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Mechanisms of the Antimicrobial Activities of Graphene Materials

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ABSTRACT: A thorough understanding of the antimicrobial mechanisms of graphene materials (GMs) is critical to the manipulation of highly efficient antimicrobial nanomaterials for future biomedical applications. Here we review the most recent studies of GM-mediated antimicrobial properties. This review covers the physicochemical properties of GMs, experimental surroundings, and selected microorganisms as well as the interaction between GMs and selected microorganisms to explore controversial antimicrobial activities. Finally, we rationally analyze the strengths and weaknesses of the proposed mechanisms and provide new insights into the remaining challenges and perspectives for future studies.

1. INTRODUCTION

Recent advances in nanomaterials have enabled a considerable number of applications of graphene in the cutting-edge fields of biological and medical sciences.[1,2] In addition to a greater understanding of nanomaterial-triggered biosensing and therapy, the direct interaction between graphene materials (GMs) (defined as graphene, graphene oxide (GO), and reduced graphene oxide (rGO) in this review) and several cell types (i.e., bacteria, carcinoma cells, and normal mammalian cells) has been progressively recognized by biologists and chemists.[3,4] In particular, substantial efforts have been devoted to exploring the broader therapeutic applications of GMs to microbial infections on the basis of their extraordinary characteristics in electronics, mechanics, thermotics, and optics as well as their unique structures.[5,6]

With the increasing number of investigations of the antimicrobial activities of GMs in recent years, several predominant mechanisms (e.g., nanoknives, oxidative stress, and wrapping or trapping) have been proposed.[7−9] Despite substantial experimental results suggesting that the physicochemical properties of GMs, such as morphology, size, and surface functionality, might affect their antimicrobial activities, the underlying antimicrobial mechanisms remain controversial because of inconsistent experimental designs.[10−14] For instance, identical mechanisms have been used to explain opposite experimental outcomes in different studies and several mechanisms have been proposed to explain a single phenomenon.

Inspired by these fascinating and meaningful findings, this Perspective focuses on the mechanisms underlying the antimicrobial activities of GMs. To the best of our knowledge, this Perspective is the first to systematically review the proposed mechanisms according to the physicochemical properties of GMs and the interaction between GMs and selected microorganisms. Herein we aim to clarify the reasons for the disagreement among studies. Additionally, we comprehensively analyze the strengths and weaknesses of the proposed mechanisms and provide perspectives for future strategies and designs to construct robust graphene-based antimicrobial agents.

2. ANTIMICROBIAL ACTIVITIES DERIVED FROM THE PHYSICOCHEMICAL AND STRUCTURAL CHARACTERISTICS OF GMS

Compared with zero-dimensional fullerenes and one-dimensional carbon nanotubes (CNTs), two-dimensional graphene presents extraordinary physicochemical properties leeched by the synthesis method.[15−18] Among these synthesis methods, the chemical vapor deposition (CVD) and epitaxial growth approaches can create graphene with a flawless crystal structure, whereas other methods (e.g., mechanical cleavage, chemical synthesis, and chemical exfoliation) generally produce graphene with some extent of defects.[19−21] The introduction of various defects, such as oxygen-containing groups and destruction of the basal plane, provides more active sites for enhanced interaction between GMs and other molecules, ions, or other materials.[22−25] In addition, these defects alter the intrinsic properties of GMs, such as lateral size, layer number, morphology, and dispersibility.[26−29] An increasing number of reports have revealed the close relationship between antimicrobial activities and the physicochemical or structural properties of GMs through both experiments and theory.[5−7,9,30,31] A comparison of antimicrobial investigations is summarized in Table 1, and the detailed findings are presented below.

2.1. Lateral Size. The lateral size of GMs is essential in determining their antimicrobial activity because the size can strongly influence the adsorption ability, dispersibility, and the numbers of corners and sharp edges, which are critical to the physicochemical interactions between GMs and microorganisms.[32] Although size-dependent antimicrobial activities of GMs have been reported, inconsistent results were observed among these studies. It is well-known that graphene sheets as well as graphene derivatives with larger lateral sizes can possess stronger adsorption abilities due to their higher surface energies. Indeed, an investigation reported that GO sheets with larger sizes (higher adsorption abilities) demonstrated stronger antimicrobial activity toward *Escherichia coli* than did smaller sheets (Figure 1a−c).[13] However, defects are always introduced when GMs are synthesized via the redox method. In general, more defects are produced with a decrease in the lateral size of GMs. Perreault et al.[10] found that the increased number of defects in smaller GO nanosheets can enhance the

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Table 1. Basic Toxicities of GMs

property	bacteria/ mammalian cell	type of GM	toxicity	ref
size	*E. coli*	GO dispersions (average sizes of GO sheets are 0.753, 0.127, 0.065, 0.035, 0.013, and 0.010 μm^2)	GO sheets with larger sizes presented stronger antimicrobial activities	13
	E. coli	GO-coated membrane (average sizes of GO are 0.65, 0.29, 0.10, and 0.01 μm^2)	the survival rate of *E. coli* in the GO-coated filters significantly decreased when the area of the GO sheet decreased from 0.65 to 0.01 μm^2	10
	human stem cells	rGONP dispersions (average lateral dimensions of rGONPs are 11 ± 0.44 nm and 3.8 ± 0.4 μm)	significant cell destruction at 1.0 μg/mL for 11 ± 0.44 nm rGONPs; significant cytotoxic effect only at a high concentration of 100 μg/mL for 3.8 ± 0.4 μm rGONPs	4
number of layers	*E. coli*	GO LB films deposited on PET (one, two, or three layers of GO)	GO LB films on PET present layer-dependent antimicrobial activities	14
shape	*E. coli*, *S. aureus*	GONWs and RGNWs with sharp edges	both GONWs and RGNWs with sharp edges had strong antimicrobial activities, more resistance of *E. coli* against the direct contact interaction with the nanowalls compared with *S. aureus*	11
	P. aeruginosa, *S. aureus*	graphene films with different edge lengths and different angles of orientation	graphene films with smooth sides were effective in killing both *P. aeruginosa* and *S. aureus*, whereas those with rough sides efficiently inactivated only *P. aeruginosa*	7
surface modification	*E. coli*, *S. aureus*	GONWs and RGNWs (89%, 84%, and 69% peak area ratio of the oxygen-containing groups such as C−OH, C−O, and O═C−OH were reduced in the RGNWs relative to the GONWs)	GONWs reduced with hydrazine exhibited more antibacterial activity than unreduced GONWs	11
	E. coli	GO films and bacterially reduced GO films (69% of the oxygen-containing groups were removed in the bacterially reduced GO films relative to the GO films)	GO films were biocompatible for microorganisms, but bacterially reduced GO films showed an inhibition for proliferation of the bacteria	40
	E. coli, *B. subtilis*	GO dispersions with or without the modification of BSA and Trp	GO sheets adsorbed BSA and Trp with low antimicrobial activities	30
agglomeration/ dispersion	*E. coli*	Gt, GtO, GO, and rGO dispersions	the GO dispersion showed the highest antibacterial activity, sequentially followed by rGO, Gt, and GtO	3
	E. coli	GO and rGO dispersions	rGO exhibited stronger trapping ability and inactivation activity against *E. coli* than GO	8

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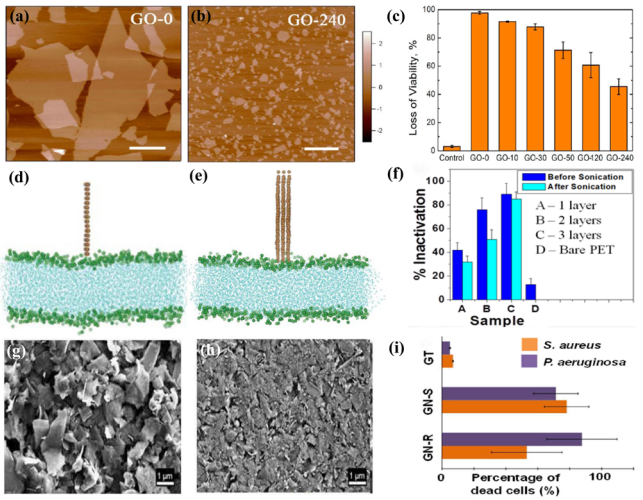

Figure 1. (a, b) AFM images of (a) GO-0 and (b) GO-240.[13] (c) Viability of *E. coli* cells after incubation with GO suspensions for 2 h with a shaking speed of 250 rpm at 37 °C.[13] (d, e) Snapshots of (d) monolayer and (e) three-layer graphene nanosheets entering a lipid bilayer membrane.[33] (f) Comparison of the antibacterial effect of varying the number of layers of GO LB films.[14] (g, h) Scanning electron microscopy (SEM) images of (g) graphene nanorough (GN-R) and (h) graphene nanosmooth (GN-S) surfaces.[7] (i) Quantity of nonviable cells present on the GN-R and GN-S surfaces.[7] (a–c) Reproduced from ref 13. Copyright 2012 American Chemical Society. (d, e) Reproduced with permission from ref 33. Copyright 2013 Royal Society of Chemistry. (f) Reproduced with permission from ref 14. Copyright 2015 Royal Society of Chemistry. (g–i) Reproduced from ref 7. Copyright 2015 American Chemical Society.

