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Identification and Optimization of Carbon Radicals on Hydrated Graphene Oxide for Ubiquitous Antibacterial Coatings

Ruibin Li^{§,†}, Nikhita D. Mansukhani[‡], Linda M. Guiney[‡], Zhaoxia Ji[⊥], Yichao Zhao[‡], Chong Hyun Chang[⊥], Christopher T. French[#], Jeff F. Miller^{⊥,‡}, Mark C. Hersam[‡], Andre E. Nel^{†,‡,⊥,*}, and Tian Xia^{†,‡,⊥,*}

[§]School for Radiological and Interdisciplinary Sciences (RAD-X), Collaborative Innovation Center of Radiation Medicine of Jiangsu Higher Education Institutions, Soochow University, Suzhou, 215123, China

[†]Division of NanoMedicine, Department of Medicine, University of California, 10833 Le Conte Ave, Los Angeles, CA 90095, United States

[‡]Center for Environmental Implications of Nanotechnology, University of California, Los Angeles, California 90095, United States

[‡]Departments of Materials Science and Engineering, Chemistry, and Medicine, Northwestern University, Evanston, Illinois 60208, United States

[⊥]California NanoSystems Institute, University of California, 570 Westwood Plaza, Los Angeles, CA 90095, United States

[#]Departments of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095, United States

Abstract

While the antibacterial properties of graphene oxide (GO) have been demonstrated across a spectrum of bacteria, the critical role of functional groups is unclear. To address this important issue, we utilized reduction and hydration methods to establish a GO library with different oxidation, hydroxyl, and carbon radical ($\bullet\text{C}$) levels that can be used to study the impact on antibacterial activity. Using antibiotic-resistant (AR) bacteria as a test platform, we found that the $\bullet\text{C}$ density is most proximately associated with bacterial killing. Accordingly, hydrated GO (hGO), with the highest $\bullet\text{C}$ density, had the strongest antibacterial effects through membrane binding and induction of lipid peroxidation. To explore its potential applications, we demonstrated that coating of catheter and glass surfaces with hGO is capable of killing drug-resistant bacteria. In summary, $\bullet\text{C}$ is the principle surface moiety that can be utilized for clinical applications of GO-based antibacterial coatings.

*Corresponding Author: Tian Xia, Ph.D.; and Andre Nel, Ph.D., Department of Medicine, Division of NanoMedicine, UCLA School of Medicine, 52-175, CHS, 10833 Le Conte Ave, Los Angeles, CA 90095-1680., Tel: (310) 983-3359, Fax: (310) 206-8107, txia@ucla.edu, Anel@mednet.ucla.edu.

Supporting Information Available: Antibiotics that tested in resistant *E. coli*, detection of residual oxygen radicals on GO, Raman spectra of GO samples, bactericidal effects of GO in silver-resistant *E. coli*, TEM images of GO in bacterial media, durability test of carbon radicals in hGO-2, spin coating process, Raman and AFM characterization of hGO-2 films, scheme for CFU test. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Graphical Abstract

Keywords

Graphene oxide; Super bugs; Antibacterial coating; Antibiotic Resistance; Functionalization

Chemically modified graphene has been widely studied for various applications, such as polymer composites, energy-related materials, and catalysis¹⁻². Of particular interest is graphene oxide (GO), an oxygenated form of graphene decorated with abundant functional groups. GO is widely used due to its wide availability, facile synthesis, and outstanding electronic, optical, and chemical properties³⁻⁵. A major use for this material is in biomedical applications for drug delivery, biosensors, and tissue engineering due to its high dispersibility, two-dimensional (2D) planar structure, large surface area and surface functionalities⁶. In addition, GO has broad-based antibacterial effects that require detailed structure-activity relationships (SARs) to be established⁷⁻⁹.

There are two major schools of thought on the mechanism of GO-induced bactericidal effects. One is relying on the physical interaction between the unique GO structure and bacterial membranes, including direct physical puncturing of bacteria membrane⁸, or destructive extraction of lipid molecules from bacterial membrane⁹. However, this hypothesis is mostly premised on computer simulation, and there is a paucity of experimental evidence regarding the details of damage to the bacterial surface. Another mechanistic explanation, with supportive experimental data, is oxidative damage to the bacterial membrane by the generation of reactive oxygen species and charge transfer¹⁰⁻¹². While surface functional groups are assumed to play a critical role in mediating GO oxidative damage, the complex chemistry related to this material has made it difficult to discern the exact surface functional groups that may be involved in this outcome.

As-prepared GO has different oxidation levels and surface functional groups such as the presence of epoxy (-COC-), hydroxyl (-OH), and carboxyl (-COOH) moieties at different densities and combinations¹³. In addition, isolated electrons in the carbon p orbitals are often conjugated by π bonding, which could form carbon radicals ($\bullet\text{C}$) at discrete sites on

the material surface¹⁴. Although attempts have been made to explore the role of oxidation level^{13, 15}, lateral flake size¹⁶ or catalytic capability¹¹ on bacterial killing, results have been inconclusive and even contradictory. One reason is the interlinked complexity of the functional groups, such that a change in one surface group will also affect others, often in a non-predictable fashion. To overcome this challenge, a new approach is required to change the surface functionalities in a more systematic fashion to elucidate SARs. For instance, it has been shown that solvothermal reduction could quantitatively adjust GO oxidation levels¹⁷, while hydrolysis by alkalized aqueous solvents could open epoxy rings and quantitatively adjust the hydroxyl density¹⁸. While it is also possible that the •C levels could also be impacted by these processes¹⁹, this aspect has not been studied. These evolving concepts prompted us to hypothesize that quantitative adjustment of GO surface functionalities may allow us to establish SARs for bacterial killing by the various surface groups.

Herein, using reduction and hydration methods, we successfully synthesized a library of GO with different surface functionalities and compared their antibacterial effects in *Escherichia coli* (*E. coli*), which is a popularly used gram-negative bacterium to explore new antibacterial agents and study antibiotic resistance^{9, 12}. Through detailed characterization and toxicity testing, we identified •C as the functionality that is best associated with the antibacterial effects. The hydration method could quantitatively modulate the •C density, allowing hydrated GO (hGO) with the highest radical levels to exert the most effective antibacterial effects. To explore its potential applications, we coated the surfaces of glass slides and silicone catheters with hGO for proof-of-concept experimentation. Immobilized hGO provided highly effective bactericidal effects in antibiotic-resistant (AR) *E. coli*, commensurate with the coating density. These results demonstrate the important role of •C and potential use of GO coatings on medical devices to combat antibiotic resistance.

Results

Establishing a well-characterized GO material library with varying surface functional groups

Recent developments in the functionalization of the GO surface present the opportunity for chemical tuning of these groups for the purpose of directed work functions. Oxidation levels can be modified through the use of reduction processes based on solvothermal or chemical methods for the production of reduced graphene oxide (rGO), which is chemically similar to graphene, albeit not identical (Scheme 1). During reduction, the variety and evolution of oxygenated species on the GO surface can be tracked by methods such as Raman spectroscopy²⁰, X-ray photoelectron spectroscopy (XPS)²¹ and electron paramagnetic resonance (EPR)¹⁴. It is also possible to use hydration chemistry for opening epoxy rings through hydrolysis, with the ability to change the density of surface hydroxyl groups; this can be achieved by heating GO in an alkaline environment (Scheme 1).

