (by EDX) in the sample was 57.9 wt.%; a mix of GO intercalation by Ag nanoparticles  
292 and GO coating of Ag nanoparticles may therefore be likely. The level of carbon (20.3 wt.%)  
293 can be accommodated by the proposed structures of the hybrid. The oxygen in the Ag<sub>2</sub>O will  
294 have contributed to the amount of overall oxygen identified in the sample (21.8 wt %).

295 The DRIFTS spectrum of graphite was largely featureless as expected. GO showed the  
296 expected broad envelope of hydrogen bonded OH stretching vibrations. AgGO appeared to  
297 remove the latter OH stretching band and generally attenuated the hydrogen bonded OH  
298 stretching. This may be due to the interaction of the silver with weakly hydrogen bonded OH  
299 groups (phenolic OH and other OH) of the GO. The more general attenuation of the hydrogen

300 bonded O-H bands, within the region above  $3350\text{ cm}^{-1}$ , may be related to reduced water content  
301 in the AgGO and / or interaction of the silver with the hydrogen bonded OH groups of the GO.  
302 There were some small aliphatic C-H stretching vibrations. These may be due to residual  
303 ethylene glycol from the compound synthesis and to a lesser extent, residual ethanol from  
304 washing. The C-O vibration was split and consisted of a blue shifted component ( $1078\text{ cm}^{-1}$ )  
305 and a red shifted component ( $1037\text{ cm}^{-1}$ ). These observations indicated a significant interaction  
306 of the GO platelets with the silver. The latter is further supported by a blue shift in the aromatic  
307 C-H bending and C-OH stretching band. Interaction of the silver with carbonyl species and  
308 with the residual *p*-electrons in the GO would lead to the observed red shifts as the bond  
309 vibration was damped by interaction with the electron orbitals of silver atoms. This would also  
310 result in shortening of the aromatic C-H bonds and phenolic and carboxylic acid C-OH bonds,  
311 hence giving rise to the observed blue shift. The split in the C-O vibration indicated silver  
312 interactions having varying effects on the different ether linkages in the GO. It may be  
313 speculated that the ether groups at the platelet edges would be blue shifted and those actually  
314 pendant from a platelet surface may be red-shifted due to their interaction with the silver atoms.  
315 These observations are supported by the XRD data which indicated that the usual relatively  
316 ordered structure of the GO had been destroyed by its interaction with the silver. It may be that  
317 the silver atoms / particles had intercalated the layers resulting in highly non-uniform stacking.  
318 This would lead to the significantly attenuated and broadened GO related reflections in the  
319 XRD data for the AgGO.

320 The features observed using the DRIFTS analysis were expected in the ZnO that had been  
321 synthesised via this route since the carbonate and carbon dioxide would be decomposition  
322 products of the starting materials (Selim et al., 2015). The carbonate would have been  
323 converted to  $\text{CO}_2$  as the annealing temperature increased, resulting in the  $\text{CO}_2$  becoming  
324 trapped within the structure. Interestingly, the OH stretching bands were more intense in the

325 ZnOGO, and it may be that these were related to the GO, though the associated carbonyl and  
326 C-O bands could not be resolved. This may be explained by the strong association between the  
327 GO and ZnO resulting in attenuation of these vibrations. The GO may have coated the surface  
328 of the synthesised ZnO particles and / or could have become interleaved within the synthesised  
329 ZnO structures. In either case, the relatively ordered stacking of the GO platelets had become  
330 disrupted. The XRD data supports the latter proposition. The other area of interest in these  
331 spectra was the Zn-O bending vibrations at ca. 440 and 520  $\text{cm}^{-1}$ . In the ZnOGO, the ZnO band  
332 at 520  $\text{cm}^{-1}$  was stronger than in the synthesised ZnO (Fig. 5b). To the authors knowledge, such  
333 observations have not been reported elsewhere, but it may be related to a difference in the  
334 chemical environment and possibly due to the interactions with the GO.

335 SEM demonstrated that the compounds were heterogeneous and irregular in size and shape.  
336 The ZnOGO particles had the greatest size range (0.05  $\mu\text{m}$  – 30  $\mu\text{m}$ ), whereas AgGO and  
337 ZnOGO had the smallest sized particles (0.01  $\mu\text{m}$  and 0.05  $\mu\text{m}$  respectively) demonstrating the  
338 availability of both nano- and micron sized particles.