antimicrobial activity of GO, as evidenced by the observation that the survival rate of *E. coli* in GO-coated filters significantly decreased when the area of the GO sheets decreased from 0.65 to 0.01 μm^2. A similar result was observed in a study in which the cytotoxicity of rGO nanoplatelets (rGONPs) toward human stem cells was notably greater in smaller rGONPs (11 ± 0.44 nm) compared with larger-sized ones (3.8 ± 0.4 μm).[4] Oxidative stress and cellular membrane damage are considered to be main factors that lead to the cytotoxicity of rGONPs. In fact, rGONPs also exhibit genotoxicy toward stem cells through DNA fragmentation and chromosomal aberrations. That is, rGONPs may pass through the cellular membrane. A theoretical simulation using coarse-grained molecular dynamics (MD) validated this viewpoint that smaller graphene nanosheets penetrated phospholipid bilayers more easily, whereas larger nanosheets mainly lay flat on the surface of the membrane.[12]

2.2. Number of Layers. Similar to CNTs, whose antimicrobial activities can be altered by fabricating them as either single- or multiwalled, the antimicrobial activities of GMs can also be modulated by controlling the number of graphene layers. Increasing the number of layers can increase the thickness of GMs, weakening the "nanoknife" effect, and decrease the dispersibility of GMs, resulting in agglomeration, which will reduce the occurrence of contact between GMs and microorganisms. The theoretical results of Wang et al.[33] showed that the energy barrier for three-layer graphene sheets with corner sites to pierce through the lipid bilayer is greater than that for monolayer sheets of the same lateral size; this finding suggested that few-layer graphene sheets might possess a strong ability to cause membrane damage (termed high antimicrobial activity) (Figure 1d,e). In contrast, in an experiment conducted by Mangadlao and co-workers, increasing the number of layers in GO Langmuir–Blodgett (LB) films resulted in stronger antimicrobial activity against *E. coli* (Figure 1f).[14] This observation has been explained by the antimicrobial activity of the basal plane induced by surface properties that are affected by the number of layers. In other words, both the surface and edges of GMs have key functions in antimicrobial activities. Regardless, additional efforts should be made to prove this conclusion.

2.3. Shape. The shapes of GMs were found to have a considerable influence on their antimicrobial activities, consistent with previous studies showing that CNTs and Ag nanoparticles can present shape-dependent cytotoxicity.[34,35] A theoretical simulation found that the shapes of nanoparticles are crucial to the interaction between nanoparticles and the lipid bilayer in translocation processes, which are believed to be directly related to antimicrobial activities.[36] It was also found that GO nanowalls (GONWs) and rGO nanowalls (RGNWs) with sharp edges obviously decrease the survival rates of both *E. coli* and *Staphylococcus aureus*.[11] To verify the effect of shape, graphene films with various shapes on the top and bottom sides were employed to kill *Pseudomonas aeruginosa* and *S. aureus*.[7] Interestingly, a graphene film with a smooth top side (GN-S) was found to be effective in killing both rod-shaped *P. aeruginosa* and round-shaped *S. aureus*, whereas a rough bottom side (GN-R) could efficiently inactivate only rod-shaped *P. aeruginosa* (Figure 1g–i). A theoretical simulation also revealed that graphene sheets with sharp corners and edge protrusions, which may have a low energy barrier, can easily permeate into the cell membrane.[37] Thus, it can be concluded that the sharp edges of these GMs are important to their marked antimicrobial activities, though an explicit mechanism has not been presented.

2.4. Surface Modification. Because pristine graphene is prone to agglomeration, thus potentially decreasing the opportunities to contact other biomolecules such as DNA and protein, studies have explored changing the surface or edge properties of graphene to prevent agglomeration through covalent and non-covalent modifications, which are believed to play a vital role in determining the antimicrobial activities of GMs.[38,39] In a comparison of GO- and rGO-induced cytotoxicities, researchers found that rGO possessed stronger antimicrobial activity against *E. coli* and *S. aureus* than did GO.[11] Another study found that rGO inhibited the proliferation of *E. coli*, whereas GO was biocompatible with the bacteria.[40] Since the difference between GO and rGO mainly originates from the surface modifications, these studies revealed that the GM-mediated antimicrobial activities may be subject to the impact of covalent modifications with oxygen-containing groups. The introduction of oxygen-containing groups can alter the properties of GM surfaces and edges, affecting the amphipathy and blade effect of GMs and leading to changes in their antimicrobial activities. In addition, GMs can act as strong adsorbents because of their high surface energies. Non-covalent adsorption of bovine serum albumin (BSA) or tryptophan (Trp) onto the GO surface also substantially weakens the antimicrobial activity[30] because the adsorbed substances can prevent GO from interacting with microorganisms. In addition, GMs modified with a metal (Ag), metal oxides (ZnO, TiO_2), certain molecules (chitosan), or a polymer (poly(*N*-vinylcarbazole) (PVK)) via electrostatic adsorption, hydrogen bonding, π–π stacking, and coupling

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have presented various antimicrobial activities, further validating the theory that surface modification can significantly influence the antimicrobial activity of GMs.[41−48] Thus, it may be deduced that GMs can affect the survival of microorganisms by means of the adsorption interactions of GMs for different molecules, ions, and other materials. Therefore, it is possible that altering the microenvironment of these microorganisms can inhibit their proliferation.

2.5. Agglomeration and Dispersion. Both GMs (especially graphene and rGO) and CNTs tend to agglomerate because of their high surface energies, which can inevitably alter their surface and edge properties to modulate their antimicrobial activities. With regard to CNTs, agglomeration is one primary factor in determining their antimicrobial activity because it can alter the shape and reduce the surface area of the nanomaterials.[49] Agglomerated GMs can show weakened intrinsic dispersibility and adsorption ability, changing the blade effect of GMs and reducing the incidence of interactions with microorganisms. In a comparison of the antimicrobial activities of several GMs, GO dispersion delivered the strongest antimicrobial activity against *E. coli*, sequentially followed by rGO, graphite (Gt), and graphite oxide (GtO).[3] This result was explained by the discrepant dispersion statuses of these nanomaterials because GO can be well-dispersed to produce thin sheets that wrap bacteria easily, whereas rGO tends to form large aggregates if it is not fully exfoliated, causing the antibacterial activity to be reduced. Interestingly, different results were observed by Akhavan's group, who claimed that rGO possesses stronger trapping ability and inactivation activity against *E. coli* than GO due to the gradual wrapping of bacteria during the formation of rGO aggregates in the suspension.[8]

Consequently, the antimicrobial activities of GMs are strongly related to the lateral size, number of layers, shape, surface modification, and agglomeration, though sufficient proof regarding the main factor is lacking. More importantly, it is well-known that redox methods (e.g., Hummers' method) always simultaneously affect the lateral size, number of layers, and surface and edge properties of GMs. Unfortunately, some studies were unable to strictly control other factors when investigating one of these factors. For example, when the effect of lateral size is being elucidated, other factors such as the degree of oxidation or number of layers should be strictly controlled to exclude their effects. Thus, more strict and systematic efforts to investigate the antimicrobial activities of GMs should be conducted. Accordingly, it is important to establish a more reliable and comprehensive system to evaluate the antimicrobial activities of GMs.

3. ANTIMICROBIAL ACTIVITIES AFFECTED BY EXPERIMENTAL SURROUNDINGS AND SELECTED MICROORGANISMS

Aside from the intrinsic properties of GMs, the experimental surroundings (i.e., liquid or solid state, aerobic or anoxic conditions, and in vitro or in vivo environment) and selected microorganism genera (i.e., Gram-positive or Gram-negative, round- or rod-shaped) should also be considered in assessing the antimicrobial activities of GMs. Determining the survival of microorganisms depends on their ability to grow under specific physicochemical conditions, and understanding these conditions enables us to manipulate the growth of microorganisms in real situations in a controlled manner. Studies have also revealed that the antimicrobial activities of GMs are largely subject to the influence of experimental surroundings and selected microorganisms (Table 1).

