1 **Implications of Chemical Reduction Using Hydriodic Acid on the**
2 **Antimicrobial Properties of Graphene Oxide and Reduced Graphene**
3 **Oxide Membranes**

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19 **Keywords:** graphene-based membranes; wastewater reuse and desalination; bacterial viability,
20 contact inactivation; hydriodic acid

Abstract

The antimicrobial properties of graphene-based membranes such as single-layer graphene oxide (GO) and modified graphene oxide (rGO) on top of cellulose ester membrane are reported in this study. rGO membranes are made from GO by hydriodic acid (HI) vapor treatment. The antibacterial properties are tested after 3 h contact time with selected model bacteria.

Complete bacterial cell inactivation is found only after contact with rGO membranes, while no significant bacterial inactivation is found for the control (i) GO membrane, (ii) the mixed cellulose ester (MCE) support, and the (iii) rGO membrane after additional washing that removed the remaining HI. This indicates that the antimicrobial effect is neither caused by the graphene nor the membrane support. The antimicrobial effect is found to be conclusively linked to the HI eliminating microbial growth, at concentrations from 0.005%.

These findings emphasize the importance of caution in the reporting of antimicrobial properties of graphene-based surfaces.

Keywords: membrane surface modification; reduced graphene oxide; Wastewater reuse and desalination; bacterial viability, contact inactivation; hydriodic acid

1. Introduction

Membrane-based water treatment technologies have proven to be able to combat the problems of shortage and safety of drinking water. However, the growth and development of microorganisms on the surface of these membranes have led to the problem of membrane fouling, especially biofouling.^[1-4] Biofouling is the most challenging problem faced by membrane-based water treatment processes because of the ability of the microorganisms to (i) multiply at the expense of biodegradable nutrients passing membrane pretreatment^[5, 6] and to (ii) produce extracellular polymeric substances (EPS) which are very sticky and form their immediate surrounding. The EPS produced by these microorganisms enables the biofilm's stability, survival (to cleanings) and also their adhesion to the membranes by serving to help immobilize bacterial cells on the membrane surfaces.^[7, 8] EPS is considered the cause of biofouling problems.^[9]

Most reports regarding trying to control the effects of biofouling in membrane-based processes focus on interventions aimed at killing microbial cells in the already formed biofilms present on the membranes. Nevertheless, such approaches have limited efficiencies and efficacies because of incomplete killing and biomass removal from the membrane system^[10] and the buildup of microbial resistance over time. The problems of biofouling are closely related to the properties of the surface of the membranes; among such properties are roughness, charge, morphology, chemical groups, and hydrophilicity which tend to play a significant role in membrane fouling.^[11] To enhance the antifouling properties of membranes, the use of new materials for membrane fabrication or the incorporation/coating of membranes with such materials (triclosan, silver, copper, gold, polydopamine and amphiphilic copolymers) has been pursued without success in biofouling control.^[12-15] Novel promising materials for biofouling control include carbon nanotubes,^[16] metal oxide nanoparticles^[17] and more recently, graphene materials.^[18]

The graphene-based membrane (GBM) not only serves to produce ultra-high permeate water flux in membrane-based water treatment systems but also, the antimicrobial properties of graphene materials (GO and rGO) can serve as a means of controlling biofouling on the membrane.^[19] The unique structure of graphene-based materials provides an opportunity for fouling/biofouling control. Several reports have indeed shown the antimicrobial properties of graphene materials. The antimicrobial effect of single and multiple layers GO and rGO was investigated against both Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*.^[20] The authors reported a loss in cell viability of both test microorganisms; thus, it was concluded that GO and rGO materials have cannot only inhibit bacterial adhesion but also display some killing/inactivation effect. Other studies have likewise reported the antimicrobial properties of graphene surfaces.^[21-23] Recent extensive reviews on the antimicrobial effects of GO and rGO and their mechanisms of action are available in literature ^[24-26]

Graphene materials as antimicrobial agents have received quite many contradicting results and conclusions. While some studies have reported the antibacterial property of graphene material,^[22, 27-33] others have reported the lack of antimicrobial ability^[34] and also the enhancement of microbial growth due to graphene material.^[35, 36] In all of those studies that have reported the antibacterial properties of graphene materials, the primary form of interaction between graphene nanosheets and bacteria include piercing by the sharp edge of the graphene nanosheets, the production of reactive oxygen species (ROS), or the wrapping of the bacterial cells by the graphene materials or the trapping of bacterial cells within the graphene nanosheets (suspension form) resulting into the killing effect (leakage of cytoplasm, membrane stress, and oxidative stress), or bacteriostatic effect or even the enhancement of microbial growth. It is noteworthy that aside from the contradicting results available on the antimicrobial property of graphene materials, the exact mechanism of action is not clear.