#### 339 *4.2 Microbiology*

340 The zone of inhibition assays demonstrated that none of the compounds had any effect against  
341 *Enterococcus faecium* or *K. pneumoniae*. This may be due to the zone of inhibition method  
342 being carried out using a semi-solid media; this combined with the thick capsule of the *K.*  
343 *pneumoniae* and the insusceptible nature of the *Enterococcus faecium* may have resulted in the  
344 reduced antimicrobial effect demonstrated. Further, the bacteria in this method were growing  
345 on the agar in colonies. These ‘communities’ of bacteria may have been more resistant to the  
346 antimicrobial effects of the compounds, similar to the effects observed when bacteria form  
347 biofilms (Gilbert et al., 2002) rather than what was observed when the bacteria are in planktonic  
348 form as in the MIC and MBC.

349 Work by others has demonstrated the antibacterial activities of graphite and graphite oxide  
350 towards *E. coli* and it was found that a GO dispersion demonstrated an 89.7% of loss viability  
351 at 40 mg mL<sup>-1</sup> (Liu et al., 2007). In our work, we demonstrated an antimicrobial activity of GO  
352 at much lower concentrations against the four bacterial strains tested (MIC = 0.125 mg mL<sup>-1</sup> –  
353 0.5 mg mL<sup>-1</sup>; MBC = 0.25 mg mL<sup>-1</sup> – 0.5 mg mL<sup>-1</sup>). Work by Xie et al. (2011) demonstrated  
354 the MIC of ZnO nanoparticles for *Escherichia coli* O157:H7 was found to be 0.4 mg mL<sup>-1</sup>. In  
355 comparison with our work, ZnOGO was the most antimicrobial compound against *E. coli* with  
356 an MIC at the lower concentration of 0.125 mg mL<sup>-1</sup>. However, the *E. coli* used in our study  
357 was a different strain. The ZnOGO was also inhibitory against *S. aureus*, *Enterococcus faecium*  
358 and *K. pneumoniae* at a MIC of 0.25 mg mL<sup>-1</sup>. GO and AgGO were also effective against *S.*  
359 *aureus* and *Enterococcus faecium* at concentrations of 0.125 mg mL<sup>-1</sup>. Work by others also  
360 demonstrated that the MIC for ZnO nanoparticles was 1.5 mg mL<sup>-1</sup> and 3.1 mg mL<sup>-1</sup> against *S.*  
361 *aureus* and *E. coli* respectively demonstrating that in some cases our ZnOGO compound was  
362 more effective than the antimicrobial action of ZnO alone used in other studies (Franklin et al.,  
363 2007; Azam et al., 2012). The MIC against *Enterococcus faecium* and *K. pneumoniae* was  
364 optimal with the AgGO hybrid compound. ZnOGO also demonstrated the same MIC as AgGO  
365 against *K. pneumoniae*.

366 Results from the MBC assays demonstrated that *K. pneumonia* was the most difficult bacteria  
367 to eradicate. Work by others using MBC assays with 18 nm nanoparticles of ZnO demonstrated  
368 that the concentration of particles required against *E. coli* was 0.018 mg mL<sup>-1</sup> and 0.016 mg  
369 mL<sup>-1</sup> against *S. aureus* (Xie et al., 2011). However, in contrast with their results, our compounds  
370 required greater concentrations in order to obtain the MBC. This may be explained by the  
371 particle size of our compounds being generally larger. It has been suggested that the smaller  
372 the size of the compounds, the greater the antimicrobial activity of the agent, however

373 contradictory results have been reported where size dependent effects were not found to  
374 influence the antimicrobial activity of ZnO (Chen et al., 2014).

375 The antimicrobial activity of the hybrid compounds may be explained in part by either the  
376 shape of the compound particles or by the percent of active facets. The atomic structure of the  
377 particle surface will affect its interaction with the bacterial cells (Selim et al., 2015). It is  
378 expected that the adsorption of atoms and molecules as a result of the interaction of the particles  
379 with the environment will be altered on the different planes, thus the difference in the atomic  
380 structure of the particles may result in a difference in their surface properties that could affect  
381 their interaction with the bacteria, leading to different antimicrobial efficacies (Pal et al., 2007).  
382 It has been suggested that high density facets with (111) faces exhibit greater amounts of  
383 antimicrobial activity (Pal et al., 2007). This is in agreement with our work since the AgGO  
384 demonstrated the greatest numbers of (111) planes. Combined with the shape of the  
385 compounds, these crystal structures can influence their mechanism of bacterial internalisation  
386 of the cell wall (Sirelkhatim et al., 2015).