3.1. Experimental Surroundings. In an experiment exploring the influence of the surroundings on the antimicrobial activities of GMs, Ruiz and co-workers found that introducing GO into Luria−Bertani nutrient broth could efficiently expedite the growth of *E. coli*.[50] Conversely, Hu et al.[5] observed that both GO/rGO papers and suspensions could inactivate the growth of *E. coli*. Motivated by these studies, Hui et al.[30] investigated the influence of those supplements on the antimicrobial activity of GO. Interestingly, they found that the antimicrobial activity of GO was remarkably inhibited when Luria−Bertani, BSA, and Trp were added to the saline. Meanwhile, other studies have shown that GMs that exist in various experimental surroundings (dispersions, films, or other states) might have diverse antimicrobial activities or cytotoxicities.[3,31,51,52] Moreover, other parameters of the surroundings, such as pH, light, electricity, magnetic field, and sonication conditions, are also responsible for the antimicrobial activity of GMs.[9,10,13,31,53−55] In particular, it is well-known that synthesis of GO by Hummers' method always introduces some impurities such as manganese and sulfur due to careless washing.[3,10] These impurities can disturb the microenvironment of microorganisms, for example by changing the pH. Moreover, GO containing carboxyl and hydroxyl groups, which easily dissociate, can slightly increase the acidity of the microenvironment. Changes in pH can also affect the dispersibility of GMs. In addition, GMs can strongly adsorb molecules, ions, and other materials because of their high surface energies. Therefore, the introduction of GMs can have a huge impact on the survival environment of microorganisms, which may affect their proliferation.

3.2. Selected Microorganisms. An antimicrobial can be classified as either broad-spectrum or narrow-spectrum depending on how many types of microorganisms are naturally susceptible to its action. The actions that inhibit the synthesis of various bacterial components (e.g., cell wall, cytoplasmic membrane, and chromosome) are largely dependent on the shape or structure of the selected microorganisms. For most pathogenic microorganisms, their basal structures, such as cell wall/membrane, cytoplasm, and nuclear body, are alike, whereas their components and morphologies are unique. For instance, Gram-negative bacteria (e.g., *E. coli* and *P. aeruginosa*) have a thin layer of peptidoglycan (2−3 nm) between the inner and outer cell membranes, whereas Gram-positive bacteria (e.g., *S. aureus* and *Staphylococcus epidermidis*) have a thicker peptidoglycan layer (20−80 nm) in their single-unit cell wall, resulting in discrepant sensitivity to various antimicrobials.[5,7,9,30,31,44] Moreover, a bacterium can also be classified as coccus (spherical), bacillus (rodlike), spiral, or filamentous depending on its specific cell shape. Since the morphologies of microorganisms may also affect their sensitivity to certain antimicrobials, particular strategies must be considered to achieve optimized killing or inactivation effects. Therefore, the conflicting antimicrobial results reported for GMs in current studies may originate from the difference in selected microorganisms. In addition to *E. coli* and *S. aureus*, other bacteria, including *P. aeruginosa*,[7,51] *Bacillus subtilis*,[6,44,56] *Salmonella typhimurium*,[6] *Enterococcus faecalis*,[6] *Cupriavidus metallidurans* CH4,[57] *Listonella anguillarum*,[58] *Bacillus cereus*,[58] and *S. epidermidis*,[48] have been selected as models to investigate the antimicrobial properties of GMs. In particular, Pham et al.[7] found that the rough side of graphene films has a higher

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antimicrobial ability against *P. aeruginosa* (causing death of 87.6% of the bacteria) than against *S. aureus* (killing only 43.1% of the bacteria). Moreover, minimal inhibitory concentrations of 1, 1, 8, and 4 μg/mL graphene sheets have been observed against *E. coli*, *S. typhimurium*, *B. subtilis*, and *E. faecalis*, respectively.[6] These results demonstrate that selected microorganisms are essential in assessing the antimicrobial activity of GMs.

4. ANTIMICROBIAL PROPERTIES RELYING ON THE INTERACTION OF GMS WITH BACTERIAL COMPONENTS

Our understanding of antimicrobial-induced bacterial cell death is still centered on the essential cellular function being inhibited by the primary antimicrobial−bacteria interaction. Antimicrobials can be categorized on the basis of the cellular components or systems they affect in addition to whether they induce cell death (bactericidal) or merely inhibit cell growth (bacteriostatic). The killing or inactivation of microorganisms is largely ascribed to the physicochemical interactions induced by the disruption of various components, such as lipids, proteins, and DNA/RNA. The phospholipid bilayer and peptidoglycan protein, which are the main components of the cell wall and cell membrane, are accountable for the rigidity of the bacterial cell wall and for determining the cell shape. Any event that interferes with the assembly of the peptidoglycan precursor and the transport of that object across the cell membrane will compromise the integrity of the wall and lead to bacterial death. In addition, damage to DNA/RNA will inhibit the duplication of microorganisms. GMs can interact with lipids, proteins, and DNA/RNA via hydrogen bonding, $\pi-\pi$ stacking, and electrostatic adsorption.[1,9,31,59] These interactions may induce nanoknives, lipid extraction, protein disruption, and reactive oxygen species (ROS) mechanisms, leading to the death or inactivation of microorganisms. Hence, the interactions between GMs and these components of microorganisms must be understood.

4.1. DNA/RNA. Bacterial genomes typically consist of a single continuous stretch of DNA or small extrachromosomal DNAs (plasmid) that may contain genes for antibiotic resistance or virulence factors. Once bacterial DNA is corrupt or an error occurs during DNA replication, bacteria tend to mutate or die. DNA/RNA may interact with GMs through $\pi-\pi$ stacking, hydrogen bonding, and electrostatic adsorption due to the existence of a π-conjugated structure and oxygen- and nitrogen-containing groups. Strong interactions between GMs and bacterial DNA have been proven by the application of DNA/RNA-coated GMs in molecule recognition,[1,59] anticancer drug delivery,[60,61] and DNA translocation and sequencing.[62,63] Thus, it can be inferred that the permeation of GMs into microorganisms may cause the above-mentioned physical interactions, which will alter the structures and properties of DNA/RNA and affect the activity of microorganisms.[1,64] Unfortunately, this inference has not been adequately proven. Current investigations tend to support those chemical interactions that induce redox reactions and destroy DNA/RNA, which are most favorable in leading to the death or inactivation of microorganisms.[4,51]

4.2. Proteins. Various proteins that are required components of microbial cells are decentralized in the cell wall, cell membrane, and cell plasma. These proteins, such as enzyme and carrier proteins that regulate the metabolism of microorganisms, are prone to disturbances in the environment. Proteins consisting of amino acids with nitrogen-containing groups may induce hydrogen-bonding interactions with other substances. Moreover, the presence of π-conjugated structures in some amino acids can lead to interactions with other substances having π-conjugated structures through $\pi-\pi$ stacking. Amino acids can also present various electronegativities under certain conditions. These strong interactions have frequently been reported for GMs because of their large π-conjugated structures and abundant oxygen-containing groups.

By grafting of specific proteins onto the surface of GMs via non-covalent interactions, various functionalized complexes have been fabricated for applications such as pH detection, drug delivery, and glucose sensing.[2,9,65,66] However, these protein−graphene interactions may damage the raw structure, morphology, and nature of the proteins. To verify this probability, Alava and co-workers designed a graphene-modified Si/SiO_2 device to investigate the interactions between graphene and protein (concanavalin A) and found that the protein was immediately denatured when it was directly absorbed on graphene.[67] However, the protein itself may also affect the properties of GMs and alter their antimicrobial activity. Thus, Chong et al.[68] studied interactions between various GMs and four proteins and found that both GO and rGO were non-cytotoxic once they were coated by proteins through hydrophobic or $\pi-\pi$-stacking interactions.

4.3. Phospholipids. A phospholipid bilayer, as the basal plane of a cellular membrane, serves as a barrier that keeps ions, proteins, and other molecules in place and protects the entire cell from damage. In antimicrobial investigations, destruction of the phospholipid bilayer was thought to be an important factor in the death of microorganisms. GMs containing large π-conjugated structures and presenting strong hydrophobicity can interact with the phospholipid molecules of cellular membranes through hydrophobic interactions by making contact with microorganisms. The experimental and theoretical results of Tu et al.[31] proved that GO can induce the destructive extraction of phospholipids from *E. coli* and reduce its viability by means of a strong dispersion interaction between GO and the *E. coli* cell membrane. Additionally, as these interactions could rely on the size of GMs, Dallavalle et al.[12] implemented MD simulations and demonstrated that hydrophobic interactions can enable small graphene nanosheets to penetrate the phospholipid layer but force larger nanosheets to lie flat on the surface of cellular membranes, disturbing the interactions between phospholipid molecules. More importantly, Li and co-workers found that micrometer-scale graphene sheets with sharp corners and edge protrusions can also permeate into the membranes easily by overcoming a decreased energy barrier originating from the strong hydrophobic interactions.[37]

5. MECHANISMS OF GRAPHENE-MEDIATED ANTIMICROBIAL ACTIVITIES

Determining the mechanisms of GM-mediated antimicrobial properties is critical to the development of GM-enabled biomedical technologies and the management of GM-enhanced infectious disease control. Despite substantial efforts dedicated to the determination of antimicrobial activities and mechanisms, a universal mechanism remains to be found because of controversial experimental results. Therefore, a systematic elaboration of GM-mediated antimicrobial activities is urgently needed.

