Graphene Oxide: A Nonspecific Enhancer of Cellular Growth

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ABSTRACT There have been multiple conflicting reports about the biocompatibility and antimicrobial activity of graphene oxide. To address this, we conducted a study to characterize the antimicrobial properties of graphene oxide (GO) and its biocompatibility with mammalian cells. When GO was added to a bacterial culture at 25 μg/mL, the results showed that bacteria grew faster and to a higher optical density than cultures without GO. Scanning electron microscopy indicated that bacteria formed dense biofilms in the presence of GO. This was shown by a large mass of aggregated cells and extracellular polymeric material. Bacterial growth on filters coated with 25 and 75 μg of GO grew 2 and 3 times better than on filters without GO. Closer analysis showed that bacteria were able to attach and proliferate preferentially in areas containing the highest GO levels. Graphene oxide films failed to produce growth inhibition zones around them, indicating a lack of antibacterial properties. Also, bacteria were able to grow on GO films to 9.5×10^9 cells from an initial inoculation of 1.0×10^6, indicating that it also lacks bacteriostatic activity. Thus, silver-coated GO films were able to produce clearing zones and cell death. Also, graphene oxide was shown to greatly enhance the attachment and proliferation of mammalian cells. This study conclusively demonstrates that graphene oxide does not have intrinsic antibacterial, bacteriostatic, and cytotoxic properties in both bacteria and mammalian cells. Furthermore, graphene oxide acts as a general enhancer of cellular growth by increasing cell attachment and proliferation.

KEYWORDS: nanomaterials · graphene oxide · silver-coated graphene oxide · biomaterials · biocompatible · quantitative real-time PCR · growth enhancer

Graphene oxide sheets are monolayers of carbon atoms that form dense honeycomb structures with unique characteristics.[1,2] They contain a range of reactive oxygen functional groups that facilitate their application in bioengineering.[3] Among the most important properties of GO are low production costs, large surface area, good colloidal behavior, and low cytotoxicity. The solubility of GO in solvents, especially water, is important for applications in bioengineering. The maximum solubility of graphene oxide in a solvent depends both on the solvent polarity and the extent of surface functionalization imparted during oxidation. A few methods are currently available to synthesize GO, and among them, a modified Hummers method is the most popular chemical approach.[4] The surface of GO, made from the aforementioned Hummer's method, has oxygen functional groups, such as hydroxyl, epoxyl, and carboxyl, which enable GO to be readily dispersed in water.[5]

Most reports show that GO materials, including GO films (paper), are superior biocompatible materials that allow the effective proliferation of human and mammalian cells with limited or no cytotoxicity. Such characteristics seem to indicate that GO materials may be used in tissue engineering, tissue implants, wound therapy, and drug delivery applications. These particular characteristics have motivated multiple research groups to further characterize the cytotoxic and antiseptic properties of graphene oxide. Recently, several reports have shown that GO paper promotes the adhesion and proliferation of L-929 cells,[6] osteoblasts,[7] kidney cells,[8] and embryonic cells.[8] However, additional studies have shown that cellular internalization of GO nanosheets applied to the culture media at a concentration of 20 μg/mL can cause a 20% decrease in mammalian cell viability, while a concentration of 50 μg/mL can lead to a 50% loss in cell viability, indicating that some inhibitory effect can be observed if a GO suspension is applied to the growth media.[9] The same conditions caused 90% viability loss in *Escherichia coli*.[9] Even after these observations, the authors concluded that GO nanosheets were biocompatible.[9] A recent study showed that graphene and graphene oxide materials are cytotoxic to human erythrocytes and skin fibroblasts.[10] Another study showed that films developed from a suspension of reduced graphene oxide and polyoxyethylene sorbitan laurate (TWEEN) were noncytotoxic to three different types of mammalian cells.[7] These combined results appear to support that GO materials are biocompatible with

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mammalian cells by promoting cell adhesion and proliferation as effectively as commercial polystyrene tissue culture materials.[6] On the other hand, colloidal GO solutions appeared to be mildly cytotoxic at high concentrations.