Over the years, the application of graphene-based materials in the field of water treatment for biofouling control is limited. Previous studies focused on the addition of graphene material to already existing or commercially available membranes to enhance its antimicrobial property.^[37]
^{38]} The vast potential of GBMs in water filtration systems in terms of high water recovery and high salt rejection as reported in a previous study from our group,^[39] as well as the antimicrobial tendency of these membranes coupled with the conflicting reports on the antimicrobial properties of graphene material; there is an urgent need to ascertain if GBMs could truly play a major role in biofouling reduction in membrane-based water treatment systems. Perhaps if there is, there is a need to clarify the mechanisms of action.

One of the proposed modes of antimicrobial action of GBMs is their ability to increase cellular oxidative stress, which could affect the microbial process. Oxidative stress could be linked to reactive oxygen species (ROS) dependent pathway, in which oxidative stress is caused by the production of ROS by GBMs or ROS-independent pathway, in which microbial process is interrupted due to the oxidation of crucial cellular components and structures without the production of ROS. Both pathways; ROS-dependent (ROS generation) or ROS-independent (electrical conductivity) could be connected to the degree of oxygen functional groups on the GBMs. For instance, the removal of oxygen functional groups on GO decreases its band gap, thereby increasing its electrical conductivity.^[40]

In this study, the main objectives are to determine the antimicrobial potential of GBMs and to investigate whether the degree of oxygen functional groups on GBMs could impact on their antimicrobial properties. It was hypothesized that the reduction of GO membrane would reduce the band gap thereby increasing its electrical conductivity. Since the electron is vital to cellular metabolism, GBMs would act as a conductive bridge over the insulating lipid bilayer to release internal microbial electrons into their external environment which would, in turn, deactivate the microbial cell. To investigate such a phenomenon, the reduction degree of the GO

membrane was controlled by exposure to HI vapor with respect to time. Previous studies from our laboratory already showed that the reduction degrees of GO membranes could be controlled by varying the exposure time of such membranes to HI vapor.^[39, 41]

2. Results

2.1. Characterization of graphene-based membranes (GBMs)

Figure S1 presents the photographic images of the fabricated GO membranes with different reduction degree by time-dependent HI vapor treatment. The darker color obtained after HI vapor treatment could be due to the graphitic sp^2 bonding recovery by HI vapor treatment. Lateral particles size analysis showed size distribution of GO sheets used in this study before fabricating the GBMs (Figure S2). Statistically, the maximum lateral particle size distribution can be regarded as the overall representative of the GO sheets before filtering on an MCE support. The maximum size was around 271 to 816 nm while the overall average was 582.8 nm.

The GO and the reduced GO membranes after exposure to HI vapor for 5 s, 30 s and 60 s with/without washing and thermal treatment were characterized by XRD and the diffraction patterns are given in Figure 1(a) and Figure S3. The main peak of GO occurred at $2\theta = 11.5^\circ$. After HI vapor exposure for 30 s and 60 s, an observable new peak at $2\theta = 24.2^\circ$ occurred for both time intervals. It was noted that the GO peak was still observable after HI vapor treatment; this was due to the insufficient reduction of the GO layer. For the thermally reduced GO, the main peak was observed at $2\theta = 18^\circ$ because of the partial reduction of GO. To obtain a complete thermal reduction, a higher temperature is necessary. However, the product would not be suitable for the fabrication of a selective barrier.

The FT-IR spectra showed the effect of HI vapor treatment on the GO membranes (Figure 1(b)). The spectrum of GO without reduction showed characteristic peaks such as –OH stretching (around 3390 cm^{-1}), C=O (around 1718 cm^{-1}), C=C (around 1632 cm^{-1}), C-O vibrational band (around 1377 cm^{-1}), C-O epoxy (around 1219 cm^{-1}), and C-O alkoxy (around 1050 cm^{-1}). However, as the HI vapor exposure time increased, the intensities of these peaks reduced and at an extended reduction time, peaks such as O-H and C-O disappeared.^[41] These results suggested that some of the functional groups in the GO without reduction had been successfully eliminated during the HI vapor exposure.

