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Evaluation of Varying Graphene Oxides through their Physicochemical Characterizations on Erythrocytes, HS27 Cells, Escherichia Coli and Staphylococcus Aureus Bacteria Cell Lines, to Assess Graphene Oxides Cytotoxicity.

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EVALUATION OF VARYING GRAPHENE OXIDES THROUGH THEIR PHYSICOCHEMICAL CHARACTERIZATIONS ON ERYTHROCYTES, HS27 CELLS, ESCHERICHIA COLI AND STAPHYLOCOCCUS AUREUS BACTERIA CELL LINES, TO ASSESS GRAPHENE OXIDES CYTOTOXICITY.

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DEDICATION

To my mother Ms. Aldaz and my brother David

Exodus 23:25

With Love

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by

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Nonetheless, I also want to thank my colleagues, as they were part of my master's journey, as their help was very valuable to this investigation. Their experience together with their efforts, made this journey more agreeable especially during the biological assessments as is normal to witness the accumulation of problems as the investigation reaches to an end.

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Tu Puedes

ABSTRACT

Research on the toxicity of Graphene Oxides (GO) has captivated interest in the field of material science, environmental sciences, and medicine for their avail significance in biomedical engineer applications either as integrates, enhancements, or in the making of medical devices. Moreover, there is still a lack of understanding as to what characteristics on GOs causes them to be cytotoxic at a cellular level. In our study we synthesized four different GOs by varying in both the method used for oxidation (Modified Hummer's method & Improved Marcano-Tour's method), and the precursor parent graphite, for a standardization approach to aid in the making of less toxic GOs. In detail, our GOs differ from each other from lateral size (Lc and La), surface area, sp^2 hybridized carbon, interlayer spacing (d), and surface oxidation. Each GO with their individual characteristics had a different cytotoxicity intensity when in contact with biological specimens. Cytotoxicity was shown to be influenced by GO affinity onto the outer surface on cells which ultimately led to cell death. To test for toxicity, the investigation qualitatively assesses GOs effects on erythrocytes, fibroblast, E. coli and S. Aureus cells. The variation of cells aimed to further the cytotoxicity understanding of each Graphene oxide, as well as identifying a relationship between cytotoxicity and GOs physiochemical characteristics. We believe our comprehensive report is first of its kind to find a relationship between the different GOs varying by their physiochemical characteristics on biological assessments (where size and functionality were compared), and cytotoxicity, by the measurement of viable cells.

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INTRODUCTION

Graphene Oxides (GO) are two-dimensional carbon material, with a honeycomb pattern (Achawi *et al*.), synthesized from graphite usually by the Hummers method for oxidation [1]. From all the Graphene members, GOs have been considered to a certain extent hydrophilic which is an important property to suspend graphene oxide sheets in water. The hydrophilicity in GOs is considered important when in contact with biological specimens, as failure to properly disperse (in aqueous solutions), may alter toxicological properties such as agglomeration, which hinders the nutrient uptake of cells [2] (Ruiz *et al*.). It is inevitable to study GOs, as they are topic of interest in different sectors such as biotech [2] (Ruiz *et al*.), pathogen control [3] (Wang *et al*.), biomedicine[1] (Tu *et al*.), industry [1] (Achawi *et al*.) and biomedical applications. The growing interest in GOs may be attributed to their excellent mechanical strength, conductivity [1], and thermal properties [2,9] (Ruiz) (Deemer *et al*.).

To this day it is unclear what physiochemical characteristics drive GOs to exhibit cytotoxicity to cells and the in the literature on GO toxicity several competing theories have been propose. Some studies, for instance, claim that high oxidation is more cytotoxic than low oxidation while others point to a lower toxicity for highly oxidized graphene. In addition, comparing and correlating the lateral dimensions of GOs to cytotoxicity has been difficult. Ashawi *et al*. systematically reviewed 93 articles based on the graphene-based materials and extensively determined that graphene materials have not been studied enough with respect to variation in GOs.

The investigation did not identify the toxicity factors which could be correlated to the induction of cytotoxicity within the cells, hence it was inconclusive to determine the causation(s)

that contributed to the cytotoxicity induced by the GOs [1]. As mentioned, the existing toxicology research done with GOs is still developing and the research performed thus far has yet to improve guidelines for determining the various physiochemical characteristics together with a specific biological endpoint; meaning that previous research on the exposure of biological specimens to GOs only measure one physiochemical characteristic from GOs or any other graphene member, thus lacking specificity.

It is very important to clearly specify the physiochemical properties and determine how the variation of the GOs influences cytotoxicity. In addition to the characterization and the variation in GOs, the selection of a biological endpoint (to display or deduce a toxicity motif), to help understand what influences each GO to induce cytotoxicity onto cells is also important to understand how structure and oxidation is related to cytotoxicity. Although it is crucial to carefully evaluate the physiochemical properties that appear to have an impact on toxicity, it may be considered a challenge due to the limited data available (Achawi *et al*; Ruiz *et al*.). For this reason, many studies have resorted on modifying their GOs surface to evaluate only the physiochemical trait of their composites without any further variation resulting from the GOs oxidation method and size. Others may argue that by functionalizing their GOs by the means of surface modification (by adding compounds onto them to make them less toxic), and later compare them with an ordinary graphene oxide, is also a form of physicochemical characterization. Their findings may have portrayed significant compatibility results, but the question still lies if the new added functional compounds serve as enhancements that help emulate biocompatible without really testing the true toxicity of their GOs. Independently of these observations, as reported by Ruiz *et al*., there might be a tradeoff by enhancing GOs, as they run the risk of becoming defective.

Nevertheless, in our efforts to find a relationship between Graphene oxide physiochemical properties and a biological end point, it was of great importance to choose cell lines that best suited the investigation, for the cytotoxicity assessment. The regulatory guidelines of ISO-10993 (Biological Evaluation of Medical Devices), which have standardized and developed methods to measure the toxicity of nanoparticles, were considered when we started the cell exposure to our GOs in vitro. Having these guidelines in mind, we decided to test with four different cell lines, with the purpose of understanding the cytotoxicity induced from our different GOs [5].

To better understand GOs interactions with Eukaryotes, we decided to use erythrocytes, commonly known as red blood cells (RBCs), and fibroblast cells. RBCs are un-nucleated cells that pass through the circulatory system and involved in perfusion in the capillaries to deliver their oxygen content. RBCs were important in the investigation for insight on the repercussions resulting from the GOs on the cellular level. Furthermore, fibroblast cells are spindle-like, adherent cells when healthy, and are the first line of defense from harmful organisms and/or molecules. The eukaryotes used in this investigation were assessed in vitro, through incubation with the varied GOs (H4, H3, I4, I3).

As mentioned in Achawi *et al*., in vitro assessments are the best route for GOs cytotoxicity studies with mammalian cell lines. As described in Ruiz *et al*., it is mandatory to use non-toxic GOs for biological applications and in medicine if GOs are to be used for therapeutic purposes like drug delivery. Additionally in Fonseca *et al*.; Xie *et al*.; Jaworski *et al*., they mentioned that if GOs are to be used for drug delivery, it is necessary to do toxicity assessments with GOs and the cells related to the circulatory system. According to Fonseca *et al*.; Xie *et al*.; Jaworski *et al*., GOs cytotoxicity induction onto the cells may have adverse effects on organs

such as the liver as the GOs travel through the circulatory system, and/or reduce the possibility to reach the target cell if GOs are used for drug delivery.