The apparent low cytotoxicity of GO materials has led research groups to characterize these materials for antimicrobial effects. A material with low mammalian cell cytotoxicity and increased antimicrobial characteristics may become an ideal material in biomedical applications. A recent report has shown that contact of *E. coli* and *Staphylococcus aureus* bacterial cells with GO can cause growth reductions of about 51 and 61%, respectively.[11] Similar results have been obtained for these two microorganisms when exposed to GO nanowalls.[12] It has been reported that graphene oxide film (paper) can cause growth inhibition zones in *E. coli* and *S. aureus*.[11] On the other hand, recent studies have also indicated that GO is not cytotoxic and also lacks any antibacterial effect. Das *et al.*[13] showed that, when GO was placed in the center of a nutrient media plate previously inoculated with bacteria, a growth inhibition zone was not formed. Alternatively, when silver-decorated GO was used, a clear inhibition zone was formed.[13] In another study, Park *et al.*[8] reported nonspecific binding of Gram-positive and Gram-negative bacteria to GO paper, but functionalization of GO with polyoxyethylene sorbitan laurate reduced bacteria nonspecific binding to GO surfaces. In summary, the numerous conflicting reports about the antimicrobial properties of GO have led us to perform an in-depth characterization of the antimicrobial characteristics of graphene oxide with the goal of addressing the discrepancies in relation to the antimicrobial properties of GO.

The main objective of this investigation is to determine if graphene oxide presents any real antibacterial or bacteriostatic activity. In this study, bacterial and mammalian cellular growth in the presence and absence of graphene oxide materials was determined by the development of carefully designed cell growth bioassays along with the use of quantitative real-time PCR analysis to accurately determine the cells number and the effect of graphene oxide on microbial proliferation. Here, we show that graphene oxide materials do not adversely impact microbial and mammalian cell growth. Furthermore, graphene oxide materials tend to produce a dramatic increase in microbial and mammalian cell proliferation, indicating that graphene oxide is not a bactericidal or bacteriostatic material, but instead a general growth enhancer that acts as a scaffold for cell surface attachment and proliferation. This is the first report that conclusively demonstrates that graphene oxide does not have intrinsic antibacterial properties and cytotoxic properties.

RESULTS AND DISCUSSION

Bacterial Proliferation in the Presence of Colloidal Graphene Oxide (GO). To determine the effect of graphene oxide on bacterial growth, samples containing 5 mL of Luria–Bertani (LB) nutrient broth in 15 mL conical tubes were amended with GO to a final concentration of 25 μg/mL and then inoculated with *E. coli* bacterial cells to a concentration of 0.03 OD. The experimental control was produced by inoculating *E. coli* to 0.03 OD in 5 mL of LB broth without GO. At least, triplicate reactions of each condition were incubated for 16 h at 37 °C and then examined for bacteria growth. Surprisingly, the culture tubes containing graphene oxide did not visually show any apparent reduction in bacterial growth (Figure 1b). Furthermore, they appeared more turbid than the control culture (Figure 1c), and a dense dark precipitate was observed at the bottom of the tube (Figure 1b). The dark precipitate was not produced in the control cultures without GO (Figure 1c). We proceeded to determine growth level in the bacteria cultures by measuring the absorbance at 600 nm. Samples were taken from the supernatant without disturbing the dark precipitates at the bottom of the samples containing GO. The results showed that the GO-containing samples achieved an average absorbance of 1.7 in 16 h of incubation while the bacteria growing in LB broth only achieved an absorbance of 1.3 (Figure 1a). These results indicated that bacteria in the presence of GO grew faster than bacteria in LB media and were able to achieve cell saturation sooner. It was possible that the dark precipitate observed in samples containing GO was responsible for enhancing bacterial growth in the media or harboring bacterial growth itself. To address this, we analyzed samples of the dark precipitate through scanning electron microscopy.

Scanning electron microscopy (SEM) analysis showed that the dark precipitate was formed by a thick bacterial biofilm (Figure 1f,g) containing a large mass of aggregated cells (Figure 1g) and extracellular polymeric material (Figure 1f). A negative control reaction containing just LB broth and GO but not bacteria showed the formation of lower density dark aggregates that did not precipitate to the bottom of the culture tube (Figure 1d). It has been shown that, when a colloidal suspension of GO in water is added to a solution media containing salts, it aggregates and could falloff suspension producing low density aggregates.[14] It is possible that precipitation required bacterial growth. The massive amount of cells observed in the biofilm indicates that there is a direct effect of GO in bacteria proliferation when colloidal GO is added to liquid media. Our results showed that the precipitation of GO in the culture media may be acting as a scaffold for bacterial attachment, proliferation, and biofilm formation. Studies have shown that carbon nanomaterials could act as attachment surfaces where