A thorough understanding of the antimicrobial mechanisms is still in its infancy because the antimicrobial properties of GMs have only been studied since 2010.[11] However, the

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antimicrobial activities of GMs are known to stem from the physicochemical interaction between GMs and microbes. According to recent achievements, three mainstream mechanisms regarding the antimicrobial activities of GMs have been proposed, namely, nanoknives derived from the action of sharp edges, oxidative stress mediated with or without the production of ROS, and wrapping or trapping of bacterial membranes derived from the flexible thin-film structure of GMs. In the following sections, we will attempt to clarify each hypothesis comprehensively and offer some clues about the modulation of the antimicrobial activities of GMs by controlling their physicochemical properties.

5.1. Nanoknives Derived from the Action of Sharp Edges. Several cumulative studies have documented that the action of sharp edges, also called nanoknives, cutters, or blades in some references, is one of the most crucial factors affecting the antimicrobial properties of GMs (Figure 2a-A). Supporters

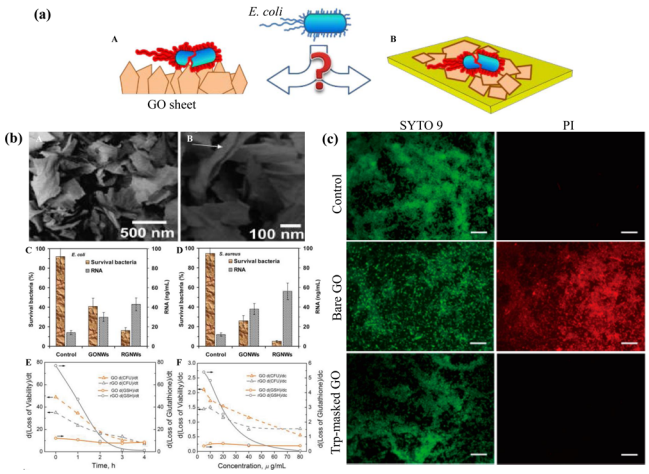

Figure 2. (a) Action of sharp edges of graphene/GO (A) and observed antibacterial activity of GO-LB films (B).[14] (b) SEM images of GONWs deposited on a stainless steel substrate (A, B);[11] cytotoxicities of GONWs and RGNWs toward *E. coli* or *S. aureus* and concentrations of RNA in PBS of *E. coli* or *S. aureus* exposed to the nanowalls (C, D);[11] time-dependent *E. coli* cell inactivation and glutathione (GSH) oxidation rates after incubation with GO and rGO dispersions (E); dependence of *E. coli* cell inactivation and GSH oxidation on the GO and rGO concentrations (F).[3] (c) Representative fluorescence microscopy images of *E. coli* treated with bare GO and Trp-masked GO (Trp:GO = 12:1) and subsequently stained briefly (15 min) with SYTO 9 (green) and PI (red); controls were assayed similarly but without the addition of GO or Trp-saturated GO. SYTO 9 is a cell-permeant green-fluorescent stain that labels both live and dead bacteria, whereas propidium iodide (PI) is a cell-impermeant red-fluorescent stain that only labels cells with compromised cellular membranes. Scale bars = 20 μm.[30] (a) Reproduced with permission from ref 14. Copyright 2015 Royal Society of Chemistry. (b-A–D) Reproduced from ref 11. Copyright 2010 American Chemical Society. (b-E,F) Reproduced from ref 3. Copyright 2011 American Chemical Society. (c) Reproduced from ref 30. Copyright 2014 American Chemical Society.

of this mechanism claim that bladelike GMs would pierce through the microbial cellular membrane, causing physical disruption of the cellular membrane and leakage of intracellular substances, which eventually lead to cell death. This mechanism, occasionally called "insertion mode" or "penetration mode", can lead to membrane stress according to results of both experimental studies and theoretical simulations.

Early in 2010, Akhavan and Ghaderi[11] conducted a preliminary study of the antibacterial activities of GONWs and RGNWs (Figure 2b-A,B) and discovered that direct contact between bacteria (*E. coli* or *S. aureus*) and extremely sharp edges of graphene nanosheets could result in loss of bacterial membrane integrity and leakage of RNA (Figure 2b-C,D). Similarly, other researchers observed the efflux of intracellular substances (e.g., RNA or DNA),[44,70−73] demonstrating that direct contact with sharp edges can lead to mechanical disruption of the cellular membrane. By systematically investigating the antibacterial activities of GO, rGO, Gt, and GtO toward *E. coli*, Liu et al.[3] found that sharp edges can induce significant membrane stress on bacteria by acting as cutters, which is similar to the observation of single-walled CNT-induced toxicity,[74] indicating a shared mechanism for these two carbon nanomaterials. Moreover, their results revealed that rGO did not present apparent antibacterial activity if direct interaction was absent (Figure 2b-E,F), indicating that membrane stress induced by sharp edges synergically contributed to the antibacterial activities through oxidative stress.[3]

The proposed mechanism of "insertion mode" has been reported in several computational simulations. It has been demonstrated by MD simulations that graphene sheets can spontaneously pierce lipid bilayer membranes at the corners or asperities (Figure 3a,b).[37,33] Depending on the degree of

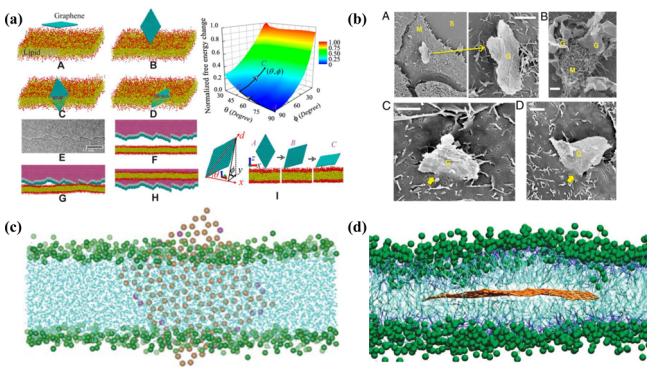

Figure 3. (a) Coarse-grained molecular dynamics simulations of interactions between a lipid bilayer and a small graphene flake (A–D) or a large five-layer graphene sheet with staggered stacking and roughened edge topography (E–H); also shown is the normalized free energy of the system as a function of the graphene orientation when one of the sharpest corners is fixed at a distance of 0.5 nm above the bilayer (I).[37] (b) Cell membrane interactions with graphene microsheets, showing edge or corner penetration for each of three cell types.[37] (c) Snapshot configurations of an edge-oxidized graphene nanosheet entering a bilayer at 16.8 ns.[33] (d) Equilibrated superstructure of a graphene sheet hosted inside the phospholipid bilayer membrane (sandwiched graphene−membrane superstructures).[69] (a, b) Reproduced with permission from ref 37. Copyright 2013 National Academy of Sciences. (c) Reproduced with permission from ref 33. Copyright 2013 Royal Society of Chemistry. (d) Reproduced from ref 69. Copyright 2009 American Chemical Society.

oxidation and the lateral size, graphene sheets can either cut across the membrane as a transmembrane object or form sandwiched graphene−membrane superstructures (Figure 3c,d).[69,33,75] Further investigations have shown that graphene with a higher degree of oxidation at the edges forms a transmembrane nanostructure because the repulsion between hydrophilic edge atoms and hydrophobic lipid tails can be

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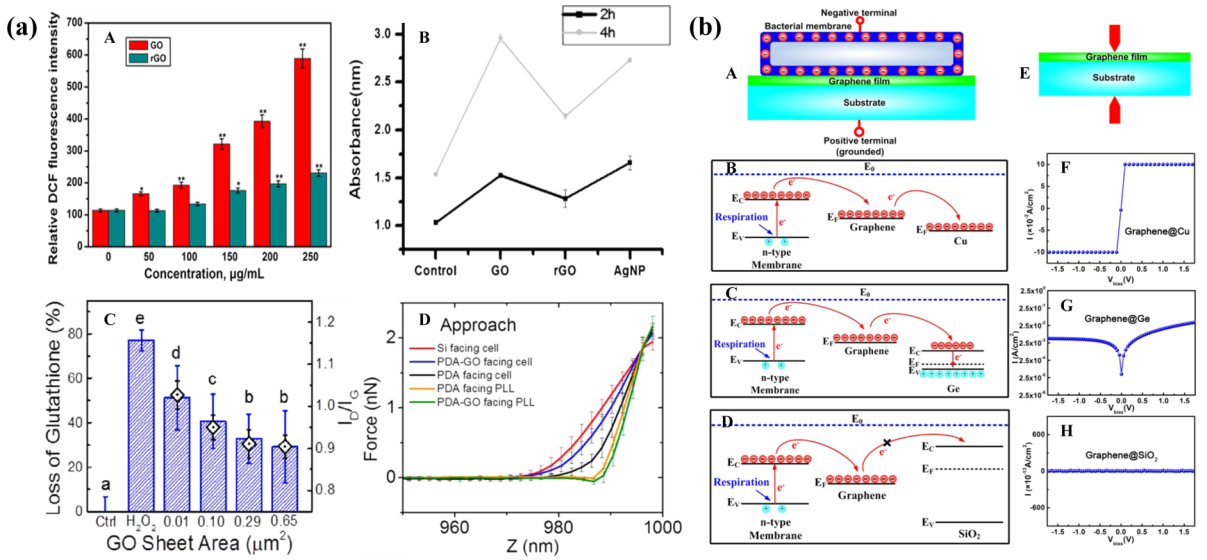