Figure 1(c) shows the Raman spectra of the membranes. The characteristic D and G peaks were observed at around 1350 cm^{-1} and 1580 cm^{-1} respectively. The D peak originated from the out-of-plane vibration of sp^2 hybridized carbon which represents the surface defect and disorder of the graphene layer. On the other hand, the G peak originated from the in-plane vibrations of sp^2 hybridized carbon which represents its symmetry and crystallizability. The intensity ratio of D peak to G peak (I_D/I_G) points out a measure of the defects in the graphene material.^[42, 43] According to the spectral analyses, the I_D/I_G ratios for GO with 0, 5, 30, 60 s HI vapor exposure time were 0.95, 0.96, 1.19, and 1.29, respectively. Similar ratios were obtained for the washed membranes while the I_D/I_G ratio for the hydrothermally reduced GO was 1.05. This trend is consistent with a previous study where hydrazine was used to reduce GO.^[44] The increasing ratio values indicated that the HI vapor treatment damaged the integrated layers and caused a significant amount of defects on the membrane surface.^[45] The Raman spectra analysis proves that washing of the membrane did not affect the order/disorder of the membrane layer as the I_D/I_G ratios for the membranes (washed and without washing) showed similar values.

The XPS analysis result is shown in Figure 1(d), (e) and (f). In the survey spectra as seen in Figure 1(d), several main peaks were observed at around 284 eV for C1s, at 531 eV for O1s, and at 625 eV for I3d5. The new peak at 625 eV was due to the residue effect of HI vapor

exposure. The C1s regions of the XPS spectrum of GO membrane correspond to carbon atoms in different functional groups: the C in C=C/C-C (284.7 eV), in C-OH/C-O (286.8 eV), and in C=O (288.4 eV)^[46]. The C1s spectra after 5 s reduction are similar to GO while after 30 and 60 s exposure to HI vapor, there is only one strong peak (C=C/C-C) and three minor peaks (Figure 1(f)). Compared to GO membrane, the content of C=O and C-O decrease significantly. The remaining C=O and C-O bands show that the reduction was not complete. The carbon-to-oxygen (C/O) ratios of GO and GO after 5, 30, 60 s HI vapor exposure are 2.0, 2.5, 4.7, and 7.4 respectively. The C/O ratio increased as the HI vapor exposure time increased.

The GBMs were examined for the electrical conductivity by using the four-point probe (FFP), a simple equipment for measuring the electrical resistivity (Figure 1 (g)). The GO membrane showed high insulating property with sheet resistance corresponding to $6.5 \times 10^6 \text{ ohm/sq} \pm 2.5 \times 10^5 \text{ ohm/sq}$. HI vapor treatment of the GO membrane based on exposure time reduced the electrical insulation of the membranes to $6.4 \times 10^2 \text{ ohm/sq} \pm 5.2 \times 10 \text{ ohm/sq}$ after 60 s of HI vapor exposure. This result proved that HI vapor treatment of GO membrane can increase the electrical conductivity of the GO membrane.

In summary, HI vapor exposure time effectively determines the reduction degree of the GO layer.

2.2. Surface antimicrobial effect of GBM

Figure 2(a) and 3(a) showed that the viability of the cells reduced as the reduction time with HI vapor increased. After reduction for 30 s, there was a complete loss of viability. This means the membranes entirely inactivated the bacterial cells. In order to investigate the possibility of bacterial growth after contact with GBMs, growth medium was put inside the wells containing

the bacterial suspension and the GBM after initial contact of 3 h. The growth of the bacterial cells was measured using the optical density measurements after 24 h (Figure 2(b) and 3 (b)).

Interestingly, there was no detectable bacterial growth observed with the cells contacted with membranes reduced by 30 s and 60 s. The membranes displayed a potent bactericidal property against a broad spectrum of bacteria, Gram-negative *P. aeruginosa* PAO1, and *Bacillus* sp. However, no significant difference was observed between the support, GO and rGO (5 s). This is because the bacterial cells were not completely inactivated and the supply of nutrient will encourage the proliferation of bacterial cells until the nutrient is depleted.

The live/dead staining is another analytical method to measure the state of both Gram-negative and Gram-positive bacterial cells. This was done to supplement the CFU counts measurement and was carried out directly on the attached bacterial cells on the membranes. The SYTO9 dye stains both the live and the dead cells green. PI only enters bacteria with damaged cell walls, and because PI has a stronger affinity for nucleic acids than SYTO9, SYTO9 is displaced by PI causing that dead cells are stained red.^[47] As shown in Figure 2(c) and 3(c), the predominance of green fluorescence (live bacterial cells) on the MCE support, GO membrane and rGO (5 s reduction) after staining with both SYTO 9 and PI indicated that irrespective of the bacteria type, the bacteria remained viable. This suggests almost no inactivation effect occurred for the mild effect seen after 5 s reduction. Nonetheless, no green fluorescence was observed on the membranes with 30 s and 60 s reduction. This explains why there was no bacterial growth when growth medium was added. rGO membranes (30 s and 60 s) exhibited a complete deactivation effect of the bacterial cells.

To further elucidate the antimicrobial mechanism of these GBMs both ROS-dependent and ROS-independent oxidative stress generation was studied since previous studies have linked the antimicrobial properties of GO and rGO to both mechanisms.