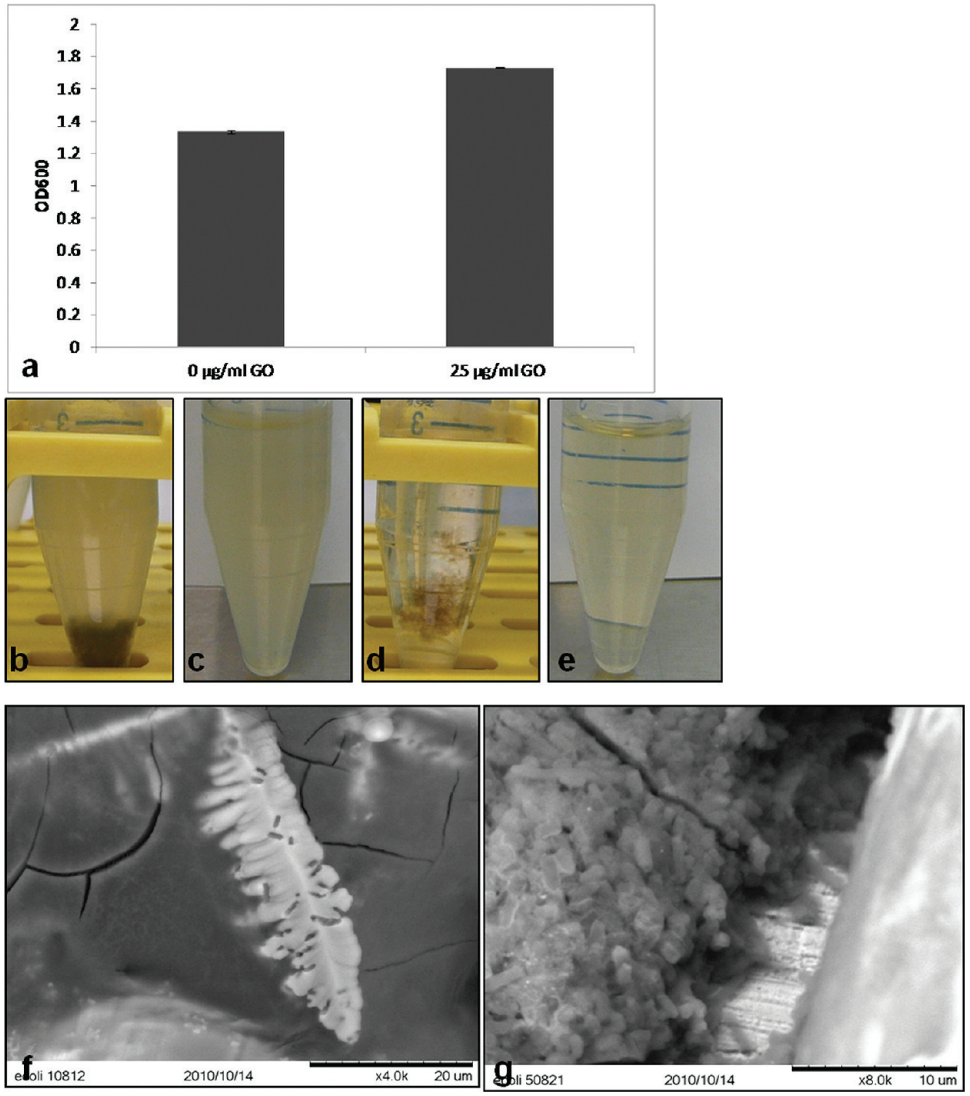

Figure 1. Bacterial proliferation in the presence of colloidal graphene oxide. Pictures showing bacterial growth in test tubes containing 5 mL of Luria–Bertani broth with 0 (c,e) and 25 μg/mL graphene oxide (b,d). *E. coli* was inoculated at a concentration of 0.03 OD_{600} (b,c) and allowed to grow for 16 h at 37 °C. Sterility controls without *E. coli* but with and without GO can be observed (d,e). Note the formation of a dark dense precipitate in the GO sample containing bacteria (b) but not in the sample without GO (c). Graph showing bacterial growth levels in the supernatant of samples containing and lacking GO (a). Scanning electron micrographs showing formation of biofilms in the presence of graphene oxide (f,g). Two characteristic regions were observed within the biofilm, one composed of mostly extracellular polymeric substance (f) and another with a very high bacteria cell density (g).

small colonies grow around tubular carbon nanostructures.[15] Further, it seems that precipitated GO induced massive cell growth, aggregation, and secretion of extracellular polymeric substance (EPS) (Figure 1f,g). In the SEM pictures, it was possible to observe void areas in the EPS with the shape of rod bacteria (Figure 1f). This indicates that bacteria were directly responsible for secreting the EPS. The rod-shape voids observed may be due to the process of bacteria dispersal, a process in which bacteria evacuate the interior of biofilms due to competition or lack of nutrients, leaving behind hollow, shell-like structures.[16]

Characterization of Bacterial Growth on Graphene Oxide Surfaces. To determine the effect of graphene oxide (GO) when coated onto a surface, we coated sterile PVDF filters with 0 (neat), 100, and 300 μL of a 250 μg/mL colloidal suspension of GO, which equaled 0, 25, and 75 μg of GO per filter. Filters were allowed to dry and were then inoculated with bacteria by submerging them into a solution containing *E. coli* at a concentration of 1×10^6 cells/mL for 1 min. The filters were recovered, allowed to dry, placed onto a sterile LB culture plate, and then incubated for 18 h at 37 °C. After the incubation period, pictures were taken and genomic DNA was extracted from each of the filters for further analysis (Figure 2c–e).