387 Work by Liu et al. (2011) focused on the interactions of GO and graphite on bacterial  
388 membranes against *Escherichia coli*. In agreement with our results, they showed that a GO  
389 dispersion had a greater amount on antibacterial activity than graphite. GO and graphite are  
390 thought to confer antimicrobial activity due to membrane stress on the bacterial cells induced  
391 by the sharp edges of the compounds (Liu et al., 2011; Chen et al., 2014). An interesting fact  
392 that was evidenced in this work was that the type of antimicrobial assay used produced a range  
393 of results and thus it may be concluded that the use of one antimicrobial assay to determine the  
394 efficacy of compounds is not sufficient. Further, the type of antimicrobial assay used should be  
395 selected in line with the proposed final application of the antimicrobials.

396 Following each of the antimicrobial tests, AgGO demonstrated the greatest overall  
397 antimicrobial efficacies. *E. coli* was the most susceptible to the compounds followed by *S.*



398 *aureus*, *Enterococcus faecium* and finally *K. pneumoniae*. This can be explained in part by the  
399 nature of the microorganisms physiology. The Gram-negative microorganisms *E. coli* and *K.*  
400 *pneumoniae* are surrounded by an outer and inner cell membrane which have between them a  
401 thin layer of peptidoglycan. However, *K. pneumoniae* also has a large polysaccharide capsule  
402 surrounding the bacterial cell; in addition, this capsule acts as a barrier to antimicrobial agents  
403 (Highsmith and Jarvis, 1985). *S. aureus* and *Enterococcus faecium* are Gram-positive bacteria  
404 that have a cell membrane, chiefly composed of thick peptidoglycan. However, Enterococci  
405 are intrinsically more resistant to many antibiotics since unlike acquired resistance and  
406 virulence traits which are usually encoded by plasmids or transposon elements, their intrinsic  
407 resistance is based on chromosomal genes (Huycke et al., 1998). Further, a number of  
408 antibiotics demonstrate bacteriostatic but not bactericidal activity against *Enterococcus*  
409 *faecium* bacteria (Huycke et al., 1998). Thus, the use of AgGO against these two resilient  
410 bacteria may be an important step in maintaining the hygienic status of areas into which the  
411 molecule is applied or incorporated.

## 412 **5. Conclusions**

413 ZnOGO and AgGO hybrid compounds were successfully produced and characterised. AgGO  
414 was the most effective antimicrobial and enhanced the activity of GO. The effect of the  
415 compounds on the bacteria did not relate to the Gram-positive or Gram-negative structures of  
416 the bacteria but rather, was due to their microorganisms overall physiology. GO-metal hybrids  
417 have the potential to be beneficially utilised as novel antimicrobials or biocides in settings  
418 where bacteria are becoming increasingly problematic.