214

215 **2.3. Antimicrobial mechanisms of GBMs**

216 The antimicrobial property of graphene-based materials has been closely linked to their
217 electrical conductivity.^[28, 48] The electrical conductivity of the GBMs in this study (Figure 1
218 (g)) also correlates well with the antimicrobial activity (Figure 2 and 3). Since no structural
219 damage was observed in the bacterial cell membrane (Figure S4), the oxidative stress pathway
220 was hypothesized as the key antimicrobial mechanism. Hence, this necessitated the need to
221 find out the exact oxidative stress that may be generated by these GBMs.

222 One of the oxidative stress paths is the ROS mediated oxidative stress. Superoxide anion is
223 believed to be a crucial example of ROS because other forms of ROS such as singlet oxygen
224 and hydroxyl radicals could be produced by them.^[49, 50] Therefore, for the oxidative stress
225 pathway, superoxide anion production using the XTT protocol as described in the methods
226 after contact with GBMs for 3, 6, 9, 12, and 24 h was measured (data not shown). No detectable
227 absorption was observed. This implies both the GO and the variously reduced GO membranes
228 could not generate superoxide anion.

229 To examine the other oxidative stress pathway, ROS-independent oxidative stress, *in vitro*
230 GSH oxidation was monitored. GSH is a natural antioxidant that promotes the degradation of
231 ROS and protects the cell from stress induced by oxidation.^[51] Several studies have used GSH
232 as a biomarker for oxidative stress mechanisms.^[52-54] Ellman's assay was used to enumerate
233 the concentration of thiol in GSH^[55] and was used to monitor the oxidation of GSH when
234 incubated with GBMs. Results show that all the GBMs could, in different intensity, oxidize
235 GSH. The loss of GSH was 26, 15, 8, and 9% for 0, 5, 30, and 60 s reduced GO membrane
236 respectively (Figure 4). This finding suggests the possibility of minor generation of other ROS.

Minor generation was implied because this ROS generation could not impact tangibly on the antimicrobial properties of GBM based on the data of this study.

Because the antimicrobial effect observed in this study does not correlate with both ROS-dependent and ROS-independent oxidative stress pathways, there was a need to further clarify the source of the antimicrobial properties. In order to do this, elemental analysis of the GBMs was carried out to include iodine, a significant component of the HI solution used for the reduction of GO membranes (Figure. 1(d) and (e)). Surprisingly, a significant amount of iodine was detected as residue on the HI treated membranes. Hence, to verify the assumption that HI residue could be the cause of the noticeable antimicrobial property on the reduced GO membranes, the membranes were thoroughly washed with DI water to remove the HI residue on the surface before the antimicrobial test. All the samples were tested for their effect on microbial cell viability and live/dead analysis (Figure 5 and 6). After washing, the potency of the rGOs after 30 and 60 s was lessened (Figure 5(a) and 6(a)). The fluorescent images of both Gram-negative and Gram-positive bacterial strains showed that the complete inactivation effect of those membranes was lost (Figure 5(b) and 6(b)). To verify this claim, XPS analysis of the washed membranes was carried out (Figure 1(d)). The analysis of the atomic percentage showed a reduction in the iodine content rather than complete removal. This could be due to the trapping of the HI within the (nanochannels of the) membrane. Put together; it can be concluded that the reduction in antimicrobial property of the washed membranes was due primarily to the removal of the HI residue from the membrane.

The result showed that residual HI has a significant effect on the antimicrobial property of the membranes. However, no amount of washing showed complete removal of the residual HI in the membranes as shown in the atomic percentage of iodine obtained from the XPS analysis (Figure 1(e)). The limited antimicrobial effect found after the membranes were thoroughly washed in DI water could also be attributed to the residual HI.

2.4. Effect of reduction methods on the antimicrobial effect of rGO

GO membrane was reduced via the thermal method in order to validate the effect of chemical residue on the antimicrobial property of rGO. Bacterial viability assessment and live/dead observation were conducted by contacting both *P. aeruginosa* PAO1 and *Bacillus* sp. bacterial cells with the GBMs for 3 h and the viability and microscopic observation after contact were conducted (Figure 7 and 8). Thermally reduced GO demonstrated no antimicrobial property against the bacterial cells while the HI vapor reduced GO showed a clear and robust antimicrobial effect.

The toxicity of HI solution was investigated by contacting *P. aeruginosa* PAO1 and *Bacillus* sp. bacterial cells with varying concentrations of HI solution for 3 h and subsequently enumerating the CFU counts (Figure S5). Results showed that HI solution is very toxic from 0.005% and above, at these concentrations, complete inactivation of the bacterial cells was observed. With decreasing HI concentration, the toxicity was also reduced.