Therefore, it was essential for this investigation to assess GOs cytotoxicity in vitro testing, as in vitro models would mimic human exposure and health effects if cells are exposed to GOs (Achawi *et al*). Additionally, the study of mammalian cell lines in this investigation is relevant in medicine, because if GOs were to be used in drug delivery via intravenous, GOs would have to prove to be non-toxic to the cells in the circulatory systemic system. If GOs toxicological assessments are not done with cells that are in relation with the circulatory system such as RBCs, it would adversely affect vital organs such as the liver, as this organ filters the blood from the circulatory system, thus carrying series implications to the organism. Consequently, assessment through the exposure of the GOs used in this investigation with mammalian cells in vitro, may help the scientific community, as the investigation may point the physicochemical characteristics that influences GOs cytotoxicity. Doing in vitro studies with human cells can be said to be the best approach to help standardize GOs, as of recent research there is no study that identifies the correlation of GOs physicochemical characteristics to a biological endpoint [1,2].

This investigation is focused on the physicochemical characteristics that influence GOs toxicity on eukaryotes and prokaryotes. To further analyze the interactions between prokaryotes and GOs TEM was used which helped visualized the effects GOs had onto the bacteria. Notably, there is a demand for an antibiotic that helps treat and contain the drug-resistant bacteria from becoming a bigger threat, hence accentuating a path for researchers to study the anti-bacterial properties of graphene oxide. In papers where Graphene Oxide nanoparticles are used as to enhance antibiotics, as discussed in Zheng. H. *et al*., have demonstrated promising results [6]

which indicate that antibiotics enhanced with GOs have proven to not only kill bacteria but to stop bacteria becoming resistant [6].

From previous research it has been sought to analyze the surface interactions from both cells and GOs to further analyze the cytotoxicity properties induced by the GOs [4,6,7]. Therefore, in this investigation, E. coli bacteria (gram-negative) was utilized to analyze its surface interactions with H4, H3, I4, I3. As discussed in Tu *et al*., the attraction between GOs and membrane lipids on E. coli are due to the strong Van der Waals interactions between the sp2 bonds and the phospholipids on the outer membrane, hence damaging the cell wall [4]. The attraction between the GOs and E. coli, result in the absorption and/or extraction of lipid molecules. Through the GOs absorption affinity onto lipids it was hypothesized in this investigation that the surface chemistry on GOs might have been the cytotoxicity source that caused bacteria to be lysed. Therefore, in this investigation the antibacterial assay on E. coli was used to help identify the physicochemical characteristics from the GOs that makes them cytotoxic to E. coli.

Following, S. aureus, a gram- positive bacteria commonly known for their thick peptidoglycan cell wall (described as the surface of the bacteria), was used to also help quantify GOs cytotoxicity induction on prokaryotes. As mentioned in Caudille *et al*., due to the S. aureus' unique membrane physiology, the point of contact on these bacteria with any molecule is on its peptidoglycan cell wall, which contains Teichoic Acid (TA). As explained in Wang *et al*., the absorption affinity of TA, onto GOs via the π-π interactions between them, was discovered to be the cause of cytotoxicity or killing mechanism of the GOs onto the gram- positive bacteria. In Caudille *et al*., it was also mentioned that due to TA being a negatively charged component in the cell wall was therefore highly attracted to cationic surfaces on molecules, contributing to S.

aureus cell death [3]. Therefore, it was deduced in this thesis report that the toxicity in the S. aureus, would have been caused by the GOs that had a hydrophobic nature, as it would facilitate its surface affinity to TA. Thus, in this investigation the antimicrobial assay was preformed onto the S. aureus, to identify from the GOs their cytotoxicity motive through GOs physicochemical characteristics.

Here in this study, cell viability and cytotoxicity of GO was measured with respect to two eukaryotes cell types: erythrocytes and fibroblasts, and two prokaryotes cell types: E. coli and S. aureus. In this thesis, GOs were compared with each other to assess their individual cytotoxicity with the selected cell lines. It was essential to compare them as the GOs were synthesized from two different parent graphite's and two different oxidation methods, differentiating in size and oxidation, respectively. Additionally in this study we also focused on the physiochemical characteristics such as the lateral sides, surface area, thickness, and surface oxidation of our GOs. In our opinion, this report is first of its kind in evaluating cytotoxicity (which we considered it our biological endpoint) [1], and the physiochemical characteristics of our varied GOs with respect to concentration of GOs.

1. METHODS AND MATERIALS

1.1. GRAPHENE OXIDE SYNTHESIS AND CHARACTERIZATIONS

The graphene oxide samples were synthesized and purified as described previously [9]. Marcano-Tour's improved method [10] was used to synthesize GO in which we abbreviated here as IX, where X denotes the parent graphite source. To assess different graphite oxide sizes we used two different parent graphites G3 and G4. Sample are labeled according to their respective oxidation method and are denoted by H or I corresponding to modified

Hummer's method [9,11] and Improved Hummer's method [10], respectively. In addition, graphene oxide sizes are varied by using different graphite starting material and are denoted by 3 and 4 according to the respective starting graphite sizes. All GOs used for the cytotoxicity experiments were prepared by diluting stock solutions. The stock solution was made by sonicating Graphite Oxide directly in 1X Phosphate Buffered Saline (PBS) with a total concentration volume of (0.5mg/mL) and into desired concentrations in 96 round bottom well plates. The variety of concentrations were prepared with 1X PBS using 0.5mg/mL as the initial and maximum concentration then decreasing to concentration as follows: 0.4 mg/mL, 0.3 mg/ml, 0.2 mg/mL, 0.1mg/mL, 0.05 mg/mL, 0.01 mg/mL, 0.005 mg/mL, 0.0025 mg/mL.

Table. 1. XRD measurement summary of the as- synthesized Graphene Oxides with 2Θ maxima, interlayer spacing (d), Length of axis (L_c), and Length of axis (L_a) (source: Deemer *et. al*. 2018)

Sample	2Θ(degree)	*d* (Å)	L_{c_c} (nm)	L_a (nm)
G3	27.04	3.39	12.8	6.7
I3	11.33	7.80	8.5	3.7
H3	11.43	7.73	6.0	2.8
G4	27.21	3.27	18.4	24.6
I4	11.55	7.65	3.1	4.2
H4	11.43	7.73	12.7	14.1

1.2. ESCHERICHIA COLI AND STAPHYLOCOCCUS AUREUS CYTOTOXICITY ASSAY

Escherichia coli and Staphylococcus aureus bacteria were grown in Mueller Hinton Broth. Optical Density was used to measure bacterial growth, O.D. of 0.8 and 1 was used. After bacteria was grown, it was transferred to a conical tube. Bacteria then was stained fluorescent using BacLight™ Green Bacterial Stain (Thermo Fisher) following the manufacturer's protocol.

After staining, the conical tube was protected from light, then 1 mL from the labeled bacteria was added to wells containing five distinct GOs with different concentrations ranging from (0.5 mg/mL to 0.0025 mg/mL) in a 96 well round bottom plate. Gentamicin antibiotic and sterile 1X PBS were used as control for both bacteria. The plates were incubated for 24 hours at 37 ° C. After incubation, all plates were read in the Fluoroskan™ FL Microplate Fluorometer and Luminometer with an excitation range reading of 485-527 emission.