Bacteria growth on filters with or without GO was determined by quantitative real-time PCR (qPCR) analysis of the bacteria genomic DNA. Bacteria growth was observed with the naked eye in all samples, but the

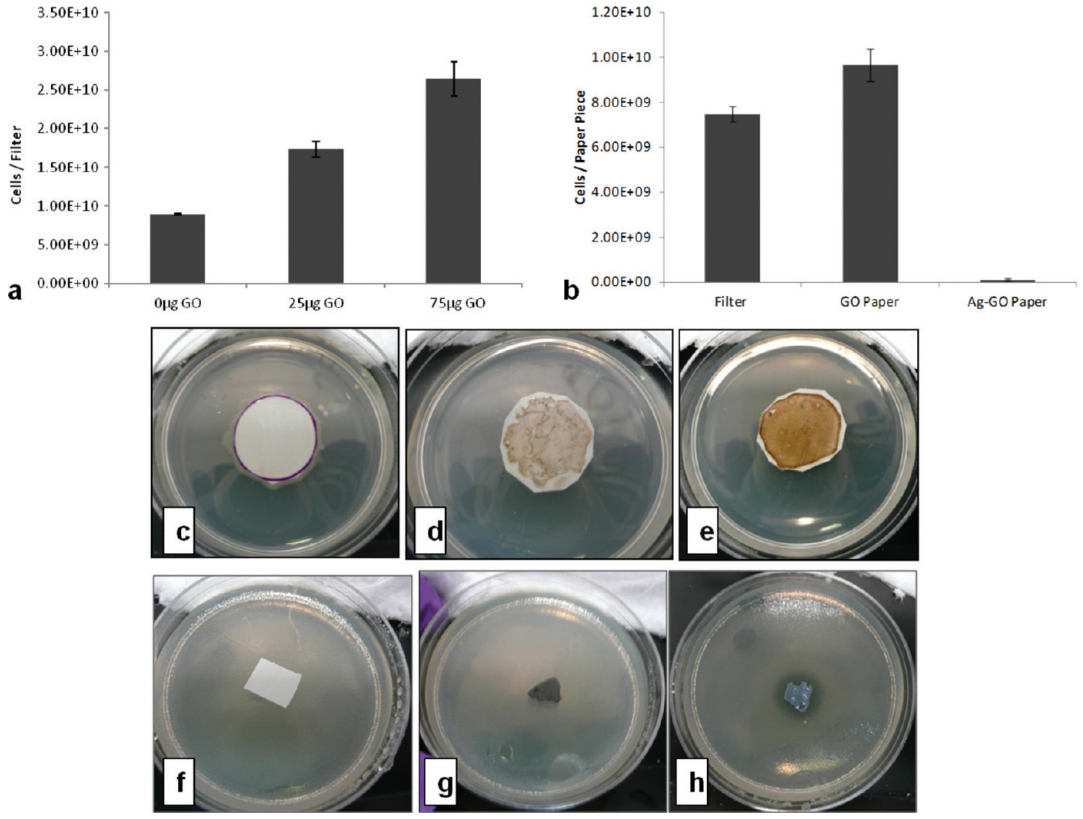

Figure 2. Bacterial growth on graphene oxide surfaces. PVDF filters coated with 0 (c), 25 (d), and 75 μg (e) of GO were inoculated with *E. coli* and incubated at 37 °C for 18 h. Quantitative real-time PCR was used to assess bacterial growth in filters with and without GO (a). Small ~1 cm^2 pieces of PVDF filter (f), GO film (g), and Ag-GO film were inoculated with *E. coli* and culture for 18 h at 37 °C. Bacterial growth was quantified by real-time PCR (b).

filters containing GO presented large bacteria colonies around specific areas that seem to contain more GO (Figure 3a–d). QPCR was chosen over other methods of analysis because it allows precise determination of the level of cellular growth. To achieve cellular quantification, a qPCR assay that targeted the 16S rRNA (*rrn*) gene, a ubiquitous gene in all bacteria, and a synthetic oligonucleotide standard for quantification that spanned the amplicon region were used. This type of analysis provided the number of copies of the 16S *rrn* gene in the sample, which was then used to determine the exact number of cells by dividing the obtained sample gene copy number by the number of 16S *rrn* genes found in the *E. coli* cell; seven copies of the 16S *rrn* gene are found per *E. coli* cell. The qPCR results showed that the bacteria levels in filters containing GO were higher than that in the filters without GO (Figure 2a). The filters containing 25 μg of GO had double the amount of bacteria than the neat filter, while the filter covered with 75 μg of GO had 3 times more cells than the neat filter. These results indicate that GO not only lacks antimicrobial properties, but that it actually enhances microbial growth when coated onto another surface (Figure 2a).