In order to assess the SARs most directly involved in bacterial killing, we established a GO material library by using reduction and hydration methods to change surface functionalities. Pristine GO prepared by the classic Hummers method was used as the base material for preparing the library. The total level of oxidized groups on the GO surface was reduced by

solvothermal reduction in N-methyl-2-pyrrolidinone (NMP), while heating in alkaline water leads to increasing the hydroxyl density by hydrolyzing the epoxy surface groups (Scheme 1). Through control of the reaction conditions (time or temperature), we prepared a series of GO samples with quantifiable differences in their oxidation states and surface functional groups. From these materials, we selected two reduced GO (rGO-1 and rGO-2) and two hydrated GO (hGO-1 and hGO-2) samples for comparative study vs. pristine GO. X-ray photoelectron spectroscopy (XPS) was used to determine the levels of oxidized functional groups on the GO surface. The C1s XPS spectra of the rGO-2, GO and hGO-2 sheets (Figure 1A), show C=C bonds at 284.6 eV, C-OH at 286.5 eV, C-O-C at 287.3 eV and C=O at 288.0 eV, as previously described^{22–23}. Analysis of the XPS peaks allowed us to estimate the atomic % of -COOH, -COC-, and -OH groups on the GO surface. This analysis showed that the NMP reduction could significantly reduce the peak intensities of the total oxygen content as well as the % of each oxidized group on the material surface. Different from the reduction process, hydrolysis mainly reduces the C-O-C content on the GO surface. The intensity of the CO-C peak decreased to 2.1%, while the C-OH peak increased from 4.5 to 13.6% for hGO-2. These changes likely result from the reaction between the epoxy groups and nucleophiles in aqueous solution, leading to the generation of -OH groups. The total oxygen levels remained stable during hydration, likely due to the fact that opening of the epoxy rings will not change the oxygen levels.

Again, we know that the isolated electrons in the *p* orbitals of the carbon atom are usually conjugated to the GO backbone by π bonding, which can result in the formation of $\bullet\text{C}^{14}$. Thus, we also characterized the $\bullet\text{C}$ density on the GO surface through an EPR method. Interestingly, these measurements demonstrated that in addition to the changes in oxygen levels and hydroxyl groups as a result of reduction and hydration chemistry, there is a dramatic change in the density of $\bullet\text{C}$. As shown in Figure 1B, all GO samples showed a single resonance peak of π -conjugated $\bullet\text{C}$ with $g=2.0029$, which is consistent with a previous report¹⁴. In addition, we ruled out the possibility that the EPR signals on GO samples resulting from the residual oxygen radicals (such as OH and O_2^-) by the 3'-(p-aminophenyl) fluorescein (APF) and 3-bis(2-methoxy-4-nitro-5-sulfophehyl)-2H-tetrazolium-5-carboxanilide (XTT) assays (Figure S1). hGO samples showed significantly higher EPR peaks than pristine GO (with hGO-2 being the highest), while rGO samples showed a lower radical density than GO. rGO-2 exhibited the weakest EPR signal. These changes suggest that hydrolysis of -COC- groups is accompanied by the generation of $\bullet\text{C}$, which stably exist on the planar GO surface as π -conjugated $\bullet\text{C}^{14}$. In addition, we demonstrated that the EPR signals used to establish a role for $\bullet\text{C}$ show the utility of reduction and hydration chemistry to quantitatively change the surface functionalities of GO sheets.

We also thoroughly characterized other physicochemical properties of the GO library materials, including primary size, shape, hydrodynamic size and surface charge in aqueous solution. Atomic force microscopy (AFM) showed that all the GO materials were irregularly shaped nanosheets with a lateral size distribution of 50–300 nm (Figure 1C). Raman spectra demonstrated that rGO, GO and hGO samples preserved the graphene structure, with maintenance of the signature G and D bands (Figure S2); this suggests that there are no major structural changes during the catalytic modification of the GO surface. We also

assessed the hydrodynamic size distribution and zeta potential of the GO materials in different aqueous media. As shown in Table 1, all of the GO nanosheets showed approximately the same hydrodynamic size distribution (400–700 nm) and negative zeta potential values (5 to –19 mV) in the presence of LB or Lactobacilli MRS (Lacto) broths (Table 1).

Carbon radical density is the determining factor in the antibacterial effects of GO

To study the killing effects of pristine GO on bacteria, we selected wildtype and antibiotic resistant (AR) *E. coli* as well as AR *Lactobacillus crispatus* (*L. crispatus*) as representative gram-negative and positive bacterial strains, respectively. The AR strains were included in light of the public health relevance of AR bacteria. The chosen *E. coli* strain is resistant to 24 different types of antibiotics, including ampicillin (Table S1). This is demonstrated by the fact that ampicillin could induce effective killing with an IC₅₀ of 10 µg/mL in wildtype *E. coli*, while the resistant strain showed no growth inhibition, even at a dose as high as 500 µg/mL (Figure 2A). In contrast, GO showed moderate antibacterial effects in wildtype and AR *E. coli*, yielding an IC₅₀ of 500 µg/mL. Similar results were found in AR *L. crispatus* strain (77% killing at 500 µg/mL), as well as in silver-resistant *E. coli* (Figure S3).

Since GO only exerts partial antibacterial effects, it would be desirable to improve the killing efficiency. This prompted us to look at the antibacterial effects of the GO library. We found that hGO sheets have significantly enhanced antibacterial effects compared to pristine GO, with hGO-2 achieving almost 100% killing at 500 µg/mL (Figure 2B). In contrast, rGO-1 and rGO-2 showed decreased bactericidal effects compared to the pristine material. Comparison of the antibacterial efficiency with the physicochemical characteristics of the library materials (including oxidized chemical content, •C density, sheet size and zeta potential), showed the strongest correlation ($r=0.99$) to •C density (Figure 2C). However, as expected, the correlation coefficient to the hydroxyl content was also strong ($r=0.94$) in light of the intimate relationship with the •C content. These results demonstrate the important role of •C in GO-induced antibacterial effects in AR bacterial strains.

GO-induced bactericidal effects involve membrane binding and lipid peroxidation

It has been shown that the hydrophobic planar properties and hydrophilic edges of GO sheets allow membrane insertion, which could be related to their bactericidal effects²⁴. In order to obtain ultrastructural resolution of the interaction of our library materials with the AR *E. coli* strain, TEM analysis was performed after bacterial exposure in culture medium for 24 h. Due to the low electron densities of GO, the suspended GO sheets could only be visualized when vertically positioned on the grid but not when they aligned with the flat surfaces parallel to the grid (Figure S4). This is consistent with other reports⁹. Following bacterial co-culture, the TEM images revealed that the GO nanosheets associate with the bacterial membrane without clear evidence of piercing or cutting of the bacterial membrane (Figure 3); this is consistent with the observations of Tu *et al*⁹. We also observed that while the pristine and reduced (rGO-2) sheets may associate with the membrane without any visible damage, AR *E. coli* treated with hGO-2 display extensive structural damage, as manifested by membrane collapse.

•C radicals are typically considered more active than other surface functional groups because they contain unpaired electrons that lead to pro-oxidative potential¹⁴. The pro-oxidative capacity of the GO library was examined by 2', 7'-dichlorodihydrofluorescein (H₂DCF), which can be converted to a fluorescent 2', 7'-dichlorofluorescein (DCF) derivative by a variety of oxidizing radicals generated by nanomaterials or small molecules^{25–27}. As shown in Figure 4A, compared to pristine GO, hGO materials induced a significant increase in DCF fluorescence, while the reactivity of the rGO materials were low. Thus, the overall ranking of the abiotic oxidative potential of the GO samples was hGO-2 > hGO-1 > GO > rGO-1 > rGO-2, which correlates well with the •C densities ($r > 0.97$). hGO-2 was also used to evaluate the stability of the carbon radicals on the material surface, using serial EPR measurements. This demonstrated limited change in the EPR peak intensity and pro-oxidative capability for hGO-2 samples stored in deionized water at 4 °C for 1 year (Figure S5). However, the carbon radical existence on GO samples in biological media will likely be of short duration due to the presence of abundant reductive molecules.