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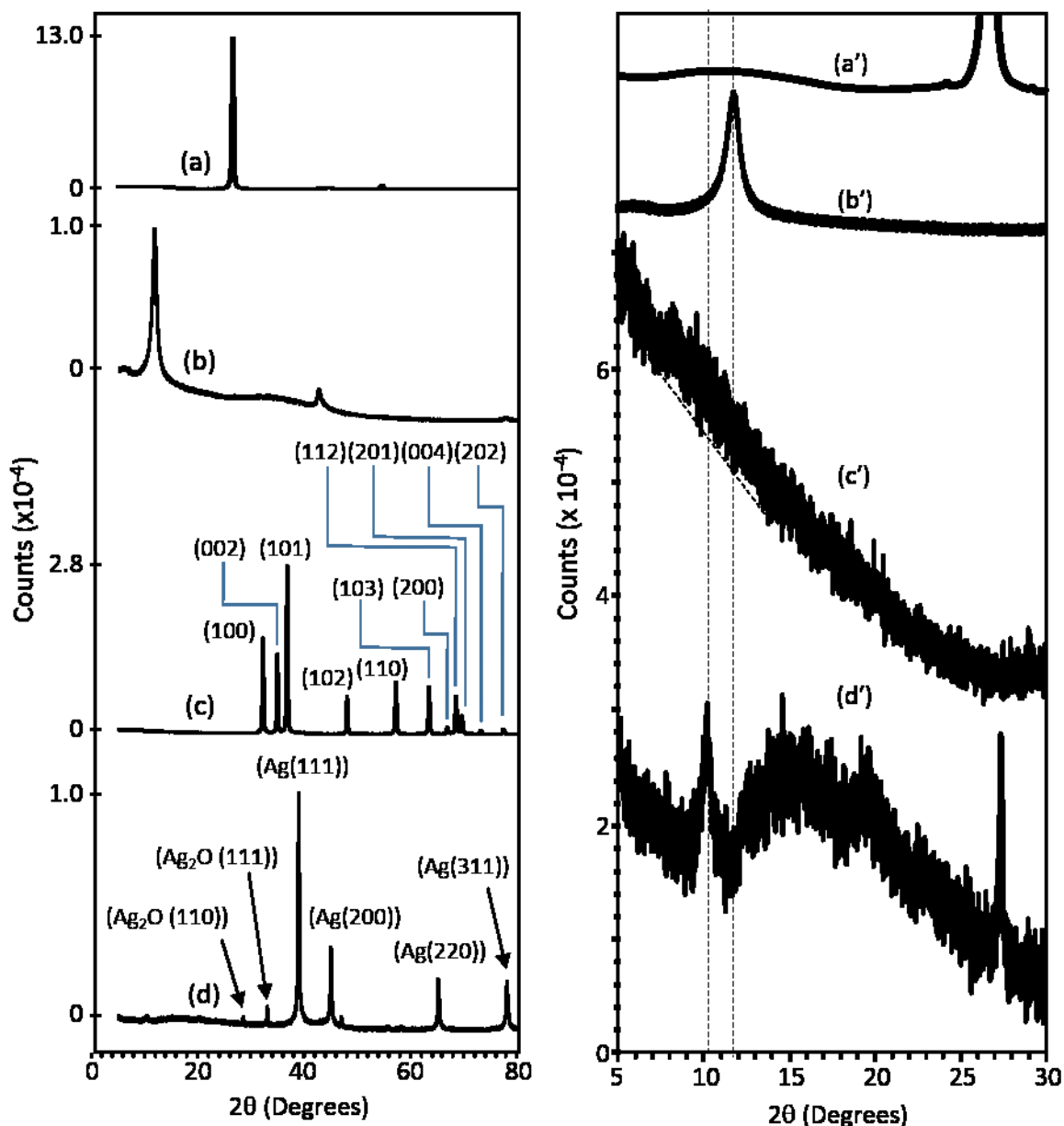
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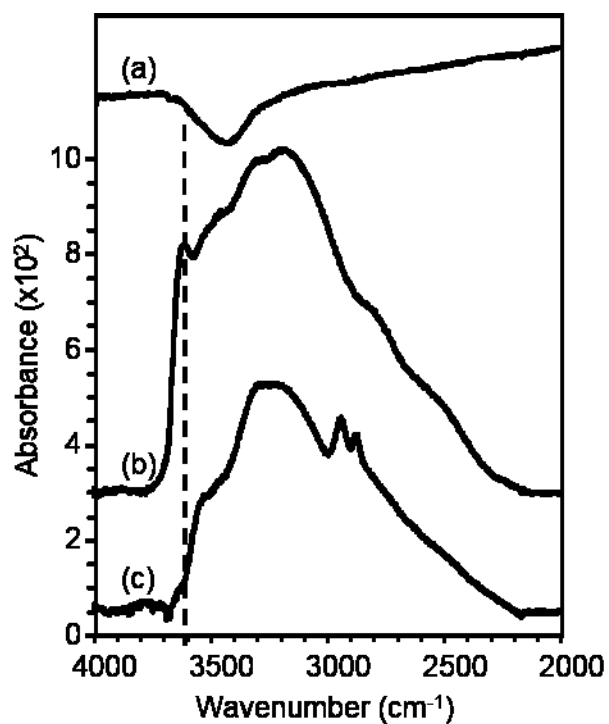
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521

522 **Fig. 1** X-Ray Diffraction patterns for; (a) graphite, (b) graphene oxide (GO), (c) zinc oxide – graphene  
 523 oxide hybrid (ZnOGO) and (d) silver – graphene oxide hybrid (AgGO). Note the individual peak heights  
 524 – patterns have been compressed to fit. In the right hand stack, patterns a' to d' correspond to those  
 525 in the left stack but are plotted over a narrower  $2\theta$  range on common counts scale (with Y-shifting for  
 526 presentation purposes). ZnOGO and AgGO have 50 x boosted counts and are Y-shifted for presentation  
 527 purposes.