Upon close inspection of the GO-coated and neat filters, we observed some interesting growth patterns

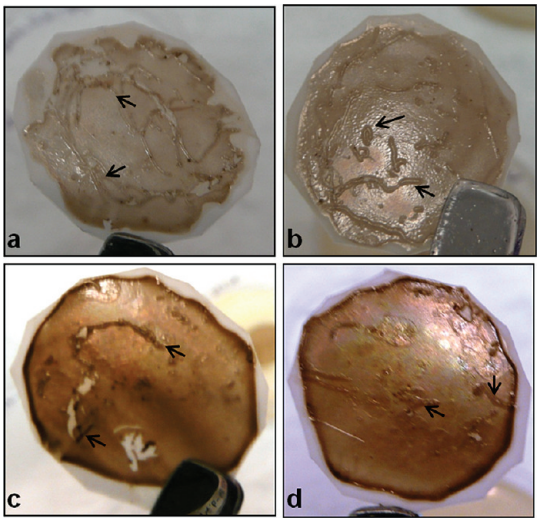

Figure 3. Bacteria interaction with graphene oxide. Black arrows indicate some of the areas with increased bacterial growth observed on filters coated with 25 (a,b) and 75 μg (c,d) of GO. Bacterial colonies can be easily observed as elongated features in GO-coated filters but not in a neat PVDF filter.

that differentiate the GO-coated filters. The GO-coated filters had easy to observe large cell colonies that follow a lined pattern around areas of higher GO content (Figure 3a–d). These areas were observed in

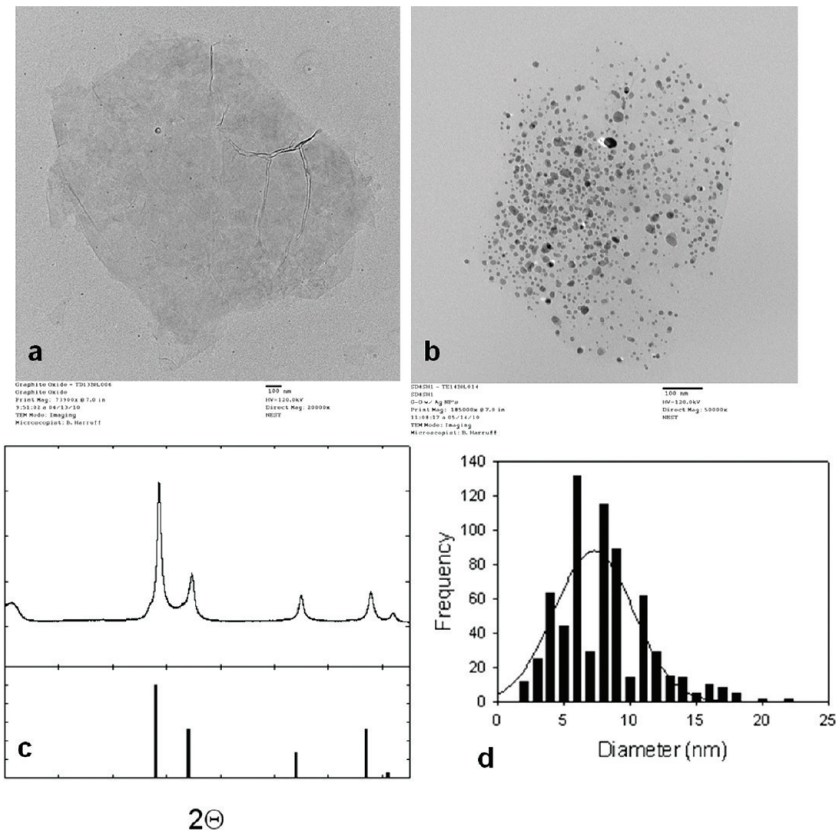

Figure 4. Graphene oxide and silver-coated graphene oxide characterization. (a) TEM image of neat GO, (b) TEM image of Ag-decorated GO, (c) XRD spectrum of Ag-decorated GO and ICDD 00-004-0783 card data for face-centered cubic Ag, and (d) size distribution studies performed using TEM for Ag-decorated GO.

filters with 25 and 75 μg of GO but not in neat filters. Also, we observed that an area of dense bacterial growth in the LB media was produced around all neat filter replicates (Figure 2c). This halo of cells was not observed in any of the GO-coated filters (Figure 2d,e). This was an interesting observation that implied that there is an inherent preference by bacteria to attach and grow in areas containing GO, especially those areas containing the highest GO levels (Figure 3).