1.3. HUMAN ERYTHROCYTES HEMOLYSIS ASSAY

Human whole blood samples were collected from different donors under the approved IRB protocol 1424274-2 Isolation of Human blood cells for pre-clinical biomedical research. Around 5 mL of whole blood was collected and immediately added to 10 mL of sterile 1X PBS in a conical tube, then centrifuged at 500 g for 10 minutes at 4 ° C in order to isolate the red blood cells (RBC's) from the serum. This process was repeated for a total of five times. Then, washed RBCs were diluted to 25 mL in 1X sterile PBS. Using a 96 well round bottom plate, dilutions of diluted RBCs were added to different concentrations ranging from (0.2 mg/mL to 0.0025 mg/mL) of the five distinct GOs compounds. Deionized water and RBCs was used as a control and PBS and RBCs was used as control as well. Samples were incubated for 3 hours at 37 ° C. After incubation, the samples were centrifuged at 10,000 g for 4 minutes at 4 °C. Supernatant from the RBCs was collected and transferred to a 96 well flat bottom plate. Hemoglobin absorbance was measured at 540 nm with 655 nm as a reference, using VersaMax™ microplate reader (VWR).

1.4. SKIN FIBROBLASTS

Cell culture

HS27 cells were grown using Dulbecco's Modified Eagle´s Medium (DMEM) media supplemented with 10% Fetal Bovine Serum (FBS) and 100 U/mL of penicillin and 100 µg/mL of streptomycin. Cells were grown under aseptic conditions in incubators at 37°C in a humidified environment with 5% CO2 and were maintained at a confluency of 80%.

WST-8 assay cell's proliferation assay.

HS27 Cells were seeded in 96-well plates at a density of 1x104 in a volume of 100 µL of media per well and were left overnight under cell culture conditions. Next, cells were treated with different concentration of the GOs used in this study (H4, H3, I4, I3), at varied concentrations ranging from 2.5 µg/mL- 200 µg/mL for a period of 24h. The media was removed, then the cells were washed three times with basal media. After the three washes the cell were incubated with WST-8 mixture following manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Following the incubation with the WST-8, the plate was read by using a spectrophotometer measured at 450 nm with 655 nm as a reference, where the results were plotted by using the OriginLab 5.0 software.

1.5. ELECTRON MICROSCOPY CHARACTERIZATION OF BACTERIA WITH GRAPHENE OXIDE

Samples were prepared for Scanning Electron Microscope (S-3400N, Hitachi) analysis by depositing 25 microliters of sample on round coverslip and submerging in 2.5% glutaraldehyde solution for 2hr in a well plate. Solutions were removed by pipetting from the wells and the samples were rinsed with a phosphate buffer solution before dehydrating in 50% alcohol solution, 80% alcohol solution and finally 100% alcohol. A 1:1 solution of 100% alcohol and HDMS was added to the samples and removed. Finally, pure HDMS was added to the samples in the well plate and

left to dry for 12 hr overnight. Prior to observation, samples were sputter coated (Desk IV, Denton Vacuum LLC) using a platinum and carbon source.

Samples were prepared for observation under the Transmission Electron Microscope (H-7650, Hitachi) by depositing 10 microliters of each sample onto lacey carbon copper grids (Ted Pella) with formvar and rinsing with DI several times. Finally, samples were stained using 10 microliters of solution of uranyl acetate (2.5%) and dried.

2. RESULTS

2.1. GRAPHENE OXIDE

Table 1 outlines GO sample's physicochemical properties as previously characterized by powder X-ray Diffraction spectroscopic methods (Deemer *et al.*). Thermal Gravimetric Analysis (TGA) and Fourier Transform Infrared (FTIR) are shown in Figure 1 along with Atomic Force Microscopy (AFM) taken of the as synthesized GO samples. Oxygen functionalities present in graphene oxide (GO) samples recovered after oxidation and purification were analyzed using X-Ray Diffraction (XRD) Thermal Gravimetric Analysis (TGA) and Fourier Transform Infrared (FTIR) spectroscopy as described previously in Deemer *et al.,* 9]. The results from FTIR spectroscopy taken of the as-synthesized GOs clearly revealed the presence of several vibrational bands which are attributed to the oxygen functionalization in the synthesized GO samples. The spectra confirmed in the as-synthesized samples peaks which are observed at 1737 cm^{-1} from C=O stretching vibration of carboxyl group, peaks at about 1390 cm^{-1} from O-H deformations, C=C stretching vibration at 1620 cm^{-1} attributed to the non-oxidized graphite sp^2 hybridized carbon vibration [12] and epoxy C-O-C stretching vibrations are observed at 1240 cm^{-1}. All the vibrational bands are consistent with previously reported GO results [13, 14]. The TGA studies performed (Deemer *et al.*) confirm the FTIR oxidations and show three distinct stages during the thermal decomposition of GO sheets. The first loss occurring around 100°C is primarily due to the loss of

H_2O molecules intercalated between the GO sheet layers. The thermal decomposition due to instable oxygen-containing functional groups occurs around 225°C. Finally, a loss occurs above 620°C as a result of combustion of the carbon skeleton [15]. Remaining mass of all the GO samples at 650°C are summarized in **Table 1**. Where any remaining mass above 625ºC is due to the remaining carbon skeleton. These results reveal the thermal stability of the sp^2 carbon structure in GO samples (prepared via both Marcano-Tour's and Hummer's methods). From these observations, it is obvious that Marcano-Tours method produced smaller and more oxidized samples by the fact that there is less mass in each I-GO sample than the H counterpart above 650ºC. H3 and H4 samples have the more remaining mass than I3 and I4, respectively, indicating a high content of sp^2 carbon that is stable as the remaining mass shows H4 > I4 > H3 > I3 and demonstrates the consequence precursor graphite size has on retaining the carbon skeleton [9]. X Ray Diffraction Analysis performed by XRD was used to determine the dimensionalities all the GO samples, including parent graphite samples G3 and G4 [9].