528

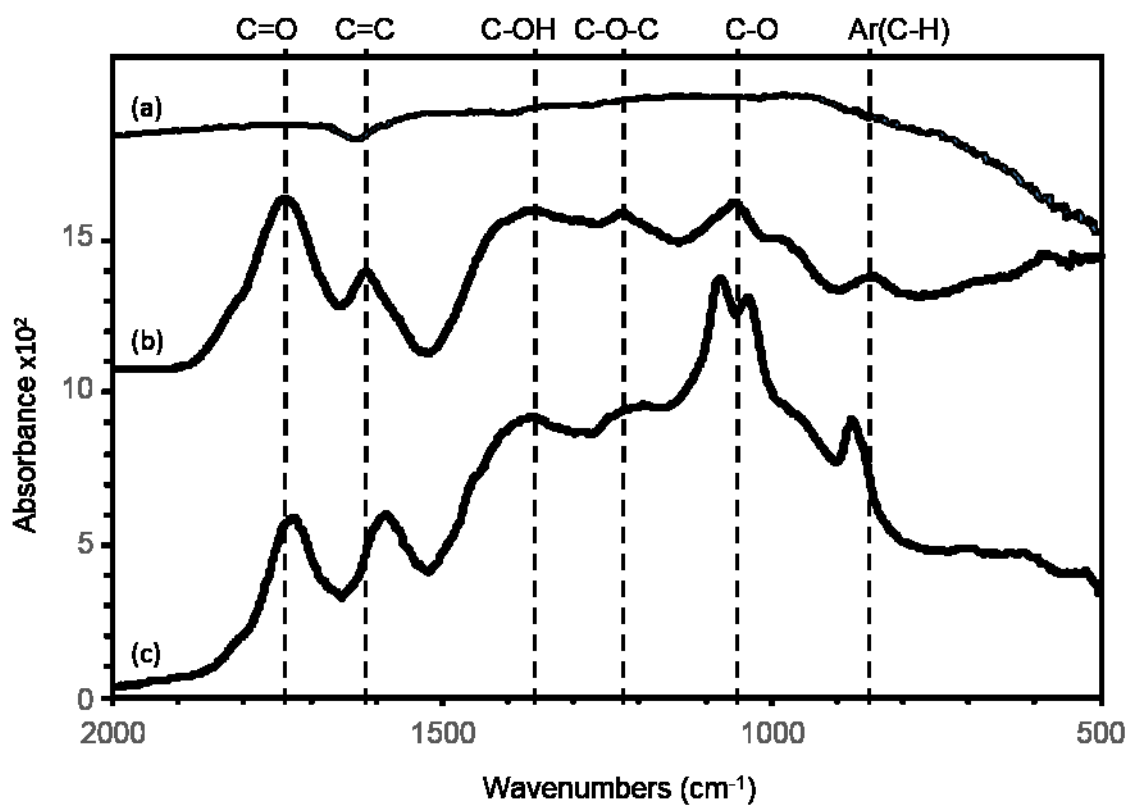


529

530 **Fig. 2** DRIFTS spectra (4000 cm<sup>-1</sup> to 2000 cm<sup>-1</sup>) of (a) graphite, (b) graphene oxide (GO) and (c) silver –  
531 graphene oxide hybrid (AgGO).

532

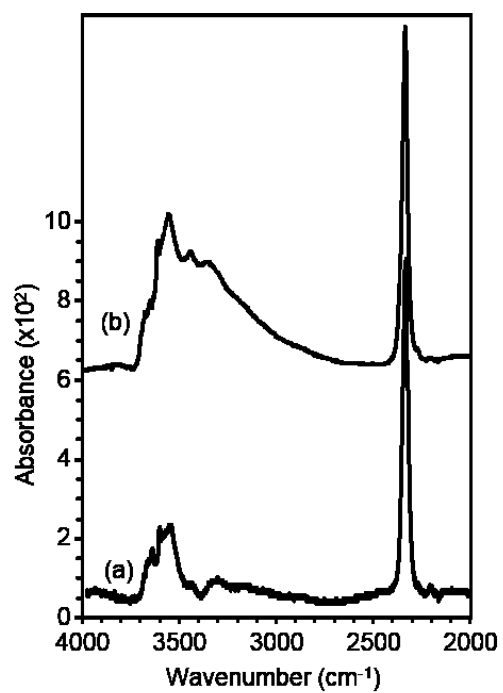




534

535 **Fig. 3** DRIFTS spectra ( $2000 \text{ cm}^{-1}$  to  $500 \text{ cm}^{-1}$ ) of (a) graphite, (b) graphene oxide (GO) and (c) silver –  
536 graphene oxide hybrid (AgGO).