To further demonstrate how the pro-oxidative effects under abiotic conditions may relate to membrane injury by hGO-2, a BODIPY® 581/591 C11 reagent was used to study lipid peroxidation in *E. coli*. The reagent shows a shift in peak fluorescence emission from red (~590 nm) to green (~510 nm) during lipid peroxidation, as demonstrated by using cumene hydroperoxide (CH) as a positive control (Figure 4B). hGO-2 induced prominent lipid peroxidation compared to pristine GO, while rGO-2 had minimal effects. These results were also quantitatively expressed by studying lipid peroxidation with flow cytometry, which showed that pristine GO induced lipid peroxidation in 12% of bacterial cells, while the comparable values for hGO-2 and rGO-2 were 21% and 5%, respectively (Figure 4C). Since lipid peroxidation could destabilize the bacterial membrane, we studied β -galactosidase release from AR *E. coli* after GO treatment. We found that hGO-2 can induce 22% β -galactosidase release from membrane-damaged bacteria (Figure 4D), which is significantly higher than GO (8%) or rGO (3%). In summary, these data demonstrate that the bactericidal effects of GO are dependent on interactions with the bacterial membrane, where surface reactive groups such as the •C could lead to membrane damage and cell death as a result of lipid peroxidation. Figure 4E shows the proposed mechanism for the induction of lipid peroxidation as a result of the •C on the GO surface interacting with unsaturated lipids in the bacterial membrane. We propose the involvement of three steps, namely: (i) electron transfer from the •C to one of the C atoms adjacent to double bonds in the lipid; (ii) electron transfer from this functionalized C atom to bystander molecular dioxygen (O₂), leading to the formation of a lipid peroxide radical, containing the attached-O-O group; (iii) generation of a lipid peroxide from the lipid peroxide radical.

Highly efficient killing of AR bacteria by hGO-2 films

Because GO is often incorporated into composites (e.g., bandages and biofilms) for antibacterial activity, we were interested to see whether immobilization of hGO to material surfaces could attain significant bactericidal effects. We performed proof-of-principle testing by depositing hGO-2 thin films on a glass substrate or covalently bonding the hGO to the surfaces of silicone catheters. hGO-2 nanosheets were attached to the surface of a glass substrate by spin coating, allowing us to prepare a series of coated substrates with

controllable GO film thickness (7~25 nm) and surface coverage. Four glass substrates (S-1 to S-4) coated with hGO-2 films (Figure S6A) were characterized through the use of AFM (Figure 5A, S6B) and Raman spectroscopy (Figure S7). The Raman spectra demonstrated the presence of signature D and G bands that are characteristic of GO, and the AFM images confirmed the varying thickness of these films, which varied from 7 nm to 25 nm. Moreover, AFM showed that hGO-2 coating exhibited interesting stacking behavior with larger sheets attaching to the substrate with their flat sides facing up, while smaller sheets were stacked on top of the larger sheets. The surface area coverage varied from 50% in S-1 to 100% in S-4.

Testing of the antibacterial effects of the coated glass surfaces was carried out by incubating S-1 through S-4 with the AR *E. coli* strain for 6 h at 37 °C. The bacterial morphology and viability were subsequently assessed by scanning electron microscopy (SEM) and propidium iodide (PI) staining, respectively. Instead of the rod-shaped appearance and intact surfaces found on non-exposed bacteria, SEM demonstrated an incremental increase in bacterial membrane damage and fragmentation during exposure to the incremental stacking density in S-1 to S-4 (Figure 5B). Assessment of the bacterial viability by PI staining and confocal microscopy showed that while there were < 10% PI-positive bacteria grown on the uncoated glass surface, there was a gradual increase in the presence of dead cells with incremental levels of coating, such that 96% of bacterial cells were non-viable in the presence of S-3 and S-4. It is also noteworthy that the killing effects of the hGO-2 films were more effective than that of the hGO-2 suspensions, suggesting that a coated film format may be superior for antibacterial applications.

The next proof-of-concept testing was covalent attachment of hGO-2 to the surface of a silicone catheter, which has relevance from the perspective of bacterial contamination of indwelling clinically used devices²⁸. A method was developed to prepare the substrate surface for coating, namely surface amination by (3-Aminopropyl)triethoxysilane (APTES), followed by conjugation of the amine groups with hGO-2 (Figure 6A). These coatings showed extraordinary durability and could stably attach to the catheter surface, even after sonication and washing procedures. This concept was initially tested on a silicon wafer, where the coating could be confirmed by the signature G and D bands in the Raman spectra that were obtained, as well as the presence of a 16 nm thick layer by AFM after 10 cycles of coating (Figure S8). The procedure was then repeated on a series of silicone catheters that were coated with hGO-2 for 1, 3, 5 and 10 cycles (denoted as 1C, 3C, 5C and 10C). Each layer of coating resulted in progressive darkening of the catheter surface. The antibacterial effects of the coated catheter surfaces were tested *in situ* by visualizing morphological changes in AR *E. coli*. As shown in Figure 6B, while the bacteria incubated with uncoated catheter surfaces exhibited rod shaped cells with intact surfaces, holes appeared in the bacterial membrane during exposure to coated catheters. We also observed bacterial fragmentation during exposure to catheter surfaces coated 5 or 10 times. These results were confirmed in a β -galactosidase assay, which reflects progressive bacterial membrane leakage (Figure 6C). We also performed a quantitative evaluation of the bactericidal effects of 10C hGO-2 coated catheter surface. As shown in Figure S9, after 1, 3 and 6 h of incubation, the bacteria settling on the catheter surface were collected, serially diluted and introduced into LB agar plates to assess the number of colony forming units (CFU). The coated catheters showed a time-dependent reduction of CFU, compared to the uncoated catheters (Figure

6D). A dramatic decrease of CFU could be seen after 1 h of exposure to the hGO-2 film, amounting to 2.2 log reduction after 6 h exposure. All considered, the above results show that hGO-2 coating of glass and catheter surfaces can be used to kill AR *E. coli*.

Discussion

In this study, we delineated the GO surface groups that are responsible for bactericidal effects and explored the potential use of GO coating on the surface of a medical device that may be contaminated by AR bacteria. We found that the •C density on the GO surface is most proximately associated the antibacterial effects of these materials compared to other functional groups. The efficacy of bacterial killing could be enhanced by a novel hydration process of the GO surface that opens the epoxy rings to generate more surface •C groups, without impacting superoxide or hydroxyl radical generation. The high •C density enhanced the oxidative potential of hGO, which allowed these sheets to induce lipid peroxidation of the bacterial membrane with significantly higher bactericidal effects in AR strains. In contrast, surface reduction decreases •C density and antibacterial killing by GO. Coating of hGO on glass and silicone catheter surfaces allows effective killing of AR *E. coli* and may be useful for preventing biofilm formation on catheter surfaces.

The most important finding of this study is the demonstration that among the surface reactive groups, •C radicals appear to play an important role in shaping the antibacterial activity of GO. The •C radicals are generated during breaking of the C=C bonds when graphite is treated with strong oxidants to prepare GO. We found that alkaline hydration could increase the •C density during hydrolysis of the epoxy groups on the GO surface. The formation of this radical group was reported to be an intermediary step during occupancy of the *p* orbitals by single electrons¹⁹. While isolated •C radicals are often unstable in association with a small molecule, the single carbon electrons on the GO surface are located in the π -network plane, allowing them to conjugate with the π electrons of neighboring double bonds to form π -conjugated •C¹⁴, which are more stable. Moreover, the mobility of •C among the conjugated C=C bonds could significantly enhance its oxidizing capability and reactivity with target molecules (*e.g.*, membrane lipids), coming into contact with the basal GO plane. This allows hGO-2, which has the highest •C density, to exert the highest bactericidal effects, in contrast to rGO that exhibits a low •C density and lack of bactericidal effects. The same characteristics are involved in the effective bactericidal effects of hGO when coated on the surfaces of catheters or a glass substrate.