3. Discussion

In order to investigate the viability of the bacterial cells after contact with GO and rGO having different reduction degrees based on HI vapor exposure time, two experimental procedures were utilized; (1) CFU assay to estimate cell viability and (2) live and dead bacterial cell observation. Also, the bacterial growth after contact with GBMs in the presence of growth medium (Figure 2 and 3). After 3 h of contact with the GBMs, CFU values were measured in the suspended bacterial cells on the surface. The inconsistency of the antimicrobial property of graphene-based materials on surfaces could be because in most cases, the attached bacterial cells on the surface are enumerated. It is important to note that bacterial attachment on a surface

is greatly influenced by the ability of the microorganism to adhere to the surface. GO and rGO have different material properties. Hence, the total attached bacteria on such surface would be different due to the interaction between bacterial cells and the surface properties. A false inactivation effect would be reported if the quantification of the total attached viable or dead cells on surfaces having different properties were used to assess the antimicrobial effect. Therefore, care should be taken when interpreting the antimicrobial and anti-adhesion properties of graphene-based surfaces. In the study, the contacted bacterial suspension was enumerated in order to reduce such misinterpretation.

The increased inactivation of rGO in this study is consistent with the result of Akhavan and Ghaderi^[20] who reported that the antimicrobial activity of GO increases after reduction by hydrazine. Although the authors suggested that the increased in the antimicrobial property was due to the sharper edge of rGO compared to GO, however, this claim could not be verified by the direct imaging of the graphene-based materials. On the other hand, the result of this study is contrary to the reports in other studies,^[22, 23] where GO material showed better antimicrobial tendencies. Complete bacterial inactivation was found as the HI vapor exposure time increased by more than 30 s.

The inability of the GBMs to produce superoxide anion agrees with the work of Liu and colleagues^[28] who reported that graphene materials could not generate superoxide anion. Nonetheless, other ROS for example, singlet oxygen, alpha-oxygen, peroxides, and hydroxyl radical which were not monitored in this study could be produced by GBMs. The different potential ROS producible by GBMs needs to be investigated in future studies. The higher depletion of GSH observed with GO and slightly reduced GO does not correspond with the antimicrobial results (Figure 4). The quest to investigate the types of ROS produced by the GBMs was not addressed as this would fall out of the scope of this study. The membrane without reduction showed better oxidation of GSH. This is because the tendency for GSH

oxidation is higher with membranes containing more oxygen functional groups. This is contrary to our hypothesis that reduced GO membranes would produce more oxidative stress because of the excellent electrical conductivity as a result of lowered band gap caused by the reduction. It was hypothesized that the reduction of GO would increase its conductivity, hence enhancing its oxidative stress which in turn would increase its antimicrobial properties. The oxidation of glutathione observed for the membrane without reduction could be because of the production of other forms of radicals other than superoxide anion. It was concluded not relevant to investigate in this study the type of radical produced by the GBMs since the pattern seen in the antimicrobial test is contrary to oxidation stress.

Both strong acid and iodine are known for their bactericidal effect. Even though the mode of antimicrobial action of HI has not been extensively studied, it could be thought that the iodine contained in the HI solution could impact the survival of the bacterial cells by acting as a powerful oxidizing agent that can easily and quickly pass the bacterial cell membrane.^[56] The mechanism of action of iodine in the cell membrane is not entirely clear. However, it is believed that once the free iodine penetrates the cell membrane, it can destabilize membrane integrity, denature nucleic acids, and can within a short time inactivate microorganisms by nonspecifically inhibiting essential cellular processes, such as cellular respiration, electron transfer and protein synthesis.^[57]

The mechanism of the antimicrobial property of the graphene-based material in suspension form and when they are aligned vertically on a surface could be somewhat different. Some reports have linked the antimicrobial effects of graphene-based material in suspension form to cellular damage caused by the sharp edge of graphene materials.^[28, 30] Recently, the orientation of the graphene-based material was controlled so that the sharp edge could be exposed in order to improve its antimicrobial property. This was done by vertically aligning graphene oxide nanosheets on a surface.^[58] The piercing effect of graphene-based materials could not be

overruled since undoubtedly the results of the study support the claim of membrane rupture. Even though HI vapor treatment increased the defects formation on the membrane, washing did not affect the amount of defects, meaning the washing process had not impact on the order or disorder of the integrated layers. Hence, the antimicrobial properties cannot be linked to the defects on the membranes. The results of this experiment showed that the antimicrobial effect of the GBM is strictly because of the residual effect of HI solution.