2.2. HEMOLYSIS

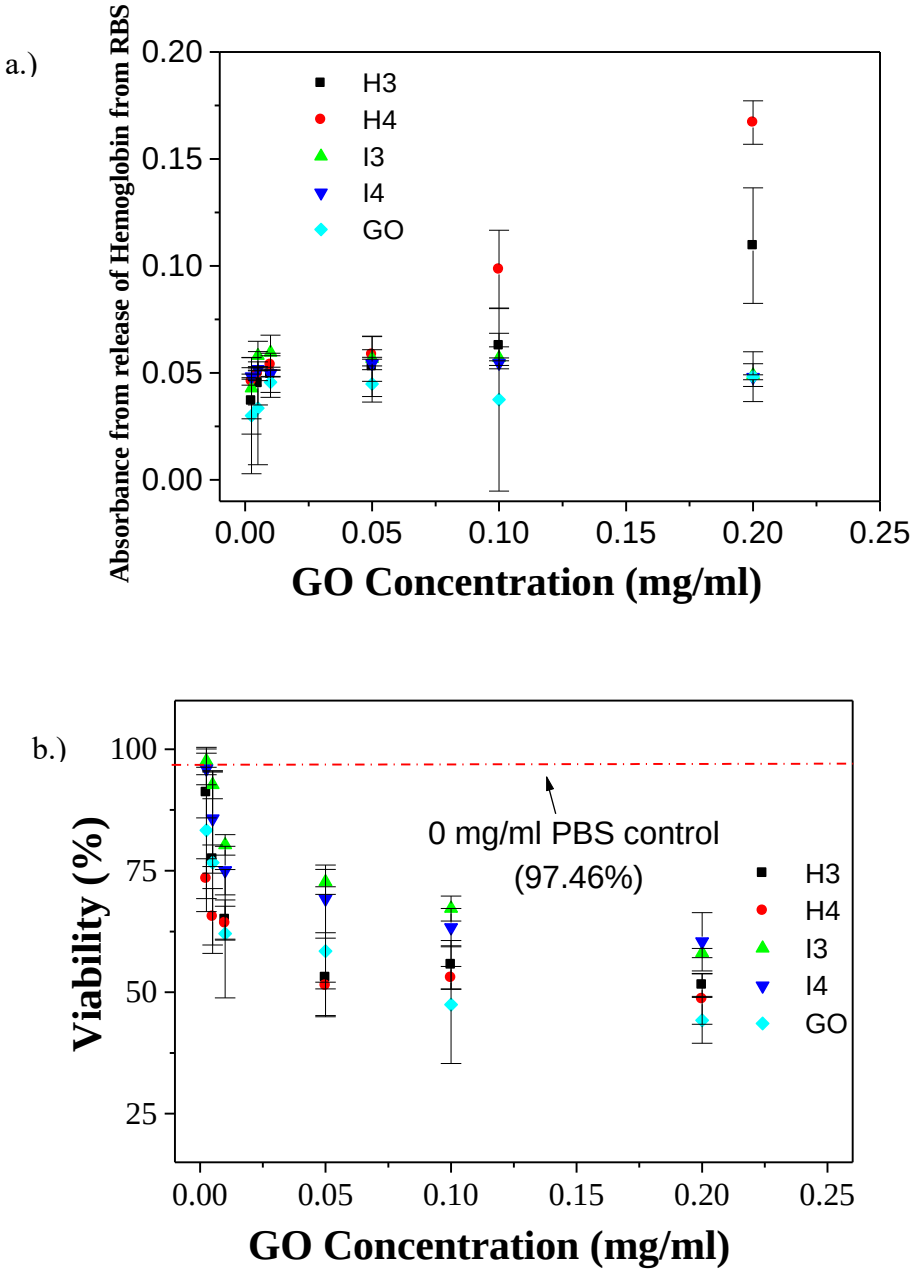


Figure 1. Absorbance from Release of Hemoglobin from Red Blood Cells (RBCs) (a) and Percent viability of Fibroblast cells (b) as a function of GO concentration exposure

As observed in **Figure 1.a**, the hemoglobin absorbance (from the RBCs) measurement from the Optical Density taken from different GOs at various concentrations, is significantly higher in H3 & H4 as compared to the other GOs from this study. The high hemoglobin absorbance indicates that there were more lysed RBCs due to the hemolytic properties exhibited from the H3 & H4. In disparity, I3 & I4 exhibited minimal hemolytic properties, as less hemoglobin absorbance was measured from the O.D. even with increasing concentrations.

By examining **Figure 1.a**, the GOs synthesized from the modified Hummers method showed higher hemolytic effects as seen in H4 and H3, only differing from each other by the precursor graphite used for their synthesis. When comparing H4 as synthesized from sieved graphite flakes (420µm in size), to H3 as synthesized from sp-1 graphite powder (<100µm in size), both obviously differed from each other, as H4 was comparably bigger than H3. This is good to note since according to our results, H3 was still more cytotoxic than H4, regardless of the size difference.

In comparison to the previous GOs, I4 and I3 synthesized from the improved Tour's method show evidence of a higher cell survival. As both abbreviated with an I for Improved Mercados-Tours (method used for their synthesis, and oxidation), they have similar sizes as previously reviewed in **Table 1.** The precursor graphite used in I4 (sieved graphite flakes, size 420µm) and in I3 (sp-1 graphite powder, size <100µm), can be suggested that precursor size when the Improved Mercados-Tours oxidation and synthesis method had no influence in RBCs survival, as seen in **Figure 1.a**. as they both showed little hemolytic activity, when compared to the negative control (PBS).

Therefore, it can be concluded from the graphical analysis that size of any graphene oxide, had a significant influence on inducing cytotoxicity on RBCs. This is supported by comparing the GOs synthesized from the Hummers method, where H3, which is relatively smaller due to a smaller starting precursor graphite, was still more cytotoxic than H4 in comparison (**Figure 1.a**). Furthermore, little hemolytic activity was observed from either I3 or I4, and the same observation can be made when they were compared to the negative control. Thus, calling to attention another

physiochemical characteristic from both GOs, as the hemolytic factor or the property in GOs that induce cell death. To further explain, size may not be the cytotoxicity factor, rather it may be inferred that oxygen content and the surface of GOs carries more weight with regard to the cytotoxicity inflicted. This can be observed through our findings, as the GOs which were synthesized from Hummer's method (H4 & H3), had the more sp^2 carbons and less oxygen-functional groups on their surface (**Table 1.**). In contrast, the GOs which were synthesized from the Improved Tour's method had more oxygen-functional groups and less sp^2 carbons (**Table 1.**). These characterizations of the GOs based on the oxidation method used, signal a probable relationship between the oxygen-functional group content on GOs surfaces, and the hemolytic effects on RBCs. Once again, H3 and H4 (differing only from their size) were the more hemolytic, in comparison to I3 &I4, as they (I3 &I4) were described as the more functional, than the GOs synthesized from the Hummers method.

As seen in **Figure 1.a**, the aliquot of 200μg/mL was observed to be the dosing concentration threshold, where cells can survive before the cells start dying rapidly. This can readily be seen in H3 & H4 which most express cytotoxicity on RBCs. This observation served as an evaluating factor on our study as this threshold concentration was similar on the results from other research papers. This threshold concentration, as seen in our results were similar to other papers from their non-functionalized GOs, making reference to the GOs that are not modified or don't have nano-compounds added to them. Nonetheless, our results do correlate with the current literature on hemolytic results [16-18].

As results point out, H3 is more hemolytic than H4 and from the other GOs (synthesized by the Improved Mercados-Tours method), giving us an order starting from the most hemolytic to the least cytotoxic GOs H3> H4> I4> I3. The low hemolytic properties presented by both I4 as well as in I3, demonstrate a much lower hemolysis rate than what the current literature denotes. This work demonstrates that I4 &I3 were even less cytotoxic than those who have done modifications to their GOs [16-18] to make them more hemocompatibility.

Thus, these results may infer that the method used to oxidize GOs, could be the determinate factor for hemolysis rates, as it can be assumed that oxygen content on GOs matter the most in comparison to size. As previously shown (Jaworski, S *et.al.*), when comparing Pristine Graphene (GN), Graphene Oxide (GO), and reduced Graphene Oxide (rGO), demonstrated the importance of both surface chemistry as well as graphene size for hemolytic determination. In their study they explain that due to GOs properties such as its hydrophilic nature, together with its stable hydrocolloids [19], results in making graphene oxide out of all graphene members, the more hemocompatibility. Hence, it can be inferred that a higher surface oxidation with minimal sp^2 carbon content (both physiochemical characteristics of GOs) on GOs, may aid with hemocompatibility on the erythrocytes (RBCs).

2.3. WST-8 ASSAY

To assess if GOs were cytotoxicity with skin fibroblast cells, WST-8 assay was used to help measure the cell metabolic activity in the cells. The WST-8 mostly used as an indicator for cell viability, served in our invitro experiment. The WST-8 was used as discussed in the methods and procedures, where the cell viability was determined by the levels of mitochondrial activity and later to be read by the optical density. The results go as follows as seen in **Figure 1.b.**, to which the data was gathered after a 24h incubation period between the different GOs and the fibroblast cell line. In the **Figure 1. b**., cytotoxicity is more prevalent on GOs synthesized by the Hummer's method (H3, H4) together with the commercial GO. It can also be said that the least cytotoxic of all GOs were determined to be the ones synthesized by the Tour's method, I3 and I4. It can also be said, and as discussed in the hemolysis assay, that the determinate factor for the viability of the fibroblast cells were to be oxygen content on GOs, as the nanoparticles tend to intercalate with water. When discussing oxygen content or functionality as the determinate factor for the viability of the fibroblast cells, the results can also be pointed out and discussed by Hussein, K. H *et al*., as

well as in other studies [20]. As mentioned by Hussein, K. H *et al*., the more functionalized or the more oxidized a graphene oxide is, the higher of viable cells there will be.