Bacteria Interaction with GO and Ag-GO Films (Papers). GO and Ag-GO films were analyzed using TEM and XRD analysis to determine the morphology, size distribution, and the crystal structure of Ag nanoparticles, as shown in Figure 4. TEM analysis clearly showed that the GO sheets were well-decorated with Ag nanoparticles (Figure 4b), and size distribution analysis performed using TEM showed that the average size of Ag nanoparticles on the surface of GO was 8 nm (Figure 4c,d). In addition, XRD analysis revealed that the silver-decorated graphene oxide (Ag-GO) spectrum closely matched the peaks seen in the face-centered cubic silver (ICDD 00-004-0783). The effect of solid GO films on bacteria growth was studied by inoculating GO films, Ag-GO films, and filter pieces with *E. coli* bacteria. Graphene oxide films were placed onto LB culture plates that were previously inoculated with 1×10^6 *E. coli* cells per plate. Then, 1×10^6 *E. coli* cells were directly inoculated on top of the film pieces and allowed to dry. The plates were incubated for 18 h at 37 °C. After the incubation period, pictures were taken and genomic DNA was extracted from each of the filters for further analysis (Figure 2f−h). The purpose of this type of inoculation was to observe growth over the GO film and also to determine if any growth inhibition zone was formed around the GO film. Growth inhibition zones around GO film have been reported in the past.[11] Inhibition areas would indicate that the material has some toxic effect on the bacteria.

Results showed that growth inhibition zones were not detected in the plate containing either GO film or filter paper (Figure 2f,g). However, Ag-decorated GO showed large growth inhibition zones characterized by a clear area with no cell growth (Figure 2h). These results clearly demonstrate that GO does not have any antimicrobial effects capable of producing a toxic effect in the area surrounding the GO film. By decorating our GO material with silver, we further demonstrate that we can replicate published results for Ag-GO material.[11,13,17] This showed that our GO material was functional and capable of supporting common antimicrobial materials including silver.

Quantitative real-time PCR (qPCR) performed to determine the growth level over the different films

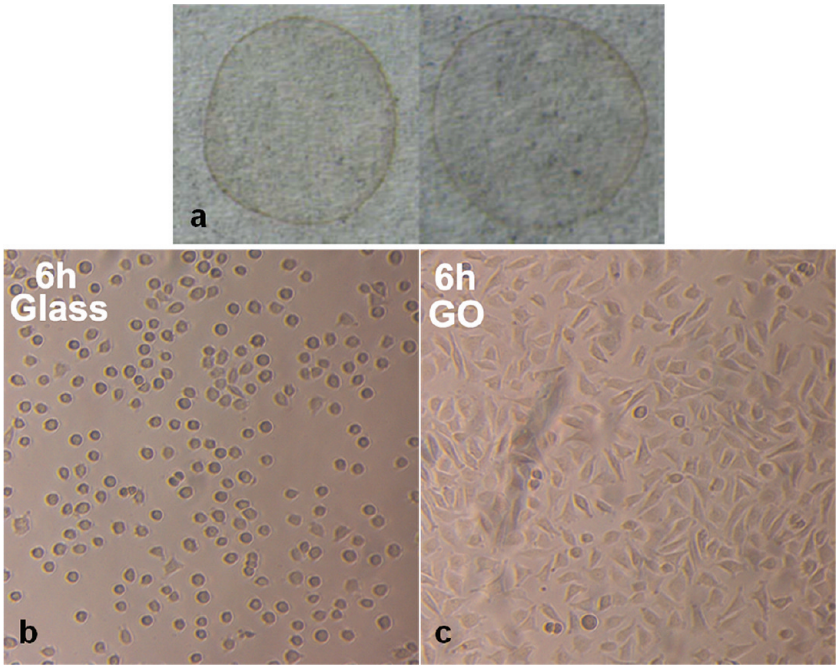

Figure 5. Mammalian cell growth on graphene oxide film. Glass slides coated with graphene oxide (a). Micrographs showing human adenocarcinoma HT-29 cell attachment and growth on glass slides (c) with or (b) without GO film. A GO-coated glass slide and a control uncoated glass slide were placed into the same culture dish, inoculated with HT-29, and allowed to incubate for 6 h at 37 °C.

revealed that GO paper supported bacteria growth more efficiently than a PVDF filter (Figure 2b). The results showed that the surface of the GO film contained 9.5×10^9 cells while the filter paper had 7.5×10^9 cells. The cell level over the Ag-GO paper was determined to be 3.5×10^4 cells, which was lower than the initial inoculation level of 1×10^6 cells, indicating that bacteria cell death was achieved. These results do not indicate any adverse or toxic effect of GO toward bacteria. Furthermore, GO seems to promote bacterial growth by enhancing attachment, proliferation, and biofilm formation.