Figure 4. (a) ROS generation after incubation of bacteria suspensions in DI water with GO and rGO dispersions at various concentrations for 2 h (A);[71] production of $O_2^{\bullet-}$ by GO and rGO dispersions (B);[88] in vitro GSH oxidation by GO sheets of different areas, with black diamond symbols representing the ratio of the intensities of the D and G bands of GO as measured by Raman spectroscopy (C);[10] plots of the force as a function of piezo position (Z) during cantilever approach to *E. coli* cells (D).[84] (b) Schematic circuitry to illustrate the proposed mechanism for the observed phenomena of different responses of bacteria to graphene films from the view of energy band diagrams for these graphene-on-substrate junctions (A–D); schematic illustration of the electrical measurements (E) to obtain the current–voltage (I–V) characteristics of three different contacts of graphene films (F–H).[9] (a-A) Reproduced with permission from ref 71. Copyright 2013 Springer Science + Business Media Dordrecht. (a-B) Reproduced with permission from ref 88 Copyright 2012 The Korean Society of Industrial and Engineering Chemistry. Published by Elsevier B.V. (a-C) Reproduced from ref 10. Copyright 2015 American Chemical Society. (a-D) Reproduced from ref 84. Copyright 2015 American Chemical Society. (b) Reproduced with permission from ref 9. Copyright 2014 Nature Publishing Group.

significantly reduced when the oxidized basal plane deviates away from the center of the bilayer membrane.[33,76] Microsized graphene prefers to adopt a near-perpendicular transmembrane configuration when penetrating the cell membrane through a driving force, which mainly originates from splay and membrane tension energies.[77] In contrast, Dallavalle et al.[12] found that smaller graphene sheets penetrated the phospholipid membrane more freely and navigated the double layer with a preferentially perpendicular orientation. Pham et al.[7] recently fabricated graphene nanofilms with different edge densities and angles of orientation. Graphene surfaces with a 37° orientation exhibited effective antibacterial activity toward *P. aeruginosa* and *S. aureus* even though an orientation of 90° was previously proven to have the maximum killing efficiency. Thus, they suggested that the density of the edges is another parameter in determining antibacterial behavior. Combining this result with theoretical evidence, they further suggested that the surface of graphene nanosheets does not act as a simple blade but rather induces the formation of pores within the cell membrane, causing a subsequent osmotic imbalance and cell death.

In contrast, other researchers have proposed that the availability of the basal planes rather than the sheet edges determines the antimicrobial properties of GMs. To prove this hypothesis, Mangadlao et al.[14] utilized an established LB technique to immobilize entire GO sheets whose edges were embedded in a poly(ethylene terephthalate) (PET) substrate so that the edge effect could be eliminated. Surprisingly, GO was still found to be able to inactivate *E. coli* without the edge effect (Figure 2a-B), and a positive correlation between the number of basal planes (by increasing the number of LB layers) and the antimicrobial activity was observed, indicating that the mechanical action of the edges is not required. In addition, non-covalent adsorption of BSA or Trp on GO basal planes can decrease the antibacterial activity of GO against *E. coli* and *B. subtilis* (Figure 2c).[30] On the basis of this information, it can be deduced that GO basal planes are important action sites and that the antibacterial activities of GMs can be weakened by masking them via non-covalent adsorption.

Some theoretical simulations have indicated that in addition to near-perpendicular membrane penetration there is another mode of interaction between GMs and cell membranes: parallel attachment to the cell membrane. Yi and Gao[77] found that as the lateral size of graphene decreased and became comparable to the cellular membrane thickness, the near-perpendicular configuration could be replaced by a more stable configuration with the entire graphene sheet positioned parallel along the midplane of the lipid bilayer. In contrast, Dallavalle et al.[12] proposed that larger sheets may generally lie flat on the top of the bilayer, where they disrupt the membrane and create a patch of upturned phospholipids. These preliminary observations indicate that direct contact with sheet edges and penetration into the membrane are not indispensable for the antimicrobial activities of GMs.

5.2. Oxidative Stress Mediated with or without the Production of ROS. GM-induced oxidative stress has been regarded as the most widely acceptable mechanism, comparable to that of CNTs, which have similar physicochemical properties.[78−80] Oxidative stress can interfere with bacterial metabolism and disrupt essential cellular functions, eventually leading to cellular inactivation or even cell death. In general, oxidative stress mainly occurs via either a ROS-dependent or a ROS-independent pathway. The former is induced by excess accumulation of intracellular ROS, such as hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), or singlet molecular oxygen ($^1O_2$). It has been well-documented that ROS induces intracellular protein inactivation, lipid

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peroxidation, dysfunction of the mitochondria, and gradual disintegration of the cell membrane, followed by necrosis/apoptosis and eventual cell death.[81] The latter involves disruption or oxidation of a vital cellular structure or component without ROS production, which may be induced by charge transfer from the cellular membrane to graphene, where graphene acts as an electron pump.[9]

5.2.1. ROS-Dependent Oxidative Stress. GMs are capable of mediating the generation of ROS by the adsorption of O_2 on the defect sites and edges of the GMs, followed by its subsequent reduction by various cellular reducing enzymes (e.g., glutathione (GSH)).[10,82,83] GSH is an important antioxidant compound that can be oxidized to glutathione disulfide (GSSG) in the presence of ROS. Thus, GSH could serve as an intracellular redox state indicator, and its depletion implies the toxicity effect of oxidative stress against bacteria.[10,44,51,84] Similar to GSH, other antioxidants such as *N*-acetylcysteine (NAC)[51] and α-tocopherol[10] or oxidant-sensitive dyes such as dichlorodihydrofluorescein diacetate (DCFH-DA)[71,85−87] can also reflect the generation of ROS. By monitoring the ROS levels using DCFH-DA, Chen et al.[71] found that ROS levels increased in a graphene-concentration-dependent manner after exposure of *Xanthomonas oryzae* pv Oryzae to graphene (Figure 4a-A). They further observed that DCFH-DA could not be oxidized directly by GO/rGO alone, indicating that the oxidative stress is induced by the mediation of ROS production. Additionally, the antibacterial activities of graphene were proven to be closely related to mitochondrial membrane depolarization, a key event associated with the accumulation of intracellular ROS, in a study showing that pristine graphene can induce a dose-dependent mitochondrial membrane potential decrease of murine RAW 264.7 macrophages.[87]

Furthermore, Kim's group found that the ROS levels in GO- and rGO-treated *P. aeruginosa* cells were 3.8- and 2.7-fold higher, respectively, than that in control cells.[51] As expected, preincubation of the cells with GSH or NAC as an antioxidant significantly reduced the levels of ROS generated by GO/rGO, confirming that cell death is mediated by ROS production. They further showed that the production of $O_2^{\bullet-}$ by GO and rGO dispersions was 2- and 1-fold higher, respectively, than that in control cells, directly proving the toxicity of ROS against *E. coli* (Figure 4a-B).[88] These results suggest that oxidative stress is a major contributor to the antimicrobial action of GMs. The oxygen-containing functional groups and defect density on the surface of GMs clearly contribute to the generation of ROS. Musico et al.[44] found that a PVK−GO-modified membrane filter exhibited greater GSH loss than one modified with PVK−G, indicating that high ROS production would lead to more oxidative stress to *E. coli* and *B. subtilis* cells. They noted that larger numbers of oxygen-containing functional groups (e.g., −COOH and −OH) in the surface of GO can facilitate the production of ROS and increase its antibacterial properties. Meanwhile, the capacity of GO sheets to oxidize GSH increased from 49% to 71% as the sheet area decreased from 0.65 to 0.01 μm^2, which could be explained by higher defect densities in smaller GO sheets (Figure 4a-C).[10] Moreover, higher oxidative stress is typically found in cells exposed to GO than in those exposed to rGO, likely because of the high defect density of GO in addition to its excellent dispersibility.[51,71,88]

Lipid peroxidation, a series of radical chain reactions initiated by the ROS-mediated oxidation of lipid molecules, is considered an important oxidative pathway because of the close interaction between GMs and cell membranes.[81,89] Lipid peroxidation can further form lipid peroxide radicals that will propagate the oxidative damage through the cell membranes. Krishnamoorthy et al.[6] adopted a lipid peroxidation assay induced by ultrasonic irradiation to determine the free-radical-modulated activities of graphene nanosheets. Using this method, they determined three reaction products (conjugated dienes, lipid hydroperoxides, and malondialdehydes) that were produced at different stages of lipid peroxidation mediated by free radicals (e.g., $OH^{\bullet}$). Moreover, Perreault et al.[10] used the lipid-soluble antioxidant α-tocopherol (which provides the most important antioxidant protection in lipid peroxidation by scavenging free radicals) to confirm the role of oxidative stress in GO-induced bacterial inactivation. They observed that preincubation of *E. coli* cells with α-tocopherol reduced the antimicrobial properties of all GO sheets, particularly larger ones. These findings partially prove the involvement of free radicals in the reaction process, hence validating the involvement of ROS in antibacterial properties.