2.4. EFFECTS OF THE VARIED GOs ONTO MAMMALIAN CELL LINES

From **Table 2**., the EC50 results on the fibroblast cell line varied, as each GO induced cytotoxicity at different concentration levels, which may have been contributed by each GO physiochemical characteristics. As seen in **Table 2**., the EC50 of I3 is observed at .05 mg/mL concentration, and in comparison to the rest of the GOs EC50 results, I3 can be said to have more viable cells, as supported by **Figure 1.b**. Subsequently, the EC50 of I4, H4, and commercial GO, can be noted to fall on .01 mg/mL concentration, hence making these GOs more cytotoxic than I3, thus corroborating to the previous findings in **Figure 1.b**. Finally in contrast to the EC50 results of the previous GOs, it was observed in H3 that its EC50 was at the concentration of .005 mg/mL, inferring that H3 had the highest cytotoxicity from I3, I4, H4, and commercial GO, thus corroborating to the results in **Figure 1.b**. Additionally on the GOs cytotoxicity assessments in mammalian cell lines, it is observe in **Figure 1.a**., that erythrocytes appeared to not titer when in contact with I3, I4, and commercial GO, therefore there was no significant EC50 results to report in **Table 2**. In comparison, the results in **Figure 1.a**, demonstrated comparable results in H4 and H3, as erythrocytes were able to survive at a higher concentration when in contact with H4 (.2 mg/mL) than in H3. As **Table 2.** suggests, the EC50 results of the erythrocytes when in contact with H3, stands at the concentration of .1 mg/mL. Hence, the results from **Table 2**., may infer that erythrocyte had a higher tolerance to H4 than when the cells were in contact with H3, thus corroborating to the findings in **Figure 1.a**, were H3 is considered the most cytotoxic of all GOs as the graph suggests.

Table 2. Cytotoxicity activity from the varied GOs based on Optical Density (O.D) to measure the EC50 from the mammalian cells for concentrations ranging from 0.2 mg/mL to 0.0025 mg/mL

GO	Erythrocytes		Fibroblast cells	
	EC50	Concentration	EC50	Concentration
I3	n.d	n.d	0.9974167 O.D	.05 mg/mL
H3	0.0732125 O.D	.1 mg/mL	1.08714167 O.D	.005 mg/mL
I4	n.d	n.d	0.9941667 O.D	.01 mg/mL
H4	0.10655 O.D	.2 mg/mL	1.11605 O.D	.01 mg/mL
GO	n.d	n.d	1.19845 O.D	.01 mg/mL

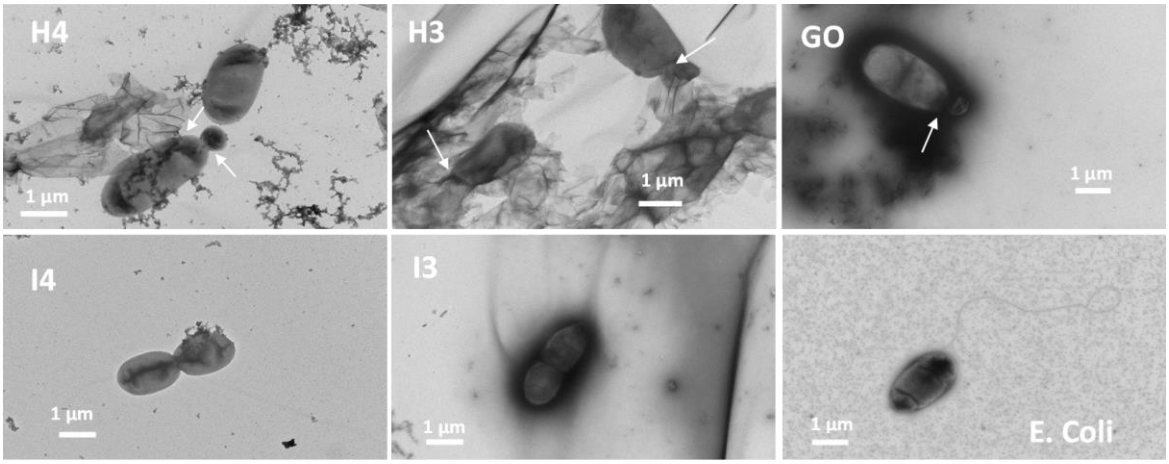
2.5. ANTIBACTERIAL ACTIVITY

In recent studies it has been demonstrated the manifestation of GOs bactericidal properties, as it successfully inhibits the growth and viability of bacteria (when in contact with a GO), and even more enthralling is that this antibacterial phenomenon is also observed in multidrug resistant bacteria (Zheng *et al*.) [3, 4, 6, 21]. By examining the surface chemistry of the GOs used in this study and study them with the bacterial surfaces, the antimicrobial properties imposed by our various GOs can be quantified.

The following experiments done to determine the cytotoxicity effects on bacteria are examined by analytical analysis and by visualization using Transition Electron Microscopy (TEM). The bacteria were incubated for a period of 24h with the as-synthesized GO materials (H4, H3, I4, I3, commercial GO) for the examination of the analytical analysis and TEM images. The data is discussed from most to least toxic Graphene oxide as shown in (**Fig. 2.b. & Fig. 3.b**.), for evaluating the antibacterial performances of each. Following the graphical analysis results, GOs bactericidal effects on E. coli and S. aureus were then visualized using the TEM shown in (**Fig. 2.a & Fig. 3.a**.)**.** The findings are explained following the E. coli results being first discussed then by the S. Aureus bacteria.

2.5.1. E. COLI

a.)



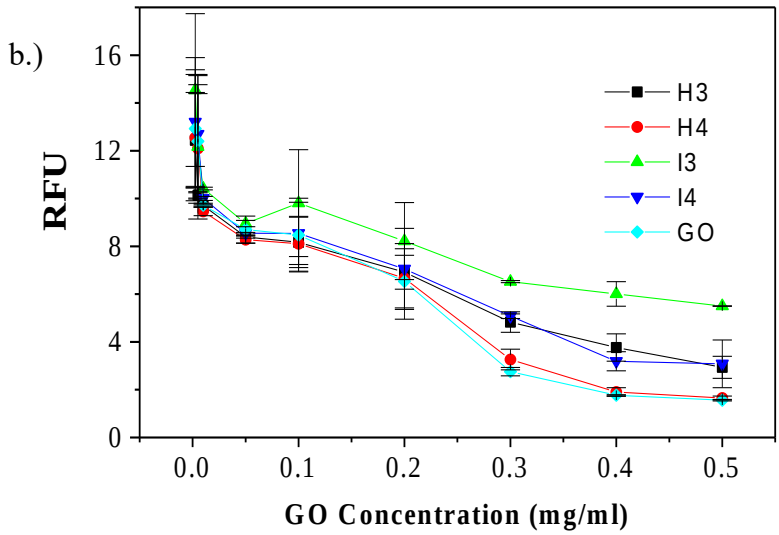


Figure 2**.** In vitro cytotoxicity analysis of E. coli exposed to varying Graphene Oxides. a) E. coli antibacterial graphical analysis after 24h incubation with concentrations ranging from 2.5 µg/mL-500µg/mL b) TEM images of E. coli's morphology after 24h incubation treatment with the varying GOs, at dosing concentration of 200µg/mL. In the TEM images, morphological changes on the treaded E. coli were tracked by the comparison of the untreated TEM images of E. coli. In the treaded E. coli the tracked changes were clarified through the white arrows, to point out the varying cytotoxicity effects from the contact with GOs which might cause blebbing, deformations, and/or agglomeration.