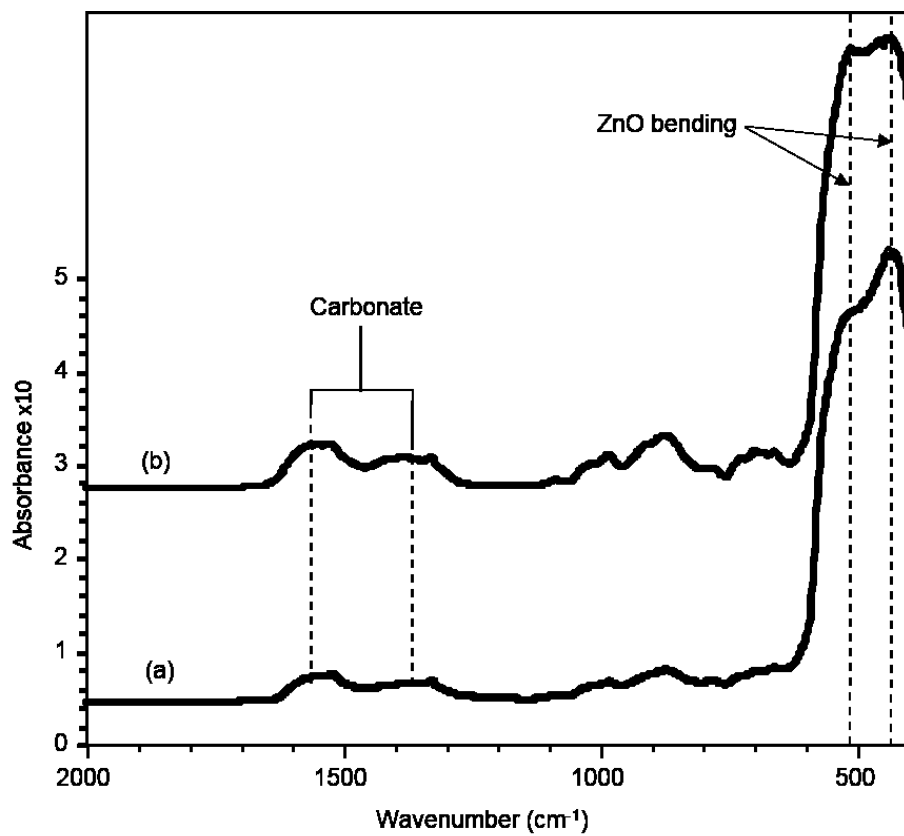
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538

539 **Fig. 4** DRIFTS spectra (4000 cm<sup>-1</sup> to 2000 cm<sup>-1</sup>) of (a) synthesised zinc oxide (ZnO), (b) synthesised zinc  
540 oxide – graphene oxide hybrid (ZnOGO).