Understanding the interactions between GMs and bacterial cellular membranes is crucial for the evaluation of their antibacterial activities. Castrillon et al.[84] studied the interaction between GO and *E. coli* cellular membranes using atomic force microscopy (AFM) to understand GO-induced bacterial inactivation. By devising a polydopamine-assisted functionalized AFM probe to measure the cellular membrane−GO interaction force, they observed that cell−GO interactions are predominantly repulsive (Figure 4a-D), suggesting that physical interactions such as membrane penetration are not indispensable. Furthermore, they demonstrated the role of oxidative pathways in GO-mediated antibacterial activity by providing evidence of the oxidation of GSH by GO.

5.2.2. ROS-Independent Oxidative Stress. ROS-mediated oxidative stress is not accepted by all researchers despite being regarded as a favorable antimicrobial mechanism. Alvarez's group previously proposed that a fullerene water suspension (nC_{60}) behaves as an oxidant and exerts ROS-independent oxidative stress against bacteria, considering that the colorimetric methods for assessing ROS production and ROS-mediated damage were prone to interference from nC_{60}.[90,91] Similarly, to better explore different pathways of oxidative stress, Liu et al.[3] employed the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) method to measure the production of $O_2^{\bullet-}$ and observed low $O_2^{\bullet-}$ levels in GO and rGO dispersions. Next, they utilized Ellman's assay to evaluate the oxidation of GSH and found that various GMs have diverse oxidation capacities toward GSH, which indirectly validated the theory that GMs are capable of mediating $O_2^{\bullet-}$-independent oxidative stress. Consequently, it was speculated that the stronger oxidative capacity for GSH possessed by rGO originates from the significantly higher conductivity of rGO compared with GO and that rGO can act as a conductive bridge over the insulating lipid bilayer to mediate electron transfer from bacterial intracellular components to the external environment.[3,71] Meanwhile, the strong oxidation of GSH by rGO suggests that conductive graphene nanosheets are capable of oxidizing thiols or other cellular components.[3] Taken together, these results indicate that ROS-independent oxidative stress can also give rise to antibacterial activities of GMs.

More importantly, Li et al.[9] released a controversial report suggesting that the antibacterial activity stems not from ROS but through electron transfer. They used both *S. aureus* and *E. coli* to investigate the antibacterial actions of graphene film on

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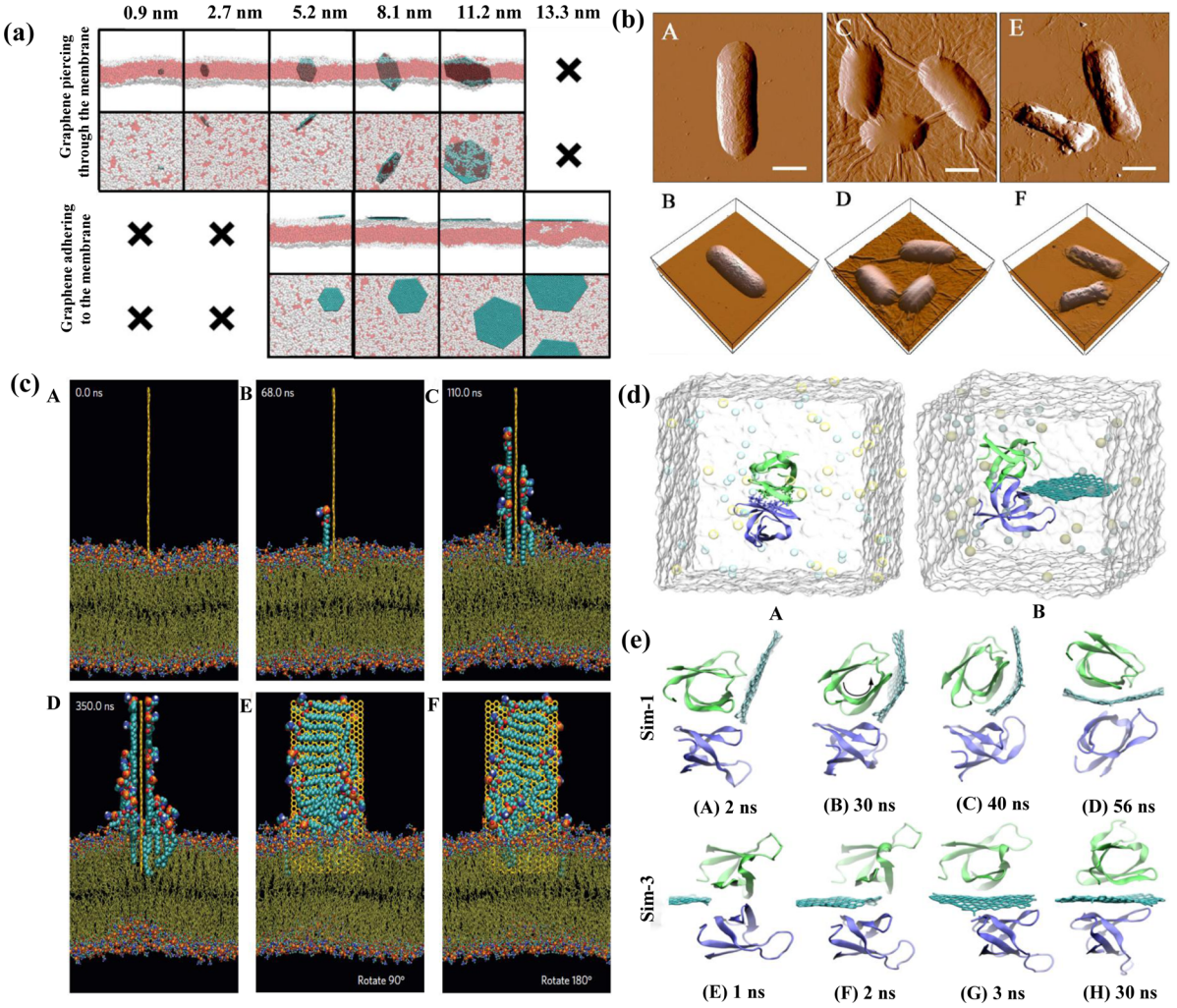

Figure 5. (a) Illustrative snapshots, at the end of the simulations, of six graphene nanosheets of increasing size (0.9, 2.7, 5.2, 8.1, 11.2, and 13.3 nm from left to right). The top two rows are different perspectives of the six sheets, as are the bottom two rows. Only the five smallest sheets pierce through the membrane. The four largest sheets adhere to the membrane. Situations not observed in the simulations are indicated by "×".[12] (b) AFM amplitudes and 3D images of *E. coli* cells after incubation with GO sheets: *E. coli* incubation with deionized water for 2 h (A, B); *E. coli* incubation with a 40 μg/mL GO-0 suspension for 2 h (C, D); *E. coli* after incubation with a 40 μg/mL GO-240 suspension for 2 h (E, F).[13] (c) Lipid extraction by graphene in docking simulations. Shown are snapshots from a representative trajectory of a fully restrained graphene nanosheet docked at the surface of the outer membrane.[31] (d) Illustration of the simulated HIV-1 integrase dimer systems without (A) and with (B) the presence of a graphene nanosheet.[93] In (A), hydrophobic residues at the dimer interface are highlighted and drawn as connected sticks. In (B), a graphene nanosheet is placed near the protein dimer.[93] (e) Dynamics of the insertion of a graphene sheet into the dimer.[93] (a) Reproduced from ref 12. Copyright 2015 American Chemical Society. (b) Reproduced from ref 13. Copyright 2012 American Chemical Society. (c) Reproduced with permission from ref 31. Copyright 2013 Nature Publishing Group. (d, e) Reproduced from ref 93. Copyright 2014 American Chemical Society.

the conductor Cu (Graphene@Cu), the semiconductor Ge (Graphene@Ge), and the insulator SiO_2 (Graphene@SiO_2). Interestingly, both the Graphene@Cu and Graphene@Ge films inhibited the growth of both bacteria, whereas the Graphene@SiO_2 film could not. This discrepancy was rationally explained by electron transfer theory. According to the schematic circuitry and the corresponding energy band diagrams, by the formation of a circuit electrons can easily be transferred from the microbial membrane to the graphene film and then to the underlying conductor Cu (or semiconductor Ge) substrate, whereas they cannot be transferred to the underlying insulator SiO_2 substrate (Figure 4b). On the basis of the above analysis, they proposed a plausible mechanism from the viewpoint of charge transfer to explain the distinctive responses of bacteria to various graphene films, in which graphene acts as an electron acceptor that pumps electrons away from the bacterial membrane. This supported the theory that the generation of ROS-independent oxidative stress and the surface of graphene, rather than ROS or edges, may be primarily responsible for the antimicrobial activities.