4. Conclusion

Due to the success recorded in the performance test of GBMs for water treatment and desalination by our group, follow-up research was done to assess the antimicrobial effects of these membranes because biofouling is a major drawback faced by membrane-based technology for desalination and water treatment. However, the results in this present work demonstrated that; (1) the stacked GO and rGO membranes did not show notable antimicrobial property sufficient for biofilm control in membrane-based water treatment systems. This is in contrast to some studies but in agreement with others. GBMs fabricated following the methods reported in this work did not show a significant antimicrobial effect, and (2) the antimicrobial effect of the GBM in this study is strictly caused by very low residual concentrations of the hydriodic acid solution used in the graphene oxide reduction process.

Additional studies on the effects of graphene-based membranes on bacterial adhesion and biofouling are currently ongoing in our laboratory. We would strongly recommend that care should be taken when reporting the antimicrobial properties of graphene-based and other modified surfaces.

5. Experimental Section

Fabrication of Graphene-based Membranes: The fabrication procedure of GBMs was as previously described in the research from our group with little modification.^[39] Briefly, a single layer graphene oxide (GO) aqueous solution (6.2 mg mL⁻¹) purchased from Graphene Supermarket (Calverton, USA) was diluted with deionized (DI) water to make a final concentration of 6.2 mg L⁻¹. The GO solution was then sonicated for 2 h and then centrifuged at 10,000 rpm for 1 h. The GO supernatant was collected and sonicated for another 1 h, and then 100 mL was filtered through a 0.2 µm pore-sized mixed cellulose ester membrane (MCE; 47 mm diameter, Advantec MFS, Inc., Japan) using a vacuum-assisted filtration system. The GO on the MCE was dried at 40 °C for 24 h. In order to reduce the GO layers on the MCE, the GO was exposed to hydriodic acid vapor (HI; Sigma Aldrich, USA) for different time to control the reduction degree (5, 30, and 60 s). The reduced GO membranes were left in the laminar flow hood for more than 12 h and stored in a desiccator until use. To obtain a thermally reduced rGO membrane, GO aqueous solution (6.2 mg mL⁻¹) was used as a precursor and sonicated for 2 h. Subsequently, the GO suspension was transferred into a Teflon-lined stainless steel autoclave and placed in a furnace at 220 °C for 3 h. The synthesized rGO was washed in deionized (DI) water by centrifugation at 10,000 rpm for 30 mins and dried in the oven at 70 °C. The resulting powder was dissolved in N-Methyl-2-pyrrolidone (NMP; Daejung Reagent Chemicals, South Korea) to make 0.5 mg mL⁻¹. The solution was then sonicated for 48 h, centrifuged at 3,000 rpm and the supernatant was collected. To fabricate the hydrothermally reduced GO membrane, 5 mL of the solution was filtered through a polytetrafluoroethylene (PTFE; JHWP 04700, pore size: 0.1 µm, diameter: 47 mm, Merck Millipore Ltd., Ireland). After which the membrane was washed in DI water and allowed to dry in the desiccator.

Membrane Characterization: The particle size of the GO dispersions was analyzed using the dynamic light scattering (DLS) technique (ELS-Z3.600/2.30, Otsuka Electronics Co., Ltd, Japan). The chemical structure and structural properties of the membranes were analyzed using

385 Electron spectroscopy for chemical analyzer, X-ray photoelectron spectroscopy (ESCA, XPS;
386 VG Microtech MultiLab ESCA 2000, Thermo VG Scientific, United Kingdom), Raman
387 spectroscopy (NRS-5100, Japan) and Fourier transform infrared spectroscopy (FT-IR; Frontier
388 FT-IR/NIR, Perkin Elmer, USA). The size and formed nanochannels between GO (or rGO)
389 nanosheets in the membranes were characterized using X-ray diffraction (XRD; X'pert PRO,
390 PANalitical, Netherlands) (ambient condition: temperature: 25 °C and humidity: 50%). The
391 sheet resistivity of the membranes was analyzed using a four-point probe (FPP, CMT-SR2000,
392 Changmin Tech Co., Ltd Korea).

393 *Model bacteria:* Gram-negative; *Pseudomonas aeruginosa* PAO1 and Gram-positive; *Bacillus*
394 sp. were used as model bacteria in this study. The pure strains of these bacteria were grown
395 separately. Both microorganisms were cultured for 16 h in Luria-Bertani (LB) medium (Becton
396 Dickinson, USA) at 37 °C with shaking at 150 rpm. The bacterial cultures were centrifuged at
397 8,000 rpm for 10 min to pellet cells, and the pellets washed three times with PBS to remove
398 the remaining culture medium. The pellets were resuspended in phosphate-buffered saline
399 (PBS), and the final concentration was adjusted to an optical density (OD) at 600 nm (OD₆₀₀)
400 of 0.2.

