Graphene Oxide Mimics Biological Signaling Cue to Rescue Starving Bacteria

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There is extensive debate about how 2D nanomaterials such as graphene oxide (GO) affect bacteria. Various effects of GO are proposed, including bacterial growth inhibition or enhancement, killing, and no activity. Herein, we report that GO protects Staphylococcus aureus bacterial cells from death in starvation conditions with up to a 1000-fold improvement in cell viability. Transcriptomic profiling reveals that bacterial cells in starvation conditions generally shut down metabolic activity, while only cells incubated with GO increase production of specific enzymes involved in the glyoxalase detoxification pathway along with repressed autolysis. The oxygen-containing functional groups of GO resemble the molecular structure of methylglyoxal, which bacteria produce to adapt to nutrient imbalances and is detoxified by glyoxalase enzymes. The ability of GO to enable bacterial cell survival in starvation conditions and accompanying cellular responses support that bacterial cells perceive GO as a methylglyoxal-mimicking nanomaterial cue to reshuffle cellular metabolism and defenses.

1. Introduction

The interaction of bacteria with nanomaterials is relevant to various health and environmental issues such as bacteria killing,[1–3] antifouling surfaces,[4–6] antibacterial drug resistance,[7] microbial ecology,[8,9] bio-recycling,[10] water filtration,[11,12] and sensing.[13,14] Two-dimensional graphene oxide (GO) sheets are a well-studied nanomaterial example, which have long been observed to kill, inhibit, or enhance the growth of bacteria.[15–18] More recently, it has been suggested that high-purity GO has no effect on bacteria and chemical impurities related to GO synthesis, not GO itself, are the cause of antibacterial activity.[19] As such, there is extensive interest in clarifying how 2D nanomaterials such as GO affect bacteria[20–22] and such insights can guide next-generation nanomaterial design and surface functionalization. The reactive oxygen-containing functional groups present on the GO sheet surface are presumed to influence biological activities, including bacterial cell interactions.[23] Specifically, these functional groups are implicated in GO-mediated oxidative stress, which occurs when a biological system is unable to cope with the quantity of reactive oxygen species (ROS) that is being produced.[24] Excessive oxidative stress can lead to deleterious outcomes such as impaired cell function or death, while bacterial pathogens such as Staphylococcus aureus (S. aureus) have evolved the ability to withstand high levels of...
oxidative stress.[25] To evade oxidative damage, *S. aureus* has developed various strategies for cellular protection, detoxification, and repair.[29] Such capabilities help to explain why *S. aureus* is a leading cause of human infections worldwide, especially with its ability to overcome host cellular defenses involving the release of reactive oxygen and nitrogen species.[27,28]

Since *S. aureus* elicits distinct cellular responses depending on the chemical source of oxidative stress,[29,30] we sought to investigate how *S. aureus* bacterial cells in nutrient-limiting starvation conditions respond to oxidative stress-inducing GO sheets. In addition to determining the empirical effect of GO on *S. aureus* bacterial cells in starvation conditions, we sought to unravel the molecular mechanisms behind cellular responses.

2. Results and Discussion

Current models of how bacterial cells interact with GO nanomaterials are presented in Figure 1a and our experimental strategy to characterize the interactions between oxidative stress-inducing GO sheets and *S. aureus* bacterial cells, involved a combination of nanomaterial characterization, microbiological testing, and transcriptomic analyses (RNA-seq) (Figure 1b).

We fabricated GO sheets with varying degrees of oxidizing activity according to the 1) Staudenmaier, 2) Hoffman, 3) Hummers, and 4) Tour methods, as previously described.[31] The Staudenmaier and Hoffman methods involve chloride oxidants that result in a higher carbon/oxygen (C/O) atomic ratio and mainly hydroxyl and epoxy functional groups on the GO sheet surface while the Hummers and Tour methods involve permanganate oxidants that yield a smaller C/O ratio and a greater density of carbonyl and carboxylic acid functional groups. The GO sheets were extensively rinsed with water until neutralized and are referred to as GO samples 1–4 according to the preparation methods listed above. Aqueous dispersibility of the GO samples was observed and is consistent with hydrophilic surface properties due to the presence of oxygen-containing functional groups, which led us to evaluate pertinent solution-phase behaviors.