5.3. Wrapping or Trapping of Bacterial Membranes Derived from the Flexible Thin-Film Structure of Graphene. Wrapping or trapping of bacterial membranes has been proposed as the third mechanism that contributes to the antimicrobial activities of GMs, after nanoknives and oxidative stress. This is mainly because graphene is the world's thinnest film and thus can provide a unique flexible barrier to isolate bacteria from their surrounding ambience as a result of its unique two-dimensional lateral structure, which consists of a single layer of sp^2-bonded carbon atoms arranged in a hexagonal crystal structure.

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Microorganisms, like all living organisms, require sufficient nutrients and specific physicochemical conditions for survival. Bacterial growth inactivation or bacterial death would occur if these conditions were hampered. Recently, Rodrigues' group reported that both GO and a well-dispersed PVK−GO nanocomposite could deliver strong antimicrobial activity toward both Gram-positive (*B. subtilis* and *Rhodococcus opacus*) and Gram-negative (*E. coli* and *Cupriavidus metallidurans*) bacteria by wrapping around the bacteria to inhibit bacterial proliferation.[43] Unlike other findings revealing that CNTs and GONWs/RGNWs would penetrate the cell membrane and induce cell disruption,[11] they observed that both the original bacterial shape and integrity remained. Instead, reduced microbial metabolic activity and cell viability were observed by measuring the turnover of nicotinamide adenine dinucleotide hydrogen and/or nicotinamide adenine dinucleotide phosphate using a cellular metabolic assay, which verified the function of wrapping in the antibacterial properties.[43]

With slight differences, several studies observed that wrapping or trapping of bacteria could also induce bacterial cellular membrane perturbation to some extent. For example, Chen et al.[70] demonstrated that GO sheets intertwined with the pathogens once they made contact. Their scanning electron microscopy results showed evidence of membrane integrity perturbation on pathogens (*Pseudomonas syringae* and *Xanthomonas campestris* pv undulosa), though no clear injury was indicated. They further showed through membrane potential experiments that membrane depolarization causes serious structural damage to wrapped bacterial cells. Similarly, Kanchanapally et al.[92] designed a three-dimensional porous GO membrane (pore size ∼ 300 nm) and found that this membrane was able to kill *S. aureus* by inducing membrane damage via mechanical wrapping or trapping. The wrapping/trapping hypothesis has been upheld by observing the size-dependent antimicrobial activity through both theoretical simulations and experimental studies. Using simulations, Dallavalle et al.[12] investigated models of the interaction between bacterial membranes and graphene nanosheets ranging from 0.9 to 13.3 nm. Their findings showed that graphene nanosheets larger than 5.2 nm could attach to bacterial membranes and partially wrap the bacterial surface as a result of the strong hydrophobic interaction between the graphene and lipid layers. Consequently, the bacterial membrane would be undermined with a piece of phospholipids upturned. However, graphene nanosheets smaller than 5.2 nm would penetrate the cellular membrane (Figure 5a). Moreover, by evaluating the antimicrobial activities of well-dispersed GO nanosheets with different lateral sizes varying from nanometers to micrometers, Liu et al.[13] demonstrated that larger GO sheets can wrap cells easily and block the active sites on membranes, thereby inhibiting bacterial proliferation. In contrast, smaller GO nanosheets showed weaker antimicrobial activity due to incomplete bacterial surface wrapping because the uncovered membrane surfaces could still take up nutrients from the environment for survival (Figure 5b). This was also supported by Perreault et al.,[10] who showed that the antimicrobial effect was enhanced with increasing GO sheet area in a suspension assay. In addition, Akhavan et al.[8] recently suggested that a graphene/GO suspension presented only slight bacterial inactivation effects, whereas secondarily added melatonin (a biocompatible reductant) significantly improved the antimicrobial activity because more bacteria were wrapped during the process of graphene sheet aggregation in a dynamically reductive process. These authors also emphasized that the pH of the solution is an important factor that can impact the aggregation state of GMs and is therefore likely related to the different wrapping effects of GMs.

In contrast, several researchers believe that graphene or GO itself does not show antimicrobial activity simply by wrapping or trapping of bacteria.[3,8] For example, Tan's group observed strong antibacterial activity toward *Xanthomonas perforans* from DNA-directed silver nanoparticles (AgNPs) grown on the GO surface. The cell viability assay indicated that the GO nanosheet alone showed no direct antimicrobial activity, although it can attach to and wrap bacteria. Instead, it was the AgNPs on the GO surface that caused direct and irreversible damage to the cell membrane by denaturing cellular membrane proteins and then entering the bacterial cells. Here the GO nanosheet simply played a role in enhancing the antibacterial activity of AgNPs by reducing the degree of aggregation of AgNPs and exposing more active sites.[94]

In summary, more studies need to be conducted to determine the wrapping mechanism of GM toxicity toward bacteria, as the GM toxicity depends on the pH, concentration, size, exposure time, and cell type.

5.4. Other Mechanisms. *5.4.1. Extraction of Lipid Bilayers.* The interactions between graphene and bacterial cells are far more complex than initially expected. Recent experimental and theoretical studies have demonstrated that graphene's unique two-dimensional structure with all sp^2 carbons contributes to a strong interaction between graphene and membrane lipids, exceeding the attraction among lipid molecules within the membrane. Consequently, large amounts of phospholipids can be vigorously extracted from the lipid bilayers onto the graphene surfaces, eventually leading to the loss of cellular membrane integrity and then bacterial death (Figure 5c).[31]

The strong hydrophobic interaction between the lipid molecules and graphene, called "nanoscale dewetting", was previously found by other researchers to provide a significant driving force for the membrane collapse and system stability.[95−97] Inactivation of bacteria via the extraction of lipid bilayers was a mechanism completely different from those that already existed, such as penetration or wrapping of the cellular membrane. As the mechanism of lipid extraction is still in its infancy, more detailed analyses and rational designs are needed to explain the diverse phenomena that exist in different experimental settings.

5.4.2. Interference of Protein−Protein Interactions (PPIs). Diverse biological processes in a microorganism cell, such as signal transduction and cellular metabolism, are conducted through PPIs. Abnormal PPI activity typically leads to metabolic or biological dysfunction and would bring about carcinogenesis and degenerative diseases. Therefore, forced separation of two functional proteins would destroy normal PPI activity, disturb the cellular metabolism, and trigger programmed cell death.

To explore graphene-mediated antimicrobial activity at the molecular level, large-scale all-atom MD simulations were utilized to investigate the potential toxicity of graphene toward microorganisms by observing the disruption of PPIs. In a study conducted by Luan et al.,[93] the C-terminal DNA-binding domain of human immunovirus-1 (HIV-1) integrase, which can form a dimer via hydrophobic interactions among six interfacial residues of each monomer, was taken as a hydrophobic PPI model (Figure 5d,e). They found that graphene could easily

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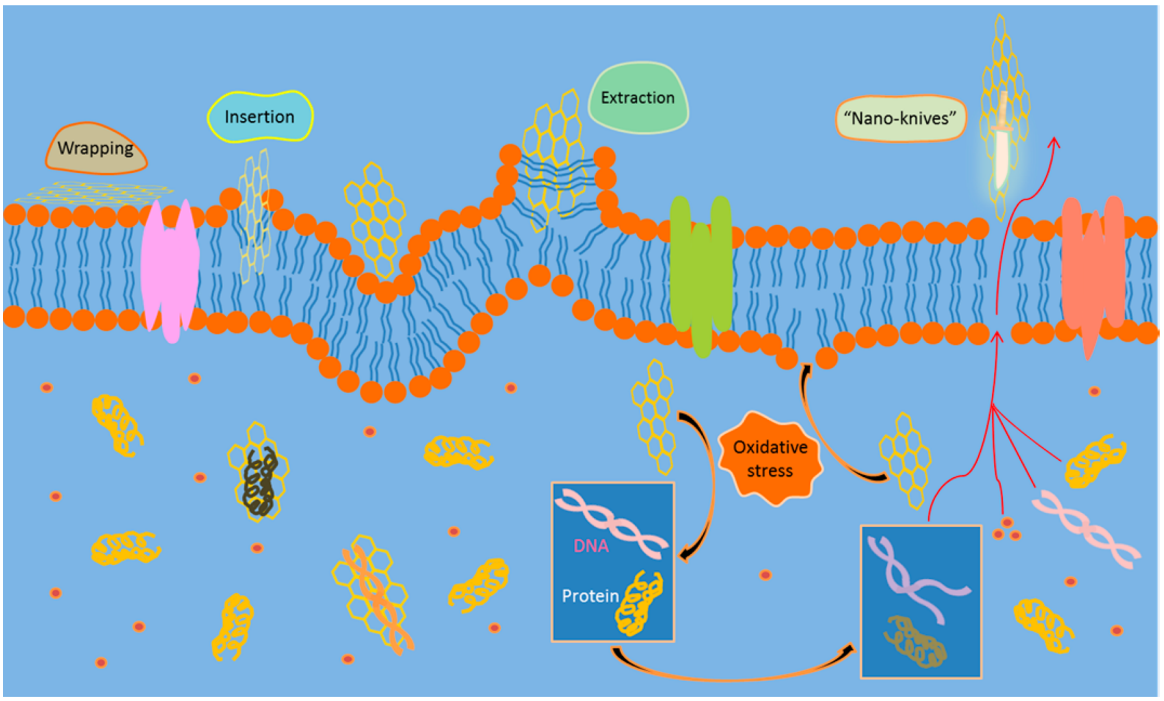