401 *Graphene-based Membranes Antimicrobial Assay:* Bacterial inactivation propensity of the
402 fabricated GBMs was investigated using the colony forming units (CFU) counts and confocal
403 scanning electron microscopy (CLSM). CFU counts were determined in the bacterial
404 suspension and CLSM was used to quantify the surface attached live-dead bacterial cells. The
405 surface antimicrobial activity of the fabricated GBMs was assayed following a previously
406 established protocol.^[59] Briefly, 50 µL of both *P. aeruginosa* PAO1 and *Bacillus* sp. in PBS
407 were contacted with different 1 × 1 cm² GBM surfaces in a 24-well plate and incubated at 25 °C
408 for 3 h. A 10 µL aliquot of the bacterial suspension was then withdrawn, serially diluted as
409 necessary in PBS and plated on an LB agar plate overnight for colony forming unit counts. A

410 2 mL LB broth was added to the remaining bacterial inoculum, together with the corresponding
411 membranes and incubated for 24 h at 37 °C and 150 rpm. To measure the bacterial growth in
412 the presence of GBMs, optical density measurements (OD₆₀₀) were performed at different time
413 intervals.

414 LIVE/DEAD baclight bacterial viability test: The LIVE/DEAD™ BacLight™ Bacterial
415 Viability Kit (Invitrogen Life Technologies, USA) was used to visualize the bacterial cells on
416 the GBMs. The bacterial cells stain green or red depending on the membrane integrity of the
417 cell. Cells with a compromised membrane are stained red, while cells with intact membrane
418 are stained green. A working solution of the fluorescent stains was prepared by adding 3 µL of
419 green stain SYTO9 and 3 µL of red stain propidium iodide (PI) to 1 mL of autoclaved deionized
420 water. 1 × 1 cm² GBMs were placed in the wells of a 24-well plate. A 50 µL suspension of
421 each model bacterium (*P. aeruginosa* PAO1 and *Bacillus* sp) as prepared above was added
422 onto the surface of the GBMs in the 24-well plate and was then incubated at 25 °C for 3 h.
423 subsequently, the bacterial suspension was removed and 200 µL of the staining solution was
424 added onto the surface of the GBMs. The 24-well plate was covered with aluminum foil and
425 incubated for 30 min at room temperature. After staining, the fluorescent images were observed
426 using an Olympus FV1000, Confocal Laser Scanning Microscope (Olympus Inc., USA).

427 *Detection of Reactive Oxygen Species Produced by GBMs:* The reduction of tetrazolium dyes
428 to their soluble formazans by the superoxide radical anions have been widely used as an indirect
429 method to detect the produced radicals in biological systems.^[60] The formazan produced by
430 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) is freely
431 soluble, allowing accurate spectroscopic analysis for their production.^[60] The potential for the
432 generation of superoxide radical anion by GBMs was investigated by monitoring the absorption
433 of XTT at 470 nm.^[28] GBMs were attached to a 24-well plate and 2 mL of 0.4 mM of XTT

dissolved in PBS was contacted with the active side of the graphene membranes and incubated at 37 °C for 0, 2, 4, 6, 8, 10, 12, and 24 h. 250 µL of the solution in each well was put in a 96-well plate. The change in absorbance at 470 nm was observed using a plate reader (Thermo Scientific Multiskan GO, UV/Vis microplate spectrophotometer).

Glutathione oxidation by GBMs: Glutathione (GSH) is a thiol and tripeptide which acts as a vital factor in the cellular metabolic protective functions, such as the reduction of hydroperoxides, and the quenching of free radicals.^[61] The thiol groups in GSH can be oxidized to glutathione disulfide in the presence of oxidative stress. GSH has been widely used as an oxidative stress indicator.^[52, 53, 62] The Ellman's assay is used to quantify the concentration of thiol groups in GSH.^[55] The concentration of thiol in glutathione was quantified using Ellman's assay. 1 × 1 cm² GO membranes reduced by HI vapor exposure for 5 s, 30 s, and 60 s were attached to the bottom of a 24-well plate and 2 mL of 0.8 mM glutathione in bicarbonate buffer was added to each well to make contact with the active sides of the membranes. The 24-well plate was covered with aluminum foil to prevent exposure to light. The plate was incubated at room temperature for 3 h. After incubation, 130 µL of 0.05M Tris-HCl and 10 µL of DNTB (Ellman's reagent, 5,5'-dithio-bis-(2-nitrobenzoic acid); Sigma Aldrich, USA) were put in a 96-well plate to yield a yellow product. A 75 µL of the glutathione contacted with the GBMs was added to the yellow product and the absorbance at 412 nm was measured using a microplate reader. Glutathione without contact with any membrane was used as a negative control. The loss of glutathione was calculated using the equation below (Equation 1).

$$\text{loss of glutathione (\%)} = \frac{\text{Absorbance of negative control} - \text{Absorbance of the sample}}{\text{Absorbance of negative control}} \times 100 \quad (1)$$

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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579 (a)

(b)

Figure 1 (a) XRD patterns of the GBMs, (b) FT-IR spectra of the membranes, (c) Raman spectra of the GBMs, (d) Survey spectra of GBM, (e) Atomic percentage of elements obtained from XPS patterns with and without DI washing, (f) C1s XPS spectra of GBMs exposed to HI vapor for (i) 0 s (ii) 5 s (iii) 30 s (iv) 60 s, (g) Sheet resistance of the GBMs fabricated.