Multidrug resistance in a growing number of infectious agents is considered as one of the greatest threats to human health in a recent World Health Organization report²⁹. For example, fluoroquinolone-resistant *Shigella* has caused more than 1 million deaths in 2012, while multidrug resistant *Mycobacterium Tuberculosis* resulted in an estimated 170,000 deaths in the same timeframe. Moreover, bacterial antibiotic resistance now impacts most pharmaceutical antibacterial agents. Among the major pathways leading to antibiotic resistance are acquired mechanisms that block antibiotic access to intracellular targets, either through the reduction of membrane permeability³⁰ or increased drug efflux by membrane pumps³¹. Another resistance mechanism involves the mutation of antibiotic targets in bacteria³² or their modification³³ to prevent antibiotic binding. A third mechanism is the

inactivation of antibiotics by enzymatic activities such as hydrolysis³⁴ or post-modification³⁵. In addition, these diverse resistance mechanisms often work synergistically to establish a formidable defense against antibiotics. In contrast, GO is capable of bacterial killing by anchoring to the membrane and inducing lipid peroxidation without the necessity of cellular uptake. This circumvents traditional AR mechanisms, with the ability to overcome antibiotic and even silver resistance pathways.

To take full advantage of the antibacterial effects of GO, we postulated that it is possible to enhance its 2D material effects through modification of its surface reactivity and immobilization on the surface of a medical device that can be colonized by AR bacteria. Indeed, GO has often been integrated into products such as antibacterial paper³⁶, bandages¹¹ and ultrafiltration membranes³⁷. Another consideration is the reported toxicity of GO to mammalian cells and animals after intravenous, intraperitoneal, subcutaneous, or intramuscular injection^{38–39}. Immobilized GO films on device surfaces would minimize direct exposure of GO to mammalian cells and thus reduce the potential toxicity. Herein, we report the preparation of hGO-2 films by noncovalent and covalent attachment to glass and catheter surfaces, respectively. Since silicone catheters are widely used in medical devices and serve as the substrate for bacterial biofilms²⁸, we explored stable, covalent attachment of hGO-2 that has potent antibacterial effects. hGO-2 also serves the same purpose if non-covalently attached to glass substrates. Both types of coatings are dependent on coating density and thickness for the efficacy of bacterial killing. This result could be attributed to two advantages of films over GO sheet suspensions: the first is enhanced binding to the bacterial surfaces and the second involves stronger exposure and shorter traveling distance for the reactive $\bullet\text{C}$ radicals on the hGO surface. In terms of the stability of spin coating versus covalent attachment of hGO-2 films on a glass substrate or silicon catheter surface, respectively, we do observe some differences. While spin coating can resist scratching or gentle washing, covalent attachment is more durable, capable of withstanding sonication for 30 minutes. There were also no observed differences between fresh-made and aged hGO-2 films in terms of the attachment stability over 6-months. In addition to the surface coating of medical devices, hGO-2 has the potential to be incorporated into bandages for wound healing, antibacterial additives in dental devices or water filtration membranes to prevent biofilm formation. Obviously, further research is required on each of these fronts.

Conclusions

Our study indicates that $\bullet\text{C}$ plays a critical role in GO-induced bactericidal effects. Hydration of pristine GO generates a high $\bullet\text{C}$ density on the GO surface, which significantly enhances antibacterial effects, including in AR and silver-resistant bacteria. The mechanism of bacterial killing involves GO contact with the bacterial membrane without cellular uptake. The subsequent induction of lipid peroxidation in the bacterial membrane leads to a lethal effect. To explore the potential use of these structure-activity relationships for medical devices, hydrated GO with a high $\bullet\text{C}$ density was deposited onto the surfaces of glass substrates and silicone catheters, with the ability to kill AR *E. coli*. These results demonstrated the potential of GO with superior bacterial killing ability to be considered for their antibacterial effects in the clinical setting of antibiotic resistance.

Materials and Methods

Chemicals

Ampicillin, N-Hydroxysuccinimide, Ethyl(dimethylaminopropyl) carbodiimide, 1-methyl-2-pyrrolidinone, erythromycin and AgNO₃ were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Beta-Glo® Assay System was purchased from Promega (Madison, WI, USA); graphite flakes were provided by Asbury Graphite Mills; Hoechst 33342, Propidium iodide and Image-iT® Lipid Peroxidation Kit were purchased from Life Technologies (Grand Island, NY, USA).

Acquisition and synthesis of a surface functionalized GO library

GO production—GO was prepared using a modified Hummers method as described previously⁴⁰. Briefly, graphite flakes (Asbury Mills 3061 grade) were oxidized and then filtered and centrifuged to remove any residual contaminants. The oxidized graphite was then re-dispersed in N-methyl-2-pyrrolidone (NMP, ACS reagent grade 328634, Sigma-Aldrich) and ultrasonicated using a Fisher Scientific Model 500 Sonic Dismembrator for 1 h at 50% power (~55 W) in an ice bath. The GO was then centrifuged using a Beckman Coulter J26-XPI at 5000 rpm for 10 min (~4,620 g). The top 80% of the supernatant was retained as the final GO sample for further processing of the reduced graphene oxide samples. The concentration of the GO solution was determined by optical absorbance at 330 nm.

Synthesis of reduced GO—GO was dispersed in NMP by ultrasonication for 1 h at 50% power (~55 W) as described before⁴¹. The solution was heated to 150 °C with constant stirring in a silicone oil bath for 1 hour (rGO-1) or 5 h (rGO-2).

Preparation of hydrated GO—10 mL GO suspension (5 mg/mL) was diluted with 90 mL deionized (DI) water and mixed with 80 mg NaOH (0.02 M), using dispersal by a sonication probe (Sonics & Materials, USA) at 32 W for 10 min. The GO mixture was transferred into a round flask and refluxed at 50 or 100 °C in an oil bath with constant magnetic stirring for 24 h. 1 M HCl solutions were used to neutralize the reaction. The mixture was centrifuged at 50,000 rpm for 30 min to collect the hydrated GO pellets. After washing with DI water three times, the hydrated GO samples were dispersed in DI water and stored at 4 °C.

Physicochemical characterization of GO samples

To obtain AFM images, Si wafers were pretreated by 2.5 mM (3-aminopropyl) triethoxysilane (APTES) aqueous solution for 30 min to functionalize the surface with a monolayer⁴². The wafers were rinsed with DI water and dried under N₂. A drop of 10 µg/mL GO solution was placed on the wafer, followed by washing twice with DI water (~5 s) and drying under N₂. Then the GO AFM sample underwent heat treatment for 30 min at 250 °C. AFM images were obtained by an Asylum Research Cypher ES AFM. Images were taken at random locations in the sample and showed little variation. All images were obtained with the same tip and scanning conditions.

X-ray photoelectron spectroscopy (XPS, AXIS Ultra DLD, Kratos, UK) has been used to investigate the chemical state and calculate the atomic concentration of oxidized groups on GO surface with monochromatic Al K α at 15 kV and 10 mA. For sample preparation, suspension of GO samples was dropped on the silicon substrate and dried at room temperature. The data analysis and curve fitting were performed with the CasaXPS program (Casa Software Ltd., UK).

The molecular structure of all GO samples were characterized using Raman spectroscopy (Renishaw inVia Reflex, Wotton under Edge, UK) with a 785 nm near-infrared diode and a 50X objective lens. Spectra were obtained for 10 seconds exposure time with an accumulation of 2 scans in the wave number region 500–2000 cm⁻¹.

The EPR measurements were obtained with an X-band Bruker ELEXYS 580 spectrometer. 5 mg GO nanosheets were dried through vacuum, and allowed to settle to the bottom of the 2 mm ID quartz EPR tubes prior to data collection. The field was calibrated using a standard sample with a known *g*-factor (2,2-diphenyl-1-picrylhydrazyl, DPPH). The EPR spectra were detected at room temperature with frequency at 9.785845 GHz, center field at 3480 G, attenuator at 13.0 dB and *g* value at 2.0029.

Zeta-potential and hydrodynamic size measurements of the GO suspensions were performed using a ZetaSizer Nano-ZS instrument (Malvern Instruments, Worcestershire WR, UK).