541

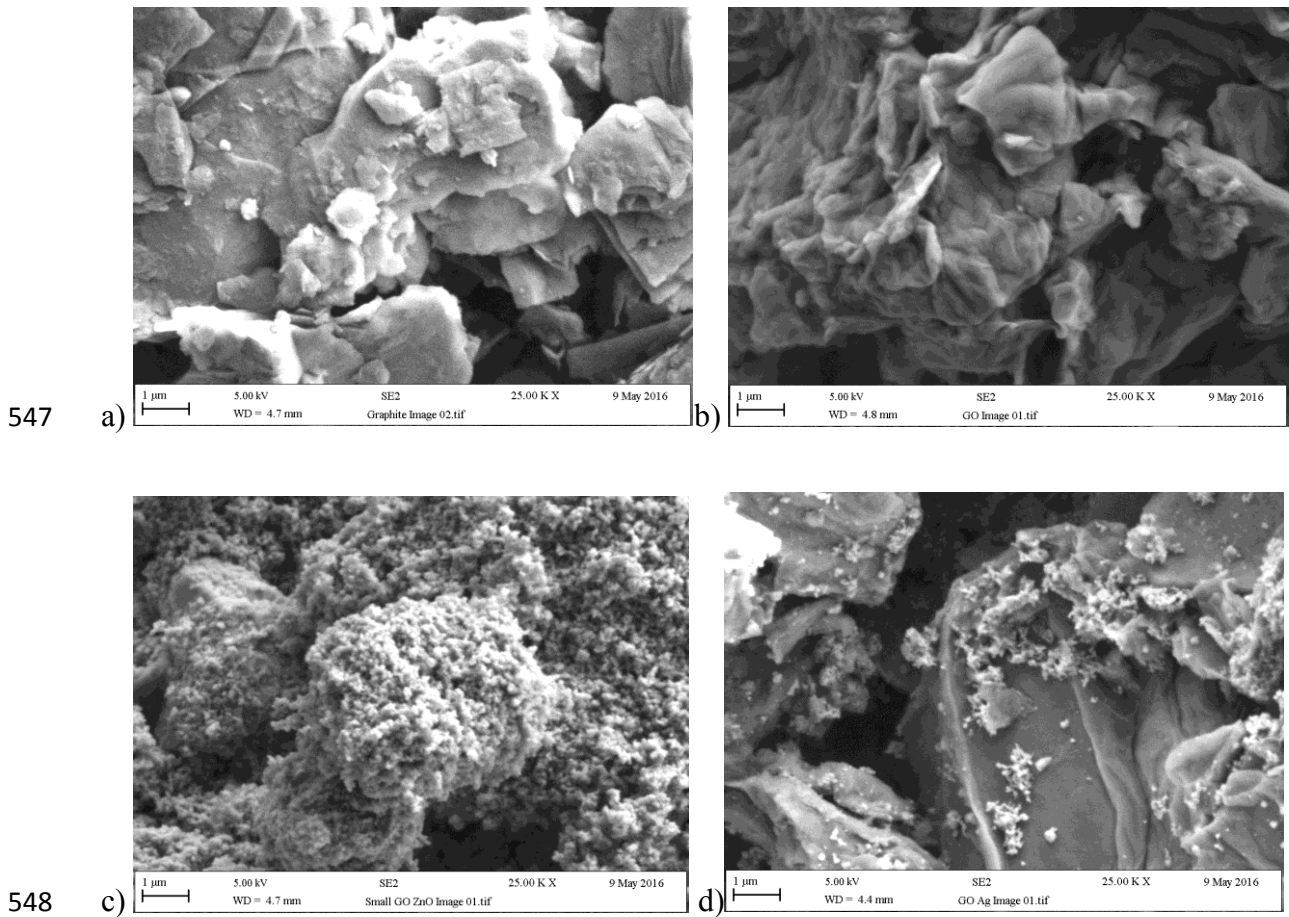


542

543 **Fig. 5** DRIFTS spectra (2000 cm<sup>-1</sup> to 400 cm<sup>-1</sup>) of (a) synthesised zinc oxide (ZnO), (b) synthesised zinc  
544 oxide – graphene oxide hybrid (ZnOGO).