Mammalian Cell Attachment and Proliferation onto GO Film. A study was performed to test the role of GO film on mammalian cell attachment and proliferation. Control glass slides and glass slides coated with 10 μg of GO (Figure 5a) were placed onto a culture dish to which culture media and 6×10^5 mammalian colorectal adenocarcinoma HT-29 cells were added. The cells were allowed to attach and develop on the slides. At various time intervals, cell attachment was assessed by light microscopy. Shown in Figure 5b,c are representative images of cell morphology after incubation for 6 h. The results indicated that the mammalian cells attached more efficiently to the GO-coated glass slides and grew (Figure 5c). The micrographs showed marked morphological changes and cell enlargement and spreading on the GO-coated slides, which are characteristic of effective cell attachment and cell growth (Figure 5c). However, very few cells became attached to and developed on the plain glass slides (the control) as observed by the round shape of cells, which indicated lack of cellular enlargement and growth (Figure 5b). These results clearly showed that the GO film, beyond not exerting any cytotoxic effects on the cells, actually promotes mammalian cell attachment and proliferation. During the course of this investigation, several literature reports focusing primarily on biocompatibility of GO films were published,[6−8,18,19] from which the results are generally consistent with what is shown here on the GO film enhancing mammalian cell attachment and proliferation. Taken together these results indicate that GO is a great support for mammalian cell attachment, growth, and proliferation. As shown, GO film coated on glass slides enhances cell attachment, growth, and proliferation. These results compare positively against carbon nanotube materials which have been shown to be cytotoxic at various concentrations.[20−28]

CONCLUSION

The results of this study clearly demonstrate that graphene oxide does not have antibacterial properties. Furthermore, graphene oxide lacks any bacteriostatic property as shown by the prolific growth observed on all forms of GO tested. It seems that GO acts as an enhancer of life, increasing not only mammalian cell growth but also bacterial growth. In the past, several studies have shown that GO is noncytotoxic to mammalian cells, but that it somehow acts as an antibacterial material. In reality, there is no clear reason for which an inert carbon material such as GO could be beneficial

to mammalian cell growth and detrimental to bacteria, which are usually less susceptible to biotic and abiotic factors than mammalian cells. It is possible that contaminants retained from the GO preparation or underestimations of GO concentrations might be responsible for some of the detrimental effects on bacteria growth observed in previous reports. Our graphene oxide production process, which includes long-term dialysis, dilution, and sonication, is one that takes great care on removing carryon impurities that could affect the properties of the material. More studies are required to determine which GO synthesis processes are producing the best quality GO material. This study significantly advances our knowledge on the biological properties of graphene oxide and its uses in biomedical and biotechnological application.

MATERIALS AND METHODS

Preparation of Graphene Oxides (GOs). The Hummers method with minor modification was used for the preparation of GO (Figure 4a) from the same graphite sample.[4] Briefly, concentrated H_2SO_4 (10 mL) in a 500 mL flask was heated to 80 °C, to which $(NH_4)_2S_2O_8$ (0.9 g) and P_2O_5 (0.9 g) were added. The mixture was stirred until the reagents were completely dissolved. The graphite sample (1 g) was added, and the resulting mixture was heated at 80 °C for 4.5 h. Upon being cooled to room temperature, the reaction mixture was diluted with water (250 mL) and kept for ~12 h. It was then filtrated and washed repeatedly with water, followed by drying in a vacuum oven. The solid sample was added to concentrated H_2SO_4 (40 mL) in a 500 mL flask cooled in an ice bath. The mixture was added slowly to $KMnO_4$ (5 g over 40 min), during which the temperature was kept at <10 °C. The reaction mixture, with a change in color from black to greenish brown, was heated at 35 °C for 2 h, followed by dilution with water (85 mL; *Caution:* the temperature must be kept at <35 °C throughout) and further stirring for 2 h. The reaction mixture was poured into a large beaker, to which water (250 mL) and then aqueous H_2O_2 (30%, 10 mL) were added. Bubbles from the aqueous mixture along with a color change to brilliant yellow were observed. After the mixture was allowed to settle for ~12 h, the clear supernatant was decanted, and the sediment was washed repeatedly with aqueous H_2SO_4 (5 wt %)-H_2O_2 (0.5 wt %) and HCl solution (10 wt %), followed by washing repeatedly with water until no layer separation was observed after centrifuging. The sample was then dialyzed (MWCO ~ 3500) against water for 7 days to yield a clean aqueous dispersion of GOs. The aqueous GOs thus obtained (acid form) were titrated by aqueous NaOH (0.1 M) until pH reaches 9. The resulting GOs (sodium form) were again dialyzed (MWCO ~ 3500) for 7 days to reach neutral pH. Finally, the aqueous suspension of GOs was diluted (~0.2 wt %) and sonicated for 30 min to achieve complete exfoliation.