Assessing the pro-oxidative capability of GO by DCF, XTT and APF assays

The oxidation capacity and oxygen species of GO samples were determined as described before²⁵. 2',7'-dichlorodihydro- uorescein diacetate (H₂DCF-DA) was used to evaluate the total oxidation capacity of GO, while the 3'-(p-aminophenyl) fluorescein (APF) and 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assays were used to determine OH and O₂⁻ on GO surface, respectively. The DCF working solution was prepared by mixing 50 μ g of H₂DCF-DA with 280 μ L 0.01 M NaOH. The resulting solution was incubated for 30 min, and diluted with 1720 μ L of a sodium phosphate buffer (25 mmol/L, pH = 7.4) to form 25 μ g/mL DCF solution. 95 μ L aliquots of 25 μ g/mL DCF, 100 μ M XTT or 10 μ M APF working solutions were added into each well of a 96 multiwell black plate (Costar, Corning, NY). A 5 μ L amount of 5 mg/mL nanoparticle suspension was subsequently added to each well, followed by 2 h incubation. DCF uorescence emission spectra in the range of 500–600 nm were collected using a SpectraMax M5 microplate reader with an excitation wavelength of 490 nm. APF uorescence emission spectra were collected at 480–600 nm with an excitation wavelength of 455 nm, while XTT absorbance spectra were recorded in the range of 410–550 nm.

Determination of bacteria killing by GO

Five bacterial strains were used in this study: a wildtype *E. coli* strain (ATCC 25922), an AR *E. coli* strain (ATCC BAA-2452), a silver-resistant *E. coli* strain (kindly donated by Dr. Susanne Stterlin from Department of Clinical Microbiology, Uppsala University, Sweden), a sensitive *L. crispatus* strain (ATCC 53545) and an AR *L. crispatus* strain (ATCC 55221). Bacterial growth was carried out in LB broth (Lennox, Sigma-Aldrich, USA) for *E. coli* and Lactobacilli MRS Broth for *L. crispatus* (BD, Franklin Lakes, NJ, USA), respectively. To

assess the bactericidal efficiency of the GO nanosheets, a growth inhibition curve was constructed. In detail, 5 mg/mL stock solution of each material was dispersed in LB and Lactobacilli MRS media at 500 µg/mL. The resulting mixture was sonicated using a sonication probe (Sonics & Materials, USA) at 32 W for 15 s. The GO suspensions were diluted stepwise with the culture media to obtain a series of gradients (7.8, 15.6, 31.3, 62.5, 125, 250 and 500 µg/mL), of which 50 µL of materials suspension was pipetted into 384-well polystyrene microplates. Nine replicate measurements were performed for each concentration. Using a separate plate, 50 µL of a log-phase bacterial culture (OD₆₀₀ between 0.5~0.7) was pipetted into a 384-well plate, before a plastic 384 pin replicator (Genetix Molecular Devices, USA) was used to inoculate bacteria from the plate to the plate containing the serial dilution of GO samples. Sterility and blank controls (bacterial media with no inoculation) were also included for each concentration (3 replicates). After 24 h incubation at 37 °C, a SpectraMax M5 microplate reader was used to monitor OD₆₀₀. A growth curve was constructed using the following equation:

$$\% \text{ Growth} = \frac{A_{NP,B} - A_{NP,M}}{A_B - A_{B,M}} \times 100\%$$

where $A_{NP,B}$ is the absorbance of the bacterial culture in the presence of GO nanoparticles (NPs); $A_{NP,M}$ is the absorbance of the GO NPs at the respective concentrations which contain no bacteria; A_B is the absorbance of the bacterial culture in blank (no GO NPs) media, and $A_{B,M}$ is the absorbance of media with no bacteria⁴³.

TEM imaging of GO interaction with bacteria

After exposure to 125 µg/mL GO for 24 h, *E. coli* were washed with PBS and fixed with 2% glutaraldehyde in PBS. After post-fixation staining in 1% osmium tetroxide in PBS for 1 h, the cells were dehydrated in a graded series of ethanol, treated with propylene oxide, and embedded in Epon. Approximately 50–70 nm thick sections were cut on a Reichert-Jung Ultracut E ultramicrotome and picked up on Formvar-coated copper grids. The sections were stained with uranyl acetate and Reynolds lead citrate and examined on a JEOL transmission electron microscope at 80 kV in the UCLA BRI Electron Microscopy Core as previously reported⁴³.

Assessment of bacterial membrane lipid peroxidation

AR *E. coli* bacteria were treated with 250 µg/mL GO samples for 6 h or 10 µM cumene hydroperoxide (positive control) for 1 h. The cells were then washed twice, incubated with 10 µM Image-iT® Lipid Peroxidation Sensor and Hoechst 33342 in culture media for 30 min. After staining, the cells were washed three times with PBS, and fluorescence readings were obtained for the reduction and oxidation of the dye at excitation/emission wavelengths of 581/591 nm (Texas Red® filter set) and 488/510 nm (traditional FITC filter), respectively. The ratio of the emission fluorescence intensities at 590 nm to 510 nm gives a read-out for cellular lipid peroxidation. The flow cytometry analysis was carried out on a FACS Vantage SE flow cytometer from BD (Franklin Lakes, NJ). The flow cytometric data were collected and processed using Flowjo Software (Ashland, OR). The confocal laser scanning microscopy analysis was performed by a TCSSP2 confocal laser scanning microscope (Leica, Wetzlar, Germany).

Assay for β -galactocidase release from bacteria

Permeabilization of the *E. coli* plasma membrane was evaluated by measuring β -galactosidase activity with a Beta-Glo® Assay System (Promega)^{44–45}. In detail, 250 μ L aliquots of AR *E. coli* in log phase growth ($A_{600} \sim 0.7$) were centrifuged to collect the cell pellets. These pellets were resuspended in 250 μ L fresh medium or each of the GO suspensions at 250 μ g/mL. The supernatants were collected after 15,000 rpm centrifugation for 5 min. The positive control was comprised of non-treated cell pellets that were mixed with 250 μ L cell lysis buffer for the release of their β -galactosidase content. 50 μ L aliquots of the supernatant or lysis solutions were added to the wells of 96-well plate, before the addition of 50 μ L β -galactocidase substrate. Following mixing and incubation at room temperature for 2 h, the luminescence intensity was recorded in a SpectraMax M5 microplate reader. The percentage of β -galactocidase release was calculated according to the

formula: $\frac{L_{GO}}{L_{ctrl} + L_{lysis}} \times 100\%$, where L_{GO} is the luminescence intensity in supernatants of GO treated samples, while L_{ctrl} and L_{lysis} represents the luminescence values in ctrl supernatant and ctrl cell pellet lysis, respectively.

Synthesis of hGO-2 thin films on a glass substrate and on a silicon catheter surface

A Laurell WS-650S spin coater was used to fabricate noncovalent hGO-2 coatings on 18 mm $\times$ 18 mm $\times$ 0.15 mm glass substrates. The substrates were placed on a rotor followed by spinning at 1000 rpm. 2 mg/mL GO suspensions were added onto the substrate surfaces using a pipette until the desired thickness was obtained. Films were dried at 50 °C overnight. These thermal treatments imparted good film-substrate adhesion as thin films were resistant to rubbing off. Image J was used to analyze the AFM images and determine the surface area coverage.⁴⁷

To covalently coat a silicone catheter surface with hGO, silicone tubes were cleaned twice with acetone, DI water and isopropanol in a sonication bath. After 1 min plasma cleaning at high radio frequency, silicone catheters were reacted with 10 μ L APTES in 20 mL isopropanol alcohol (IPA) for 3 h, washed twice with IPA, DI water and ethanol, and placed at 105 °C for 30 min. The aminated catheters were cooled down to room temperature and immersed in 5 mL 250 μ g/mL carbodiimide GO solutions, which were prepared by stirring 5 mL GO (250 μ g/mL), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 0.123 mg/mL) and N-Hydroxysuccinimide (NHS, 0.105 mg/mL) in aqueous solution for 3 h, followed by filtration and re-dispersion in 5 mL DI water. After 24 h reaction while stirring, GO modified catheters were washed twice with DI water and ethanol. The entire process was considered as a complete coating cycle. For example, to achieve a second coating, the modified catheter is once again reacted with APTES to create an aminated surface and subsequently submerged in the carbodiimide GO solution. In this study, the GO modified catheters were coated with hGO-2 for 1, 3, 5 and 10 cycles, and designated as 1C, 3C, 5C and 10C, respectively. Both the noncovalent and covalent coated GO films were further characterized by a Thermo Microscopes Autoprobe CP-Research AFM and a Renishaw inVia Reflex Raman spectroscopy.