The in vitro oxidizing activity of the solution-phase GO samples was first characterized by measuring the extent to which GO can convert glutathione (GSH), an important tripeptide antioxidant that helps *S. aureus* cope with ROS,[12] into...
glutathione disulfide (GSSG) (Figure 1c). Greater GSH consumption is associated with more oxidizing activity. GO samples (6–200 µg mL⁻¹) were incubated with GSH and UV–vis spectroscopy experiments revealed dose-dependent oxidation in all cases. At the highest GO concentration of 200 µg mL⁻¹, sample 3 caused 51% GSH oxidation while samples 1, 2, and 4 caused 21%, 10%, and 14% GSH oxidation, respectively. We also measured the GO-mediated production level of superoxide radical anion, which is an ROS involved in oxidative stress (Figure 1d). Low concentrations of GO, up to 25 µg mL⁻¹, tended to decrease superoxide radical anion production by around 20%, while higher concentrations of GO caused dose-dependent increases in production compared to the baseline. At 200 µg mL⁻¹ GO, sample 3 caused the highest production levels with around a 57% increase while samples 1, 2, and 4 increased production levels by around 42%, 7%, and 24%, respectively. The trends across both experiments were consistent and support that GO samples caused dose-dependent oxidative stress to varying extents while low GO concentrations partially suppressed superoxide radical anion production.

In nutrient-limiting conditions, S. aureus bacterial cells experience stress and lose viability over time. The extent of viability loss depends on the environment, and cells become nonculturable in the absence of amino acids or phosphate. We initially tested the time-dependent viability of S. aureus cells that were suspended in 10 mM phosphate buffer (PB) at a density of 5.5 × 10⁵ colony-forming units per mL (CFU mL⁻¹). Viable counts were determined by plating the treated cells on Mueller–Hinton (MH) agar plates overnight, followed by CFU enumeration (Figure 2a; Figure S1, Supporting Information). After 1 h incubation, the number of viable cells decreased by more than 87% and there was a >99% loss in viable cells after 2 h incubation. Accordingly, GO samples were dispersed in PB solution at a concentration of 50 or 200 µg mL⁻¹, and S. aureus cells were suspended in the GO-containing PB solution for 3 h before plating. For all samples, the presence of GO led to a marked increase in the number of viable cells and GO protected against viability loss in a dose-dependent manner (Figure 2b).

At 200 µg mL⁻¹ GO, sample 3 preserved around 80% bacterial cell viability compared to the initial inoculum while samples 1, 2, and 4 maintained viability levels around 57%, 36%, and 56%, respectively. Thus, GO protected S. aureus cell viability in nutrient-limiting conditions by over 1000-fold and the degree of cellular protection mirrored the trend in GO oxidizing capacity.

To verify if GO aids bacterial cell survival, S. aureus cells (density of 1 × 10⁷ CFU mL⁻¹) were incubated for 3 h with 200 µg mL⁻¹ GO in PB solution or phosphate-buffered saline (PBS), and the percentage of live and dead bacterial cells was determined by fluorescence microscopy (Figure 2c). Live and dead bacterial cells are indicated by green and red colors, respectively. Over the incubation period, the percentage of live cells in PB solution decreased from 97% to 33%, while GO had significant protective effects, as indicated by 89% survival (Figure 2d). By contrast, the percentage of live cells before and after incubation in PBS was nearly equivalent (~95%), and GO neither increased nor decreased bacterial cell viability, which agrees with past observations.

In both PB and PBS conditions, the microscopy images also showed that GO-treated bacterial cells were protected from stress and maintained a high percentage of viability compared to untreated controls.
bacteria appeared to coalesce, which supports that adhesive interactions between bacterial cells and hydrophilic GO sheets contribute to the observed protective effects. Taken together, the findings establish that GO protected against cell death in nutrient-limiting solution conditions in the absence of salts.

To decipher the molecular basis for GO-induced bacterial cell protection, transcriptomic profiling was conducted and we measured RNA expression levels of *S. aureus* bacterial cells incubated in PB in the presence and absence of GO (labeled as PB+GO and PB, respectively). *S. aureus* cells (density of \(\approx 6 \times 10^8\) CFU mL\(^{-1}\)) were incubated with GO (400 µg mL\(^{-1}\)) for 15 or 30 min, and equivalent control experiments were performed without GO (Figure 3a; Figures S2 and S3, Supporting Information). These time points were chosen because there was not a significant loss in cell viability up to 30 min, while the cells resuspended in PB showed >80% loss in cell viability by 60 min. After incubation, the cell-GO samples were treated with bovine serum albumin (BSA) protein to passivate the GO surface before adding lysostaphin enzyme to digest *S. aureus* cell walls, liberating cellular RNA which was collected for deep-sequencing analysis. The BSA passivation step minimized RNA attachment to the GO surface upon intracellular RNA release and hence facilitated efficient RNA extraction, which was otherwise encumbered by strong GO-RNA binding interactions (Note S1 and Figure S4, Supporting Information). To determine if there was a difference in the expression of stress-related genes for PB and PB+GO treated cultures, we quantified genome-wide differences in transcription at the different time points. Based on global analysis, 258 (130 upregulated and 128 downregulated) genes were similarly altered in expression for both the PB and PB+GO samples (Figure 3b,c).