Figure 2. *P. aeruginosa* PAO1 after 3 h GBM contact (a) Bacterial viability (CFU counts), (b) Bacterial growth after 24 h incubation, (c) Fluorescence images (green, live bacteria; red, dead bacteria) on (i) MCE support, and after (ii) 0 s, (iii) 5 s, (iv) 30 s, and (v) 60 s reduction. White bar: 175 μ m.

Figure 3. *Bacillus* sp. after 3 h GBM contact (a) Bacterial viability (CFU counts), (b) Bacterial growth after 24 h incubation, (c) Fluorescence images (green, live bacteria; red, dead bacteria) on (i) MCE support, and after (ii) 0 s, (iii) 5 s, (iv) 30 s, and (v) 60 s reduction. White bar: 175 μ m.

Figure 4. Oxidation of glutathione by GBMs reduced by HI vapor exposure for 0, 5, 30, and 60 s after 3 h.

Figure 5. *P. aeruginosa* PAO1 after contact with thoroughly washed GBMs for 3 h (a) Bacterial viability (CFU counts), and (b) Fluorescence images (green, live bacteria; red, dead bacteria) on (i) MCE support, (ii) 0 s reduction, (iii) 5 s reduction, (iv) 30 s reduction, and (v) 60 s reduction, illustrating that HI caused the bacterial inactivation. White bar: 175 μ m.

Figure 6. *Bacillus* sp. after contact with thoroughly washed GBMs for 3 h (a) Bacterial viability (CFU counts), and (b) Fluorescence images (green, live bacteria; red, dead bacteria) on (i) MCE support, (ii) 0 s reduction, (iii) 5 s reduction, (iv) 30 s reduction, and (v) 60 s reduction, illustrating that HI caused the bacterial inactivation. White bar: 175 μm .

Figure 7. Effect of reduction methods on *P. aeruginosa* PAO1 after contact for 3 h (a) Bacterial viability (CFU counts), and (b) Fluorescence images (green, live bacteria; red, dead bacteria) on (i) MCE support, (ii) GO, (iii) Thermally reduced, (iv) Chemical reduction (HI). White bar: 175 μ m.

Figure 8. Effect of reduction methods on *Bacillus* sp. after contact for 3 h (a) Bacterial viability (CFU counts), and (b) Fluorescence images (green, live bacteria; red, dead bacteria) on (i) MCE support, (ii) GO, (iii) Thermally reduced, (iv) Chemical reduction (HI). White bar: 175 μm .

805 **Keywords:** membrane surface modification; reduced graphene oxide; Wastewater reuse and
806 desalination; bacterial viability, contact inactivation; hydriodic acid

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827 Supporting Information

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829 **Implications of Chemical Reduction Using Hydriodic Acid on the**
830 **Antimicrobial Properties of Graphene Oxide and Reduced Graphene**
831 **Oxide Membranes**

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833 *Abayomi Babatunde Alayande, Hee-Deung Park, Johannes S. Vrouwenvelder, and In S. Kim**

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Figure S1. Photographic images of GO membranes after time-depended exposure to HI vapor with/without washing and hydrothermally reduced GO

Figure S2. Particle size distribution of GO solution used for membrane fabrication

Figure S3. XRD pattern of the hydrothermally reduced GO membrane.

Figure S4. SEM micrographs showing morphological damages to *P. aeruginosa* PAO1 after contact with GBM for 3 h. (a) MCE support, (b) 0 s HI solution exposure, (c) 5 s HI solution exposure, (d) 30 s HI solution exposure (e) 60 s HI solution exposure.

Characterization of the cellular morphology after contact with GBMs in the absence of growth medium was analyzed by taking SEM images. After exposure to GBMs the presence of thickening patches and flattened morphology compared to the cells deposited on MCE support was observed. It is clear that although bacterial cells can come in close contact with GBM surface there was no observable uptake of GO-based material by the cells.

Figure S5. Effect of HI solution concentration on bacterial cell toxicity. (a) Against *P. aeruginosa* PAO1, and (b) Against *Bacillus* sp.