Assessment of the bactericidal effects of hGO-2 films

Inhibition of bacterial growth on hGO-2 coated substrates was determined by a modified protocol⁴⁶. AR *E. coli* in log phase were collected by centrifugation at 15,000 *rpm* for 5 min, washed twice with PBS, and diluted in fresh LB medium with O.D. at 0.7. Bacteria were exposed to hGO-2 films by immersing the coated substrates (18 × 18 mm) or catheters (5 × 5 mm) in bacteria suspensions for 5 min. The substrates were air dried for 30 min to allow the attachment of bacteria to the surface. The bacteria settling on catheter surfaces were then placed in a Petri dish with fresh broth and incubated at 37 °C for 1, 3 or 6 h. The growing bacteria were collected to perform 10-fold serial dilutions in 1 ml PBS. Aliquots (0.1 mL) of each dilution were introduced to LB agar plates for 24 h incubation. Colonies were counted under a microscope, and the desired colony numbers (20–300) at appropriate dilutions were used to calculate CFU by following formula: $CFU (mL^{-1}) = N \times 10^n / 0.1$, where N is the colony number on agar plates at 10^n dilution. The bacteria grown on glass substrates were stained by propidium iodide (PI) and Hoechst 33342 for 30 min. Then the glass substrates were gently rinsed thrice with PBS and fixed with 70% ethanol. Bacterial cell death on the coated substrates was determined by visualizing the percentage of PI-positive cells under a Leica Confocal SP2 1P/FCS microscope. In addition, scanning electron microscopy (SEM, ZEISS SUPRA 40VP) was used to evaluate the surface morphology of *E. coli* grown on hGO-2 films. Prior to SEM analysis, the prepared samples were mounted on the SEM stubs and coated by PELCO SC-7 sputter for 30 sec at 30 mA to generate a thin Au/Pt conductive layer (2–20 nm).

Statistics

Mean and standard deviation (SD) were calculated using a minimum of 3 observations for each parameter, such as bacterial absorbance at 600 nm, intensity of •C on EPR spectra, % of PI-stained bacteria, *etc.* Comparisons between groups were evaluated by two-side Student's t test or one-way ANOVA. A statistically significant difference was assumed for $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Georgakilas V, Otyepka M, Bourlinos AB, Chandra V, Kim N, Kemp KC, Hobza P, Zboril R, Kim KS. Functionalization of Graphene: Covalent and Non- Covalent Approaches, Derivatives and Applications. *Chem Rev.* 2012; 112(11):6156–6214. [PubMed: 23009634]

2. Sun Z, James DK, Tour JM. Graphene Chemistry: Synthesis and Manipulation. *J Phys Chem Lett*. 2011; 2(19):2425–2432.
3. Chen D, Feng H, Li J. Graphene Oxide: Preparation, Functionalization, and Electrochemical Applications. *Chem Rev*. 2012; 112(11):6027–6053. [PubMed: 22889102]
4. Compton OC, Nguyen ST. Graphene Oxide, Highly Reduced Graphene Oxide, and Graphene: Versatile Building Blocks for Carbon-Based Materials. *Small*. 2010; 6(6):711–723. [PubMed: 20225186]
5. Zhao YL. Bottom-up Construction of Highly Ordered Mesoporous Graphene Frameworks. *Sci Bull*. 2015; 60(22):1962–1963.
6. Chung C, Kim Y-K, Shin D, Ryoo S-R, Hong BH, Min D-H. Biomedical Applications of Graphene and Graphene Oxide. *Accounts Chem Res*. 2013; 46(10):2211–2224.
7. Chen J, Peng H, Wang X, Shao F, Yuan Z, Han H. Graphene Oxide Exhibits Broad-Spectrum Antimicrobial Activity against Bacterial Phytopathogens and Fungal Conidia by Intertwining and Membrane Perturbation. *Nanoscale*. 2014; 6(3):1879–1889. [PubMed: 24362636]
8. Li Y, Yuan H, von dem Bussche A, Creighton M, Hurt RH, Kane AB, Gao H. Graphene Microsheets Enter Cells through Spontaneous Membrane Penetration at Edge Asperities and Corner Sites. *Proc Natl Acad Sci U S A*. 2013; 110(30):12295–12300. [PubMed: 23840061]
9. Tu YS, Lv M, Xiu P, Huynh T, Zhang M, Castelli M, Liu ZR, Huang Q, Fan CH, Fang HP, Zhou RH. Destructive Extraction of Phospholipids from Escherichia Coli Membranes by Graphene Nanosheets. *Nat Nanotechnol*. 2013; 8(8):594–601. [PubMed: 23832191]
10. Efremova LV, Vasilchenko AS, Rakov EG, Deryabin DG. Toxicity of Graphene Shells, Graphene Oxide, and Graphene Oxide Paper Evaluated with Escherichia Coli Biotests. *Biomed Res Int*. 2015; 2015:869361. [PubMed: 26221608]
11. Sun HJ, Gao N, Dong K, Ren JS, Qu XG. Graphene Quantum Dots-Band-Aids Used for Wound Disinfection. *ACS Nano*. 2014; 8(6):6202–6210. [PubMed: 24870970]
12. Zou X, Zhang L, Wang Z, Luo Y. Mechanisms of the Antimicrobial Activities of Graphene Materials. *J Am Chem Soc*. 2016; 138(7):2064–2077. [PubMed: 26824139]
13. Liu SB, Zeng TH, Hofmann M, Burcombe E, Wei J, Jiang RR, Kong J, Chen Y. Antibacterial Activity of Graphite, Graphite Oxide, Graphene Oxide, and Reduced Graphene Oxide: Membrane and Oxidative Stress. *ACS Nano*. 2011; 5(9):6971–6980. [PubMed: 21851105]
14. Yang L, Zhang R, Liu B, Wang J, Wang S, Han M-Y, Zhang Z. Pi-Conjugated Carbon Radicals at Graphene Oxide to Initiate Ultrastrong Chemiluminescence. *Angew Chem-Int Edit*. 2014; 53(38):10109–10113.
15. Akhavan O, Ghaderi E. Toxicity of Graphene and Graphene Oxide Nanowalls against Bacteria. *ACS Nano*. 2010; 4(10):5731–5736. [PubMed: 20925398]
16. Perreault F, de Faria AF, Nejati S, Elimelech M. Antimicrobial Properties of Graphene Oxide Nanosheets: Why Size Matters. *ACS Nano*. 2015; 9(7):7226–7236. [PubMed: 26091689]
17. Dubin S, Gilje S, Wang K, Tung VC, Cha K, Hall AS, Farrar J, Varshneya R, Yang Y, Kaner RB. A One-Step, Solvothermal Reduction Method for Producing Reduced Graphene Oxide Dispersions in Organic Solvents. *ACS Nano*. 2010; 4(7):3845–3852. [PubMed: 20586422]
18. Pavlidis IV, Patila M, Bornscheuer UT, Gournis D, Stamatis H. Graphene-Based Nanobiocatalytic Systems: Recent Advances and Future Prospects. *Trends Biotechnol*. 2014; 32(6):312–320. [PubMed: 24794165]
19. Khimiya. Chemical Properties of Ethylene Oxide. In: Zimakov, PV., Dyment, OH., editors. *Ethylene Oxide*. 1967. p. 57-85.
20. Bagri A, Mattevi C, Acik M, Chabal YJ, Chhowalla M, Shenoy VB. Structural Evolution During the Reduction of Chemically Derived Graphene Oxide. *Nat Chem*. 2010; 2(7):581–587. [PubMed: 20571578]
21. Shen Y, Zhang HB, Zhang H, Ren W, Dasari A, Tang GS, Yu ZZ. Structural Evolution of Functionalized Graphene Sheets During Solvothermal Reduction. *Carbon*. 2013; 56:132–138.
22. Bo Z, Shuai X, Mao S, Yang H, Qian J, Chen J, Yan J, Cen K. Green preparation of reduced graphene oxide for sensing and energy storage applications. *Sci Rep*. 2014; 4:4684. [PubMed: 24732631]