Figure 6. Mechanisms of the antimicrobial activities of GMs.

insert into the PPI dimer for two reasons: (i) the unfavorable interaction between graphene and water leads to graphene's close proximity to the dimer and (ii) the interaction between the hydrophobic residues and the graphene sheet was more energetically favorable than the interfacial interactions between the hydrophobic residues in the dimer. This agreed well with previous studies showing that the binding between CNTs and a protein or DNA (once CNTs entered into a bacterial cell) would trigger bacterial biological dysfunctions and result in cytotoxic effects.[85] It is important to explore the interactions between nanomaterials and intracellular components of microorganisms to facilitate a better understanding of the detailed mechanisms regarding antimicrobial activity at a fundamental level.

5.4.3. "Self-Killing" Effect. Focusing on the mutual interaction between GMs and bacteria during antimicrobial processes is particularly meaningful for thoroughly understanding this antibacterial activity. Akhavan and Ghaderi[40] found that viable bacteria could react with the nanomaterials and decrease oxygen-containing functional groups via a glycolysis process when the bacteria were incubated with GO. Compared with GO nanosheets, which could provide biocompatible sites for bacterial adsorption and proliferation on their surfaces, the bacterially reduced GO nanosheets showed increased inhibition of the bacterial proliferation. This finding was consistent with a recent study showing that rGO nanosheets presented significantly higher cytotoxicity than GO nanosheets.[5] Coincidentally, it was reported that *Shewanella*, a genus of marine bacteria, could reduce GO through bacterial respiration under both aerobic and anoxic conditions.[98−100] The same reduction capability was also found in *E. coli*, which can reduce CuO to Cu_2O during bacterial inactivation.[101]

According to the original references, this interesting phenomenon was figuratively termed the "self-killing" effect because it worked as if the bacteria passively killed themselves during the process of reducing the GO material when they interacted. The underlying mechanisms of why and how bacteria can reduce nanomaterials still remain unclear and deserve further study to offer insights on a better design of GM-mediated antibiotic agents or to explore broader biomedical applications.

6. CONCLUSIONS AND PERSPECTIVES

We have offered a summary of what we have learned regarding the antimicrobial mechanisms of GMs (Figure 6) and given some guidelines regarding how to modulate their antimicrobial properties. The current studies are far from exhaustive, even though they cover many aspects of the impact of GMs on bacteria. Thus, it is a rather interesting topic worthy of an in-depth investigation, and further studies to obtain a deeper understanding of the underlying molecular mechanisms are needed.

Whether the action of sharp edges or the availability of the basal planes dominates the antimicrobial activities of GMs remains controversial. Both opinions have been supported by direct evidence: the efflux of intracellular substances (e.g., RNA or DNA) in bacterial cells revealed mechanical disruption of the cellular membrane derived from the action of sharp edges, whereas masking of GO basal planes via non-covalent coating decreased the antimicrobial activity derived from the availability of basal planes. If the action of sharp edges dominates, various physical properties of GMs (e.g., size, dispersibility, shape, edge length, orientation angle, mechanical strength) imparting enhanced physical penetration would significantly improve their antimicrobial properties. In addition, the antimicrobial properties of edges may not be limited to only a mechanical blade effect but may also be related to the type of edge termination. In contrast, the antimicrobial activities derived from the availability of the basal planes can be modulated by altering the membrane stress, oxidative stress, charge transfer, or even nutrient deprivation.

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Whether oxidative stress is mediated with or without the production of ROS remains unclear. Current studies have only provided evidence that GMs exert oxidative damage, but no solid evidence of ROS production has been provided. Thus, it is unclear whether GMs behave as direct oxidants or mediators of charge transfer or produce ROS-mediated oxidative stress against bacteria because various existing oxidative-damage assays may interfere with antioxidants (GSH, NAC, α-tocopherol) or oxidant-sensitive dyes (DCFH-DA). ROS-detecting systems that depend on the oxidation of antioxidants (or dyes) may produce unreliable results if the GMs themselves are oxidants. Therefore, we must be cautious when interpreting the results of using conventional oxidative-damage assays, and additional controls are needed. Moreover, the generation of GM-triggered ROS should be cautiously deduced because of the difficulty of producing ROS, which requires photocatalysis/electrocatalysis through electron transfer to produce $O_2^{\bullet-}$ and $OH^{\bullet}$ or through energy transfer to produce $^1O_2$.[102−106] Overall, oxidative stress involves oxidation or dysfunction of cellular membranes or intracellular components (including DNA/RNA, protein, lipid, and mitochondria). Therefore, elaborately regulating the physicochemical properties of GMs (e.g., composition, doping, electronic structure, and oxygen-containing functional groups) could effectively modulate their antimicrobial activities.

Inactivating or killing bacteria by wrapping of the cellular membrane was proposed as an exclusive property of the GMs compared with other nanomaterials, resulting from its two-dimensional layer structure. However, it is still questionable whether graphene itself is capable of wrapping the bacterial membrane considering their different sizes (a graphene sheet is nanometer-scale, while a bacterium is micrometer-scale) and why the wrapped bacteria are inactivated or killed. Therefore, more efforts should be undertaken to find the intensive mechanism underlying the "wrapping" hypothesis. In addition, disrupting intracellular PPIs, extraction of lipid bilayers, and the self-killing effect are three newly proposed antibacterial mechanisms that also deserve further attention.

Additionally, whether GMs can selectively kill pathogenic microorganisms while not affecting normal mammalian cells is still unclear. Only a few investigations associated with the specificity of GMs between bacteria and mammalian cells have been conducted, although it is undoubtedly recognized that selectivity is most critical in exploring the biological and biomedical applications of GMs. The current results on the toxicity of GMs toward mammalian cells are controversial (perhaps the differences are due to the materials used), and the underlying cytotoxic mechanism has yet to be discovered. There exists different hemocompatibility as GMs encounter blood components. In one study, graphene showed little hemolysis of red blood cells.[107] However, two other studies demonstrated that GO had significant hemolytic activity and was highly thrombogenic.[108,109] Besides, dose- and time-dependent toxicity of GO on human fibroblasts (HDF),[110] normal human lung cells (BEAS-2B),[111] neuroblastoma SH-SY5Y cells,[112] and A549 cells[5] was observed. However, an opposite result was also reported, showing that GO induced only a slight loss of cell viability for A549 cells without signs of apoptosis or cell death.[113] Therefore, it is essential to compare the different types of GMs and correlate their impacts on cells to their physicochemical characteristics. Currently, no in vivo antibacterial activity has been demonstrated in an animal model for GMs, at least in a systemic mode. Some reports have suggested that GMs, as antibacterial materials, may be beneficial for wound healing[114,115] and dental care.[72] In addition, another report found that Ag@rGO nanocomposites induced no edema or erythema on injured rat skin.[47] These may be possible future applications of GMs in vivo.

In summary, exploring the nature of the antimicrobial mechanisms using intensive approaches combined with in vivo/in vitro experimental and theoretical simulations still requires a number of joint efforts because these mechanisms, which are deduced from currently obtained findings and achievements, are still controversial. However, the improvement of technology and methodology has paved the way for an accurate determination of the nature of GM-mediated antimicrobial activity. More factors such as experimental conditions (e.g., pH), adsorption of GMs, edge termination of GMs, and potential impurities should be considered. When the effect of one factor, such as the lateral size of GMs, is studied, other factors, such as the degree of oxidation, should be controlled. Additionally, studies in the future should focus on selectivity by exploring the toxicities of GMs, particularly in differentiating between bacteria and normal mammalian cells, between bacteria from different genera, and between normal and abnormal human cells. A rational design of controllable GMs according to proposed mechanisms would facilitate the exploration of broader biomedical and medical applications of various nanomaterial-based antimicrobial agents and strategies.

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Notes

The authors declare no competing financial interest.

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