545

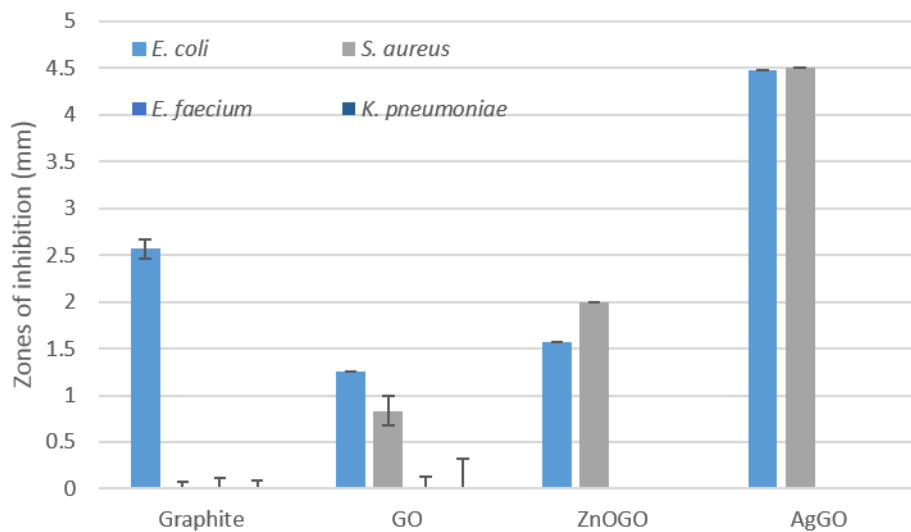
546



549 **Fig. 6** SEM images demonstrating the morphology and particle sizes of a) graphite, b) graphene oxide  
550 (GO), c) zinc oxide – graphene oxide hybrid (ZnOGO) and d) silver – graphene oxide hybrid (AgGO).

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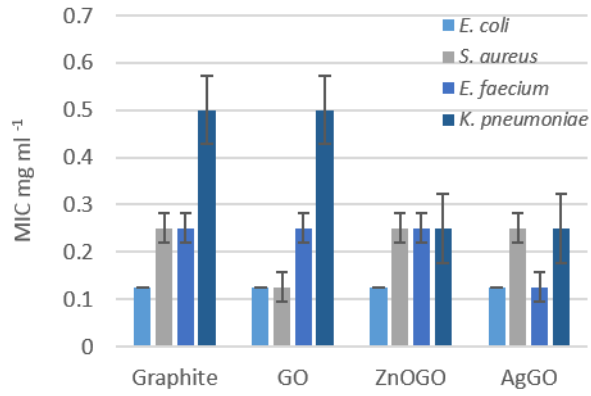


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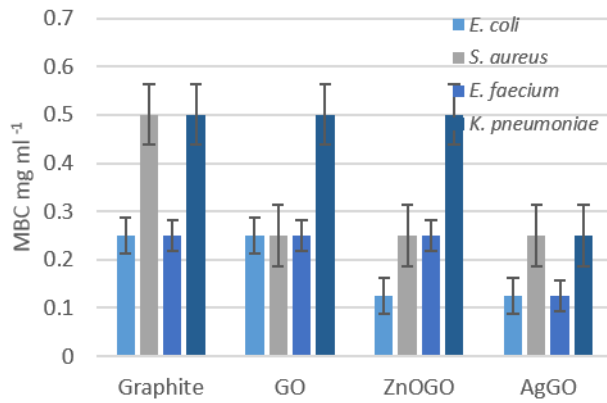
554 **Fig. 7** Zone of inhibition measurements demonstrating the antimicrobial efficacy of the compounds.  
555 The silver – graphene oxide hybrid (AgGO) was determined to be the most effective antimicrobial  
556 using this method. *K. pneumoniae* and *E. faecium* did not demonstrate inhibition by the compounds  
557 using this method.

558

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560



561

562 **Fig. 8** a) MIC and b) MBC of compounds against the four medically relevant bacteria demonstrating  
 563 that the silver – graphene oxide hybrid (AgGO) demonstrated the greatest inhibitory and bactericidal  
 564 effect.

565

566 Table 1 EDX analysis demonstrating the elemental analysis (% weight) of the compounds

	<b>C</b>	<b>O</b>	<b>Ag</b>	<b>Zn</b>
Graphite	91.12 ± 0.13	8.88 ± 0.13	N/A	N/A
GO	54.15 ± 0.79	45.85 ± 0.79	N/A	N/A
ZnOGO	8.60 ± 0.04	18.75 ± 0.17	N/A	72.65 ± 0.13
AgGO	14.50 ± 1.50	15.74 ± 1.03	69.77 ± 2.53	N/A

567 N/A Not applicable for elemental analysis

568

569

570 Table 2 Effect of silver addition on infrared absorption frequencies

Group vibration	Vibration frequency (cm <sup>-1</sup> )		Dn (cm <sup>-1</sup> )
	GO	Ag-GO	
C=O	1738	1728	-10
C=C	1615	1586	-29
C-OH	1356	1363	+7
C-O-C	1225	1220	-5
C-O	1056	1078	+22
		1037	-19
Aromatic C-H	849	879	+30

571

572



573 Table 3 Minimum to maximum size range of the particles

574

	Smallest size ( $\mu\text{m}$ )	Greatest size ( $\mu\text{m}$ )
Graphite	0.10	25.7
Graphene oxide	0.20	20.0
ZnOGO	0.05	30.0
AgGO	0.01	13.0

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