23. Zhang L, Ji L, Glans P-A, Zhang Y, Zhu J, Guo J. Electronic Structure and Chemical Bonding of a Graphene Oxide-Sulfur Nanocomposite for Use in Superior Performance Lithium-Sulfur Cells. *Phys Chem Chem Phys*. 2012; 14(39):13670–13675. [PubMed: 22968125]
24. Kim J, Cote LJ, Kim F, Yuan W, Shull KR, Huang J. Graphene Oxide Sheets at Interfaces. *J Am Chem Soc*. 2010; 132(23):8180–8186. [PubMed: 20527938]
25. Zhang H, Dunphy DR, Jiang X, Meng H, Sun B, Tarn D, Xue M, Wang X, Lin S, Ji Z, Li R, Garcia FL, Yang J, Kirk ML, Xia T, Zink JI, Nel A, Brinker CJ. Processing Pathway Dependence of Amorphous Silica Nanoparticle Toxicity: Colloidal Vs Pyrolytic. *J Am Chem Soc*. 2012; 134(38): 15790–15804. [PubMed: 22924492]
26. Yeum KJ, Aldini G, Chung HY, Krinsky NI, Russell RM. The Activities of Antioxidant Nutrients in Human Plasma Depend on the Localization of Attacking Radical Species. *J Nutr*. 2003; 133(8): 2688–2691. [PubMed: 12888659]
27. Wrona M, Patel KB, Wardman P. The Roles of Thiol-Derived Radicals in the Use of 2',7'-Dichlorodihydrofluorescein as a Probe for Oxidative Stress. *Free Radic Biol Med*. 2008; 44(1):56–62. [PubMed: 18045547]
28. Jacobsen SM, Stickler DJ, Mobley HLT, Shirtliff ME. Complicated Catheter-Associated Urinary Tract Infections Due to *Escherichia Coli* and *Proteus Mirabilis*. *Clin Microbiol Rev*. 2008; 21(1): 26–59. [PubMed: 18202436]
29. Antimicrobial Resistance: Global Report on Surveillance. http://apps.who.int/iris/bitstream/10665/112642/1/9789241564748_eng.pdf
30. Kojima S, Nikaido H. Permeation Rates of Penicillins Indicate That *Escherichia Coli* Porins Function Principally as Nonspecific Channels. *Proc Natl Acad Sci U S A*. 2013; 110(28):E2629–E2634. [PubMed: 23798411]
31. Dolejska M, Villa L, Poirel L, Nordmann P, Carattoli A. Complete Sequencing of an IncHI1 Plasmid Encoding the Carbapenemase Ndm-1, the Arma 16s Rna Methylase and a Resistancenodulationcell Division/Multidrug Efflux Pump. *J Antimicrob Chemother*. 2013; 68(1): 34–39. [PubMed: 22969080]
32. Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, Holmes NE, Rhee H-W, Hong J-I, Hartland EL, Stinear TP, Howden BP. Two Novel Point Mutations in Clinical *Staphylococcus Aureus* Reduce Linezolid Susceptibility and Switch on the Stringent Response to Promote Persistent Infection. *PLoS Pathog*. 2010; 6(6):e1000944. [PubMed: 20548948]
33. Katayama Y, Ito T, Hiramatsu K. A New Class of Genetic Element, *Staphylococcus Cassette* Chromosome *Mec*, Encodes Methicillin Resistance in *Staphylococcus Aureus*. *Antimicrob Agents Chemother*. 2000; 44(6):1549–1555. [PubMed: 10817707]
34. Queenan AM, Shang W, Flamm R, Bush K. Hydrolysis and Inhibition Profiles of Beta-Lactamases from Molecular Classes a to D with Doripenem, Imipenem, and Meropenem. *Antimicrob Agents Chemother*. 2010; 54(1):565–569. [PubMed: 19884379]
35. Wright GD. Bacterial Resistance to Antibiotics: Enzymatic Degradation and Modification. *Adv Drug Deliv Rev*. 2005; 57(10):1451–1470. [PubMed: 15950313]
36. Hu WB, Peng C, Luo WJ, Lv M, Li XM, Li D, Huang Q, Fan CH. Graphene-Based Antibacterial Paper. *ACS Nano*. 2010; 4(7):4317–4323. [PubMed: 20593851]
37. Yu L, Zhang YT, Zhang B, Liu JD, Zhang HQ, Song CH. Preparation and Characterization of Hpei-Go/Pes Ultrafiltration Membrane with Antifouling and Antibacterial Properties. *J Membr Sci*. 2013; 447:452–462.
38. Seabra AB, Paula AJ, de Lima R, Alves OL, Duran N. Nanotoxicity of Graphene and Graphene Oxide. *Chem Res Toxicol*. 2014; 27(2):159–168. [PubMed: 24422439]
39. Ji Z. Use of Compositional and Combinatorial Nanomaterial Libraries for Biological Studies. *Sci Bull*. 2016; 61(10):755–771.
40. Duch MC, Budinger GRS, Liang YT, Soberanes S, Urich D, Chiarella SE, Campochiaro LA, Gonzalez A, Chandel NS, Hersam MC, Mutlu GM. Minimizing Oxidation and Stable Nanoscale Dispersion Improves the Biocompatibility of Graphene in the Lung. *Nano Lett*. 2011; 11(12): 5201–5207. [PubMed: 22023654]

41. Chowdhury I, Mansukhani ND, Guiney LM, Hersam MC, Bouchard D. Aggregation and Stability of Reduced Graphene Oxide: Complex Roles of Divalent Cations, Ph, and Natural Organic Matter. *Environ Sci Technol*. 2015; 49(18):10886–10893. [PubMed: 26280799]
42. Green AA, Hersam MC. Solution Phase Production of Graphene with Controlled Thickness *Via* Density Differentiation. *Nano Lett*. 2009; 9(12):4031–4036. [PubMed: 19780528]
43. Kaweeteerawat C, Chang CH, Roy KR, Liu R, Li R, Toso D, Fischer H, Ivask A, Ji Z, Zink JJ, Zhou ZH, Chanfreau GF, Telesca D, Cohen Y, Holden PA, Nel AE, Godwin HA. Cu Nanoparticles Have Different Impacts in *Escherichia Coli* and *Lactobacillus Brevis* Than Their Microsized and Ionic Analogues. *ACS Nano*. 2015; 9(7):7215–7225. [PubMed: 26168153]
44. Liu H, Du YM, Wang XH, Sun LP. Chitosan Kills Bacteria through Cell Membrane Damage. *Int J Food Microbiol*. 2004; 95(2):147–155. [PubMed: 15282127]
45. Jan S, Baron F, Alabdeh M, Chaari W, Grosset N, Cochet M-F, Gautier M, Vie V, Nau F. Biochemical and Micrographic Evidence of *Escherichia Coli* Membrane Damage During Incubation in Egg White under Bactericidal Conditions. *J Food Prot*. 2013; 76(9):1523–1529. [PubMed: 23992496]
46. Kumar A, Vemula PK, Ajayan PM, John G. Silver-Nanoparticle-Embedded Antimicrobial Paints Based on Vegetable Oil. *Nat Mater*. 2008; 7(3):236–241. [PubMed: 18204453]
47. Li R, Ji Z, Qin H, Kang X, Sun B, Wang M, Chang CH, Wang X, Zhang H, Zou H, Nel AE, Xia T. Interference in Autophagosome Fusion by Rare Earth Nanoparticles Disrupts Autophagic Flux and Regulation of an Interleukin-1 β Producing Inflammasome. *ACS Nano*. 2014; 8(10):10280–10292. [PubMed: 25251502]

A

Atomic % concentration of oxidized groups on GO surface by XPS

	rGO-2	rGO-1	GO	hGO-1	hGO-2
C=O	4.9	7.3	9.97	12.6	16.3
C-O-C	3.5	12.9	20.5	11.5	2.1
C-OH	2.6	3.8	4.5	9.9	13.6
Total oxygen	11.5	24	35	34	32

B

C

Figure 1. Characterization of GO library materials by EPR, XPS and AFM

A) XPS spectra of oxidized groups on the GO surface. XPS was performed by the stepwise (50 meV) acquisition of high resolution spectra of the C 1s region. B) Detection of •C on GO surface by EPR. EPR was used to assess the •C density on GO surface by testing 5 mg of each of the dried GO samples by an X-band Bruker ELEXYS 580 spectrometer with g value of 2.0029. C) Visualizing the morphology of GO samples by AFM. AFM images were obtained by placing a drop of the GO solution (10 μg/mL) on Si wafers that were pretreated with a 2.5 mM APTES aqueous solution. After washing with water and drying with N₂, AFM images were obtained in a Thermo Microscopes Autoprobe CP-Research AFM in tapping mode with conical probes.