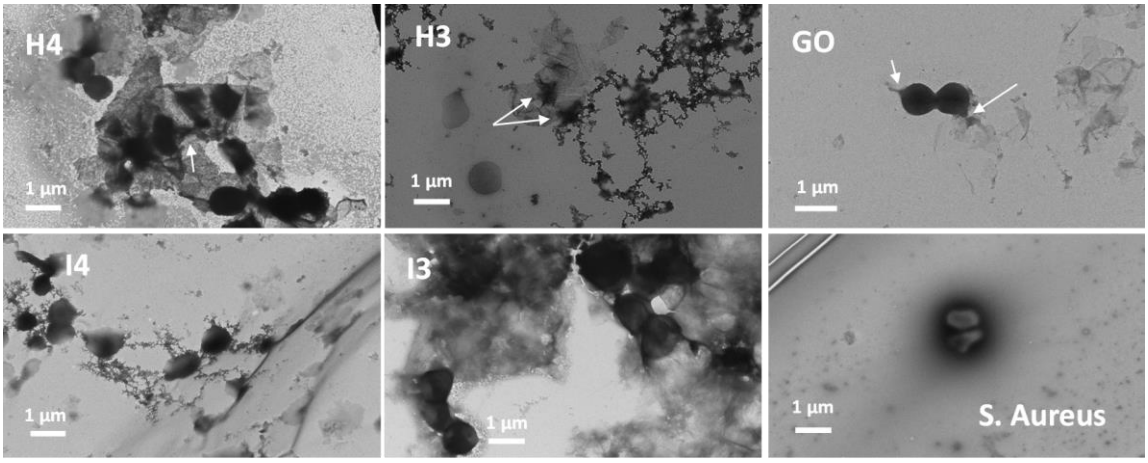
As shown in **Figure 2.b**., the E. coli (gram negative) bacteria suffered the most cytotoxic and anti-microbial repercussions out of all the GOs from H4. According to Tu *et al*., sp^2 carbons

facilitate dispersion interactions between graphene and lipid molecules, which as a result cell death or lysis in E. coli cells as the bacterial intracellular content leaks out. This corroborates our findings where H4 was shown to be the most consistently cytotoxic GO. Previously reported characterization of the as-synthesized GOs (Deemer *et al*.) determined that the content of GO mass percent above 650°C determined by TGA corresponded to the remaining sp^2 graphitic regions of the GO and established GOs sp^2 domains were in order of H4 > I4 > H3 > I3 as summarized in **Table 1**. From the observations in **Figure 2.b**., we found that the anti-microbial and cytotoxic activity of the GOs corresponded with the sp^2 content ordered from high to low, as follows H4 > I4 > H3 > I3 [4, 9] (Deemer *et al*.; Tu *et al*.).

This corresponding order of sp^2 carbons (H4 > I4 > H3 > I3), forces one to note that precursor graphite plays a major role on the remaining carbon skeleton [9] (Deemer *et al*). It can be concluded that the size from the precursor graphite has more impact in GOs toxicity when tested with E. coli. GOs synthesized from the larger precursor graphite have the most remaining mass, thus having more sp^2 carbons. Therefore, one could suggest that higher quantities of sp^2 carbons correspond to bigger, less functionalized (unoxidized) regions and which leads to a less hydrophilic GO with more hydrophobic characteristic. Such physiochemical traits from the GOs may explain the phenomena revolving lipid extraction from the bacterial membrane on E. coli. It may seem that E. coli bacteria might have experienced lipid extraction from the Van der Waals attractions between the GO and lipid molecules [4] (Tu *et al*.), resulting in bacterial lysis (cell death). The structure of a membrane lipid allows its hydrophobic region to be attracted to the unoxidized region of the GO, thus creating a strong dispersion interaction among the bacterial membrane.

2.5.2. S. AUREUS

a.)



b.)

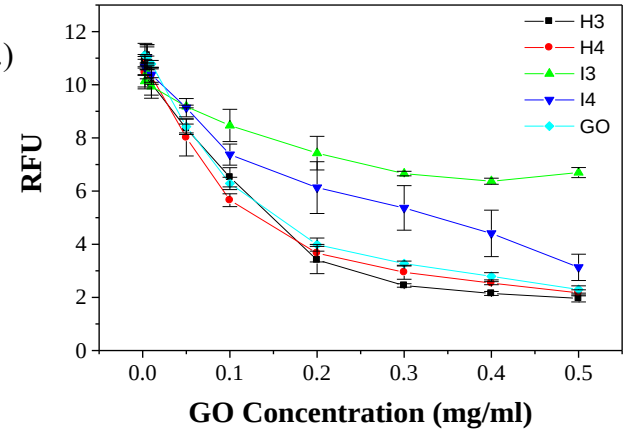


Figure 3. In vitro cytotoxicity analysis of S. Aureus exposed to varying GOs. a) TEM images of the morphology of S. Aureus after 24h treatment incubation with the varying GOs, at dosing concentration of 200µg/mL. In the TEM images, treaded S. Aureus morphology changes were tracked by the comparison of the TEM images of the untreated S. Aureus. The morphological changes of the treaded S. Aureus when in contact with the GOs were observed through the white arrows to draw attention to blubbling, explosive cell lysis, deformations, and/or agglomeration. b) S. Aureus bactericidal results by graphical analysis after 24h incubation with concentrations ranging from 2.5 µg/mL- 500µg/mL

In evaluating S. Aureus (gram-positive bacteria) post treatment with the various GOs in different concentrations, the bacteria experienced higher bactericide repercussions from H3 & H4, due to the interaction between the GOs and the S. Aureus unique membrane physiology (**Figure 3.b**). The interactions between the physical surface properties of the gram-positive bacteria

(characterized from its thick cell wall interlinked with Teichoic Acid (TA)), and the surfaces of the various GOs, indicate that oxygen-containing groups or functionalization, strongly influenced toxicity rather than GOs size. **Table 1**., highlights that H3 has a much smaller lateral (La) and perpendicular (Lc) size than H4, both having been synthesized from different parent graphites, but the oxidation performed on H3 and H4 was the same modified Hummers method. Because we used two different methods for oxidation, the quantities of oxygen-containing groups or functionalization varied on each graphene oxide, which endowed different cytotoxic results once in contact with S. Aureus when compared to E. Coli, where its viability depended on GOs size. Therefore, in **Figure 3.b**, we see in the analytical data that H3 followed by H4, exhibit high levels of cytotoxicity onto the S. Aureus, were regardless of size, as H4 being larger than all other GOs, H3 appeared to be more deadly or cytotoxic to the bacteria. This observation made from the comparison of H3 and H4, indicate that GOs size was not the main factor for the induction of cytotoxicity onto the S. Aureus. Additionally, it seems through the analysis of the graphical data in **Figure 3.b**, that higher oxygen containing groups such in I3, then followed by I4, are less lethal to the bacteria (respectively). The viability of the bacteria when in contingence with I3 appears to be higher in comparison to all other GOs (H3, H4 & commercial GO) when in association with S. Aureus. Our findings are supported by Zheng *et. al*., were high levels of oxygen-containing groups on GOs, were an influential factor in the viability of bacterium.