Synthesis of Ag-GO. GO was synthesized using the Hummers method as explained previously. Ag-GO (Figure 4b) was prepared using a sonochemical method as follows. First, 50 mg of GO, 25 mg of silver acetate, and 15 mL of DMF were mixed in a three-arm sonochemical flask (Sonics Inc., Suslick flask). The mixture was sonicated at 37% amplitude and 20 kHz for 20 min using a pulsed (1 s on, 1 s off) procedure. After the sonication, the solution turned black and was stable for a few hours without any noticeable precipitation. In the process of recovering Ag-GO, the mixture was transferred to a round-bottom flask and DMF was removed using a rotary evaporator. The remaining solid material was transferred to a centrifuge tube where it was washed with DI water and ethanol five times. The ethanol was dried by blowing nitrogen across the surface of solution, and the final Ag-GO product was recovered as a black powder.

GO and Ag-GO Film (Paper) Preparation. In the film (paper) fabrication of GO and Ag-GO, a suspension of GO or Ag-GO in DMF (0.7 mg/mL, 19 mL) was filtered through a PVDF membrane (Whatman, 0.45 μm, 47 nm diameter). The thin layer of the film formed on the membrane was then subsequently peeled away.

Preparation of GO Film onto a Glass Slide. Graphene oxide (GO) suspension was obtained by sonication of the obtained GO powder in water (~250 μg/mL). Then 40 μL of GO suspension or about 10 μg of GO per slide was spotted using a micropipet onto a glass slide, and the slide was allowed to evaporate in a fume hood to result in a thin GO film on the slide. For the blank control slide, ultrapure sterile water was spotted and allowed to dry. Glass slides with or without GO film were placed into a culture dish (10 cm in diameter) and treated with UV irradiation for 1 h.

Coating of PVDF Filters with GO. PVDF filters (0.22 μm) were coated with a 100 and 300 μL GO suspension containing 25 and 75 μg of GO, respectively. Filter coating took place by releasing the GO solution evenly using a circular motion from a micropipet. The GO-coated filters were allowed to dry in the laminar flow hood under sterile conditions. Once dried, the GO-coated filters were used in the growth bioassay experiments.

Bacterial Cell Culture. *Escherichia coli* strain JM109 was routinely grown in Luria–Bertani (LB) broth or solid media at 37 °C for 16–20 h with or without agitation depending if the study used broth or solid media. The bacteria stocks used to inoculate the different assays were produced as described and the cell level quantified by quantitative real-time PCR. Once the bacterial stocks were quantified, assays were inoculated at a specific starting cell concentration. Bacterial assays were allowed to incubate at 37 °C for 16–18 h before measurements were taken.

Mammalian Cell Culture. Colorectal adenocarcinoma (HT-29) cells were routinely cultured in Eagle's minimum essential medium (EMEM) (ATCC Manassas, VA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin). The cells were cultured at 37 °C in a humidified atmosphere with 95% air and 5% CO_2 for 48 h (reach 85% of confluency) before subculture.

In experiment, 6×10^5 cells were seeded into the culture dish which contained the glass slides (with or without GO film) and incubated to allow the cells for attachment and development on the glass slides. At various time intervals, the morphology of cell growth was taken under a microscope (Motic) supplied with a camera.

Quantitative Real-Time PCR Analysis of Genomic DNA. Real-time PCR analysis was performed on bacterial genomic DNA samples using the CFX real-time PCR system (BioRad, Hercules, CA) with a two-step amplification program with post-amplification melt curve analysis as described by Ruiz *et al.*[29] The 16S gene-specific real-time PCR primers and synthetic oligonucleotide standard were developed. The synthetic oligonucleotide was serial-diluted from 1×10^8 to 1×10^4 copies/μL and used as standards for absolute quantification purposes. Real-time PCR sample reactions were produced by preparing a master mix containing the 16S gene-specific primers, BioRad SYBR Green SuperMix, water, and the appropriate sample DNA.

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