Genes associated with genetic information processing, such as those involved in translation and ribosome expression, were generally reduced in expression for all conditions (Figure 4a). Similarly, genes involved in glycolysis, the RNA polymerase subunit delta (ID 14075), and the cell division protein ftsW (ID 09520) were strongly repressed, suggesting a general shutdown of cellular metabolism in response to stress conditions. To better understand changes in gene expression that might explain the increased survival of PB+GO treated bacterial cells, we focused on genes that were differentially expressed between the two conditions, relative to the initial culture. At 15 and 30 min, 462 (218 upregulated and 244 downregulated) and 771 (369 upregulated and 402 downregulated) genes were differentially expressed in the PB+GO condition (Data S1, Supporting Information).

One of the most highly induced genes in the PB+GO treated cultures was a putative glyoxalase enzyme (gene number 13170) that was induced 10- and 13-fold after 15 and 30 min, respectively (Figure 4b). Similarly, 13165, which is adjacent to, and may be co-transcribed with, the glyoxalase gene, was induced 15- to 17-fold in the presence of GO. This gene is annotated as a putative carboxylesterase and has no known function, but its similarly high induction in the presence of GO suggests it may work in conjunction with the putative glyoxalase. In marked contrast, neither of these genes was differently expressed in the PB condition and the 13170 and 13165 genes were induced by seven- and eightfold, respectively, in the PB+GO condition at 15 min. Three additional genes in the *S. aureus* genome were annotated as glyoxalases (01465, 07875, and 13185). Two of these, 13185 and 07875, were similarly repressed under both conditions while 01465 was also induced in the PB+GO condition. Moreover, none of the glyoxalases was induced in the PB condition.
In general, genes associated with cell wall synthesis were also repressed in the presence of GO, for example, phage infection protein, sortase, \( n\)-acetylemuramoyl-\( l\)-alane amidase, and \( dltA \), suggesting that PB+GO induces cell replication arrest, which may protect the cells from lysis. \( nrdR \) was also reduced in expression in the PB+GO incubated cells and is a repressor of genes associated with the generation of deoxyribonucleotides from ribonucleotides and may indicate that the PB+GO treated cells are reorganizing metabolism to ensure a proper balance of nucleotides. Together, the data support that PB+GO induces a general shutdown of growth and autolysis in \( S.\ aureus \) cells along with inducing a glyoxalase detoxification pathway.

Glyoxalase enzymes are involved in the detoxification of methylglyoxal (MG), which is produced as part of glucose metabolism when bacteria experience nutrient imbalances and need to reshuffle metabolic activities to survive in harsh environmental conditions.\(^{[16]} \) MG production is viewed as a high-risk strategy to facilitate bacterial adaptation because high MG concentrations are associated with DNA damage, mutation, and cell death.\(^{[17]} \) As such, bacterial cells produce glyoxalases in order to convert MG and other reactive aldehydes into less toxic by-products. It is thus striking that the PB+GO condition induces a general shutdown of growth and autolysis in \( S.\ aureus \) cells along with inducing a glyoxalase detoxification pathway.

Figure 4. GO triggers specific survival responses associated with methylglyoxal detoxification. a) Distribution of upregulated and downregulated gene transcripts in different conditions according to functional class. The classes were organized based on the KEGG pathway maps. Each bar summarizes the percentage of upregulated (blue) and downregulated (light orange) genes in each category. b) Summary of differentially regulated genes related to the glyoxalase detoxification pathway. The log2(fold-change) values are reported for genes with at least twofold significant changes compared to the negative control. Positive and negative values indicate upregulated and downregulated genes, respectively (see also Data S1, Supporting Information). c) Molecular structures of MG and GO functional groups.

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3. Conclusion
In summary, our findings demonstrate that GO can enable \( S.\ aureus \) bacterial cells to survive in starvation conditions and that the structural and functional properties of oxygen-containing functional groups on the GO sheet surface play
an important role in enabling this protective activity. While oxidative stress is typically associated with harmful effects, there is precedent that an oxidative stress response can help other microorganisms such as yeast survive in starvation conditions.\(^{[43]}\) ROS-mediated oxidative stress elicited by host cell immune responses has also been shown to induce antibiotic tolerance of \(S.\ aureus\) bacterial cells.\(^{[40]}\) Our study presents the first example of how a nanomaterial can induce an oxidative stress-related response in living cells—in this case triggered by how \(S.\ aureus\) bacterial cells perceive GO as an MG-mimicking cue—that enhances cellular function to survive in starvation conditions.

Looking forward, these results and the methodologies developed in this study open the door to exploring how the wide range of nanomaterials with reactive oxygen-containing functional groups, which are commonly associated with antibacterial mechanisms, might also enhance the function of bacterial cells and other cell types.

**Supporting Information**

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

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

Research data are not shared.

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