For the purpose concerning the cytotoxicity study, it is important to acknowledge the differences on cell wall composition of a gram-positive from a gram-negative bacterial strain, since differentiating would help understand the process taken by GOs for which they induce bacterial cell death. To further explain, GOs bactericidal effects can be attributed to the morphology of the bacteria, were its important to note that cytotoxicity from GO's disturbance on the cells, is induced differently according to the physical surface of individual bacterial cell lines [3, 6]. For instance, the toxicity element taken by the GOs onto E. coli is caried by the higher quantities of sp^2 carbons (on GOs), that allows for lipid extraction of the outer surface of the bacteria. This is due to E. coli's morphology, as is characterized for its thin peptidoglycan layer (formed by crosslinked

carbohydrate chains) [3], sandwiched by the inner membrane (formed by phospholipids) and the outer membrane (formed by phospholipids and glycoproteins), where the bacterial outer surface membrane is GOs point of contact [3]. Contrary to E. Coli, GOs are cytotoxic to the S. Aureus bacteria differently as the cell wall in gram-positive bacteria (as mentioned), depicts a unique compound called Teichoic Acid (TA) (only found in Gram-positive bacteria) which is made of polyglycerol units, situated in the thick peptidoglycan cell wall (made by crosslinked carbohydrate chains), and anchored by covalent bonds (Caudill *et al*). In this case the cell wall in S. Aureus is the epitome for antibacterial agents (further explained within this report), therefore it is of importance to acknowledge the cell's physical surface properties of each bacterial strain to understand its interactions with the various GOs.

In Caudill *et al*., it was proposed an antibacterial mechanism influenced by the S. Aureus TA component, which affects the degree of interaction with the physiochemical properties of GOs via the electrostatic forces. This interaction carried by the negatively charged TA component in the cell wall of gram-positive bacteria, is attracted to cationic surfaces of molecules [8]. Therefore, it may be inferred that TA may be attracted to the less negatively charged surfaces. Thus, for the purpose of this study, it may be inferred there will be more connections between the surfaces of the less negatively charged GOs and the S. Aureus bacteria for its gram-positive morphology.

Therefore, it can be said that our results corroborate the proposed findings in Caudill et. al., since H3 and H4, the GOs with the least oxygen functionalities, seem to be the deadliest. These two GOs would have interacted more with the TA since they have surfaces with more sp^2 hydrophobic domains that have a less negatively charged areas. After the interactions between TA and GOs, a series of events are followed from the antibacterial properties in GOs.

After the established interaction between TA with the GOs, it would then be followed by TA(s) getting absorbed onto graphene oxide flakes via π-π interactions [3] (Wang *et. al*.). TA absorption would then cause its deficiency on the peptidoglycan (cell wall), thereafter, dysregulating autolysin, which is an enzyme normally regulated by TA, followed by its activation.

Consequently, from the autolysin activation, peptidoglycan cell wall would have degraded, thus killing the bacteria as its membrane integrity would have been compromised.

According to these findings for both bacterial strains (**Fig.2. & Fig. 3**.), the least cytotoxic GO after 24h incubation with bacteria, was the I3. As seen in **Table 1**., I3 was the smallest, with the most oxidation and oxygen functionalization, compared to the other GOs tested. The physiochemical properties of the I3 may have influenced the survival of either bacteria, compared to the other GOs, as it may be that smaller GOs flakes are least likely to hinder the bacteria's ability to uptake nutrients [3,4]. The functionalization or oxidation traits in I3 might have helped bacteria survived since I3 had less sp^2 bonds (Note: unlikely for lipid dispersion to ensue) and of its hydrophilicity (as cells are majorly composed of water). According to the results it can be inferred that E. coli viability is dependent on the sp^2 bonds of GOs flakes since the sp^2 bonds quantity may have attributed to the cytotoxicity. The high cytotoxicity observed in the results rendered by the gram-negative bacteria (E. coli), is credited to larger GOs flakes, as they have higher quantities of sp^2 bonds. In contrast to a gram-negative bacterium, the toxicity in gram-positive bacteria can be attributed by the least functionalized GOs. Although both bacteria had different cytotoxicity factors, the data from the graphical analysis, was still consistent as the deadliest GOs where H4, GO, H3 and the least cytotoxic was I3.

2.5.3. EFFECTS OF THE VARIED GOs ONTO BACTERIAL CELL LINES

In **Table 3**., the EC50 determined that in E. coli and S. aureus cell survival varied, as each GO induced cytotoxicity differently depending on the cell line as seen in **Figure 2.b**. & **Figure 3.b**. It can be inferred that each GO exhibited cytotoxicity onto the two different bacterial strains, due to the varied physiochemical characteristics of each GO. The EC50 results on E. coli varied as it can be inferred that each GO had a different level of affinity onto the E. coli's outer membrane, therefore each GO varied in toxicity level. As seen in **Table 3.**, the EC50 of I3 and I4 was observed at .1 mg/mL concentration, and in comparison to the rest of the GOs, I3 and I4 had a low

cytotoxicity induction onto E. coli. Following, the EC50 of H3, H4, and commercial GO, is noted to fall on .2 mg/mL concentration, hence corroborating to our previous results in **Figure 3.b**., as theses GOs induced the highest levels of cytotoxicity onto E. Coli. Additionally in the **Table 3**., the EC50 results on S. aureus did not vary as previously though from GOs interactions with the cell wall of the bacteria, as mentioned in this investigation on the antibacterial assay result section of E. coli and in (Wang *et al.*). As observed in **Table 3**., the EC50 in I4 was .2 mg/mL, inferring that I4 was the least cytotoxic as bacteria was able to thrive within this concentration in comparison to the other GOs, as I3, H3, H4, and commercial GO, had an EC50 at the concentration of .1 mg/mL.

Table 3. Antibacterial activity from the varied GOs based on the fluorescence intensity from the bacteria to measure EC50 for concentrations ranging from 0.5mg/mL to 0.0025 mg/mL

GO	E. Coli		S. Aureus	
	EC50	Concentration	EC50	Concentration
I3	10.02025 RFU	.1 mg/mL	8.3817083 RFU	.1 mg/mL
H3	7.68825 RFU	.2 mg/mL	6.42225 RFU	.1 mg/mL
I4	8.143125 RFU	.1 mg/mL	6.89825 RFU	.2 mg/mL
H4	7.0995 RFU	.2 mg/mL	6.5329167 RFU	.1 mg/mL
GO	7.24325 RFU	.2 mg/mL	6.709125 RFU	.1 mg/mL

2.5.3.1. (E. COLI) ANTIBACTERIAL ACTIVITY CHARACTERIZED BY TEM

Additionally, GOs cytotoxicity effects on both E. coli and S. Aureus bacteria was visualized in the Transmission Electron Microscopy (TEM), to further discuss the bacterial morphology after exposure with GOs. The TEM images serve to support the graphical analysis, thus provide a clearer insight of the toxicity effects from the Graphene Oxide onto the bacterial membrane.

As described, our experiment involved incubating the bacteria with the different GOs at the set concentration of 200µg/mL. The set concentration was determined by the results gathered

from the Graphical analysis (**Figure 2.b & Figure 3.b**), as this concentration portrays the point at which maximum bacterial growth occurs before most bacteria dies due to the antibacterial properties found in GOs. The following TEM observations first discusses from the most cytotoxic to least cytotoxic GOs to the E. coli and on the S. aureus bacterial strains.