Figure 2. Bactericidal effects of the GO library

A) Comparison of bacterial killing by GO and antibiotics in sensitive (wild type) and AR bacteria. The resistance of *bacteria* was demonstrated by comparing the killing effect of antibiotics (ampicillin or erythromycin) in sensitive and AR *E. coli* (upper left panel) and *L. crispatus* (lower left panel) after incubation at 37 °C for 24 h; the bactericidal effects of GO were compared with ampicillin in AR *E. coli* (upper right panel) and erythromycin in AR *L. crispatus* (lower right panel). Cell growth was evaluated by measuring bacterial OD at 600 nm. * $p < 0.05$ compared to ampicillin or erythromycin. B) Bacterial killing effects of GO samples in resistant *E. coli*. To determine the bacterial killing by GO samples, AR *E. coli* were exposed to 8–500 μg/mL rGO-2, rGO-1, GO, hGO-1 and hGO-2 at 37 °C for 24 h. * $p < 0.05$ compared to GO. C) Calculation of the correlation coefficient between •C density and

bacteria killing. Pearson's analysis was used to evaluate the correlation between the rate of cell growth and the absorbance peak area of •C on the GO surface.

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Figure 3. TEM images showing the interaction between GO samples and AR *E. coli*
Cells were treated with 125 $\mu\text{g/mL}$ of each of the GO samples for 24 h, then washed, embedded in resin, and negatively stained before TEM imaging. Arrows show the rGO-2, GO or hGO-2 nanosheets interacting with bacteria membrane.

A

B

Figure 4. Mechanism of GO bacterial killing involves membrane association, lipid peroxidation and membrane damage

A) Determination of the pro-oxidative potential of GO samples by an abiotic DCF assay; To assess the oxidation potential of GO samples, 95 μL aliquots of 25 $\mu\text{g/mL}$ DCF were added into each well of a 96 well black bottom plate and mixed with 5 μL of GO suspensions at 5 mg/mL , followed by 2 h incubation. DCF uorescence emission spectra were recorded using a SpectraMax M5 microplate reader with an excitation wavelength of 490 nm. B) Confocal imaging of GO-induced lipid peroxidation in AR *E. coli*. To assess lipid peroxidation, AR *E. coli*, prior exposed to 250 $\mu\text{g/mL}$ GO samples or 10 μM cumene hydroperoxide, was stained by using the Image-iT® Lipid Peroxidation Sensor kit and Hoechst 33342 dye before performance of confocal microscopy. The microscopy was carried out using a Texas Red filter to visualize the reduced dye and a FITC filter to visualize the oxidized dye. C) Quantification of cells with lipid peroxidation by flow cytometry. Flow cytometry was carried out on a FACS Vantage SE flow cytometer to determine the percentage cells undergoing membrane lipid peroxidation. D) β -galactocidase release in GO-treated bacteria. The permeability of cytoplasmic membrane was evaluated using a luminescent β -

galactosidase substrate to measure the activity of released β -galactosidase from *E. coli* treated by 250 $\mu\text{g/mL}$ GO suspensions. * $p < 0.05$ compared to GO treatment. E) Schematic image to explain the bactericidal effect of GO including membrane association and lipid peroxidation.

Figure 5. Inhibition of AR bacteria growth by non-covalently coated hGO-2 films on a glass substrate

A) AFM imaging of hGO-2 coated substrates. A series of substrates (S1, S2, S3 and S4) with different GO coverage and thickness were characterized by AFM. B) SEM imaging of changes in the bacterial morphology after incubation with the hGO-2 coated substrate; C) Visualization and D) quantification of bacterial death on hGO-2 films by confocal microscopy. Following 6 h incubation of AR *E. coli* with substrates S1 to S4, the cells were stained with PI, fixed and washed with 70% ethanol to determine the percentages of dead cells on substrates. The morphological changes of bacteria were visualized by SEM. * $p < 0.05$ compared to control.

Figure 6. Inhibition of AR bacterial growth by hGO-2 covalently attached to the surface of a silicone catheter

A) Schematic to describe hGO-2 coating of catheters as well as product images. A complete coating cycle involves the amination of catheter surface by APTES and conjugation of the hGO-2 to the amine groups. A series of hGO-2 coated catheters were prepared through multicycle coating. The catheter surfaces grow darker with incremental rounds of coating.

B) *In situ* visualization of *E. coli* bacteria embedded on coated catheters. Catheters were immersed in the bacterial suspensions to allow bacterial attachment to the catheter surface. After 6 h incubation, the catheters were treated with 2% glutaraldehyde, followed by 70% ethanol washing to remove residual salts and Au/Pt coating. The morphology of *E. coli* on catheters was observed by SEM. Arrows and the dash-line areas show the damaged bacteria.

C) β -galactosidase release from bacteria grown on coated catheters. The β -galactosidase release from embedded bacteria on catheter surfaces was determined after 2 h incubation.

D) Assessing the growth of bacteria retrieved from the coated catheter surfaces by CFU assay. After settling of bacteria on catheter surfaces, they were incubated for 1, 3 and 6 h. Then the retrieved bacteria from catheter surfaces were serially diluted and spread on the agar plates

for 24 h incubation. The images show the growing colonies from uncoated or hGO-2 coated surfaces at each time point at same dilutions (left panel). CFU were calculated by desired colony numbers (20~300) at appropriate dilutions (right panel).

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Scheme 1. Scheme to show the synthesis of reduced and hydrated GOs

GO was synthesized by a modified Hummers method. rGO-1 and rGO-2 were synthesized by solvothermal reduction of GO in NMP at 150 °C for 1 or 5 h, respectively. To prepare hGO-1 and hGO-2, GO was hydrated in aqueous alkalized solution at 50 °C or 100 °C for 24 h. Reaction of the epoxy groups with nucleophiles leads to the opening of these rings and the generation of hydroxyl groups (as well as $\bullet\text{C}$ radicals shown in Fig. 1B).

Table 1

Hydrodynamic size and zeta potential of GO samples in aqueous solution

Nanoparticles	GO	rGO-1	rGO-2	hGO-1	hGO-2
Primary Size (nm)	148	152	148	157	138
Hydrodynamic Size (nm)	Water	334.1 ± 3.1	378.1 ± 3.9	1349.2 ± 8.5	307.5 ± 6.7
	LB	655.4 ± 95.4	615.9 ± 54.9	474.9 ± 14.9	412.1 ± 4.5
	Lacto	443.6 ± 4.6	654.9 ± 44.3	509.6 ± 6.7	367.2 ± 8.4
Zeta Potential (mV)	Water	-51.7 ± 0.9	-36.3 ± 1.4	-24.4 ± 0.7	-50.6 ± 0.8
	LB	-18.53 ± 0.6	-8.7 ± 2.7	-8.2 ± 2.2	-16.7 ± 0.8
	Lacto	-7.5 ± 0.5	-5.5 ± 1.2	-6.5 ± 3.6	-7.9 ± 3.9
					-6.4 ± 2.2