To further discuss the morphological changes induced by the different GOs onto the E. coli bacteria, TEM pictures were taken of untreated E. coli, to serve as a comparison to the treated bacteria (E. Coli exposure to GOs). The untreated E. coli bacteria (**Figure 2.a**), had an appearance considerable appropriate in healthy bacteria acquiescent by literature, having an intact cell wall, and a distinguishable exoskeleton rod-shape. Notably, GOs exhibit (in most cases) cytotoxicity activity particularly on the E. coli bacterial membrane, as captured in the TEM pictures. An excellent paradigm that epitomizes the GOs antimicrobial potency can be observed when E. coli bacteria was exposed to H4 and I4 (each graphene oxide in a different plate), were the membrane integrity seems to have been compromised (**Figure 2.a**). Although cell lysis is observable in the TEM pictures of either H4 or I4, each graphene oxide affects cell viability, and membrane integrity differently.

In the TEM pictures taken with H4 with E. coli presented by **Figure 2.a**, agglomerate formation can be perceived as a web-like environment. This agglomeration or web-like environment between the H4 and E. coli, would have affected the uptake of nutrients leading to bacterial starvation [6] (Zheng *et al*.). It was also illustrated in the TEM (**Figure 2.a**), outer membrane blebbing [7] (Toyofuku *et al*.), due to the absorption affinity on lipids and the hydrophobicity nature of H4, as these properties allow H4 interaction with the outer membrane of E.coli, hence disturbing the cell envelop. Due to the properties in H4, crosslinking damage would have resulted in-between the outer membrane and peptidoglycan layer (in E. coli), therefore causing the separation of these two layers. Consequently, H4 properties may had influenced the over production of outer membrane vesicles (OMV), observable in the TEM pictures (**Figure 2.a**.), as pinched off membrane vesicles [7] (Toyofuku *et al*.). In contrast, I4 seems to have made macro-cuts onto the outer membrane of E. coli, which then provoked bacterial lysis. These cuts on the

outer membrane, likely ensued from the physiochemical properties in I4, specifically its edges as they may have acted as nano-knives [4, 6]. Consequently, I4 edges may have crippled E. coli by injuring its cell envelop, therefore causing bacteria leak out its intracellular content [4, 6].

Similar to the H4 TEM results, disruption on the cell envelope on E. coli via contact of either the commercial GO or H3, would cause outer membrane hypervesiculation [7] (Toyofuku *et al*.). In the case of H3, its properties may have also caused the dissociation along with crosslinking damage, between the outer membrane and the peptidoglycan on E. coli. Also, it can be pointed out that similar to I4, H3 edges may have acted as nano-knives, compromising the cell envelop on E. coli, hence prompting cellular leakage.

Furthermore, I3 the smallest of the GOs, had the lowest induced cytotoxicity on E. Coli. It was evident that I3 was the least cytotoxic as shown in the TEM pictures (**Figure 2.a**.), where the E. coli surface looked undisturbed even when in contact or closed to the I3 graphene oxide flakes. Judging from the TEM pictures taken from the E. coli treated with I3, there was minimal outer surface distinction in comparison to the untreated E. coli. The treated E. coli had acceptable physical traits such as a distinguishable cell envelop and perhaps an intact outer membrane, thus supporting bacterial viability, as such traits are also portraited in healthy E. coli, as mentioned. Considering the physiochemical traits in I3, compared to the other GOs that manifested greater toxicity traits on E. coli, I3 may not be a favorable option for antibacterial duties.

2.5.3.2. (S. AUREUS) ANTIBACTERIAL ACTIVITY CHARACTERIZED BY TEM

Moreover, to further evaluate the morphological changes and the cytotoxicity effects from the varying GOs, S. aureus bacteria a gram-positive strain, was used for the diversification of this report. As previously described, in preparation for TEM visualization, the S. aureus was incubated (24 h) with the different GOs at the set concentration of 200µg/mL. To better visualize the morphological alterations on the treated S. aureus after being in contact with GOs, TEM pictures

were taken from healthy S. aureus (untreated) for comparison (**Figure 3.a**) The taken TEM picture of the untreated S. aureus bacteria appear to have a well- spherical shape exoskeleton (**Figure 3.a**) denoting that the bacteria are healthy as it be supported by literature [21]. The following observations are discussed from the GOs that exhibit the highest to lowest antibacterial properties onto the S. aureus bacterial strains. Among the GOs that displayed severe disruptive activity onto the S. aureus were the H3 and H4, hence it may be inferred that low levels of oxygen-containing groups (functionalization) endowed higher cytotoxic effects on the gram-positive bacteria. As noticed in the TEM pictures taken from H3 (**Figure 3.a**.), few bacteria were spotted, notably with undistinguished membranes, shrinkage appearance, and only were they visible in agglomeration with the graphene oxide (H3) flakes. The agglomeration mass perceived in the TEM pictures by the H3 can also be noted in H4 when in contact with the S. aureus, and consequently the uptake of nutrients would have been hindered by both GOs (H3 & H4), resulting in bacterial starvation6 (Zheng *et al*.). Moreover, it was perceptible that the spotted bacteria had deformed membranes, perhaps consequential from the micro-cuts induced by the H4 edges acting as nano-knives, and/or by autolysin, a peptidoglycan-degrading enzyme that triggers explosive cell lysis [3,7] (Toyofuku *et. al*; Wang *et al*). As mentioned in Wang *et al*., absorption of Teichoic Acid (TA) onto graphene oxide (H4) by π-π interactions, would have cause dysregulation of autolysin (induced by the deficiency of TA in the cell wall), which would have provoked cell wall degradation, thus resulting in cell lysis. Because of H4 absorption affinity with Teichoic Acid (TA) in the peptidoglycan layer, autolysin acts as a peptidoglycan-degrading enzyme which would have prompted explosive cell lysis and bubbling cell death [7] (Toyofuku *et. al*). Additionally, it is discernible that like H4, the S. aureus experienced explosive cell lysis (**Figure 3.a**.) and agglomeration from both commercial GO and I4. Finally, when evaluating I3 toxicity on S. aureus, the bacteria seemed to have a well distinguished membrane, like the untreated bacteria (**Figure 3.a**), and there was low abrasion observed on the membrane morphology. Lastly, when evaluating I3 toxicity on S. aureus, the bacteria seemed to have well distinguished membranes, like the untreated bacteria (**Figure 3.a**), and low abrasion was observed on the membrane morphology. Furthermore, TEM pictures reveled

there was agglomeration between the S. aureus and I3, hence obstructing nutrient uptake and thus noticing a decline of bacteria population congruent with the graphical analysis (**Figure 3.a**.)

3. CONCLUSION

In summary, we synthesized four different GOs with different Physiochemical characteristics (lateral size (Lc and La), surface area, sp2 hybridized carbon, interlayer spacing (d), and surface oxidation) varying from the method of oxidation used and precursor graphite used for their synthesis, to then test their cytotoxicity. We examined each graphene oxide on both Eukaryotes for their capability to survive, and Prokaryotes to determine GOs bactericides properties. In the study we also made observations on the cells surfaces when in contact with the GOs, especially in the bacterial strains, as it was noticed that due to their unique surface morphology having contact with the GOs surfaces, cells tended to lyse. Lysis on the tested prokaryotes happened when the selective surface of the bacteria made interaction with the surface of the GOs, unleashing a series of events before cell death. As we summarize, it can be suggested that depending on the physiochemical characteristics of each graphene oxide, cell viability outcome is variable, as some cells may not survive GOs interaction. This investigation aimed to find a relationship from the diversified GOs differing from each other by their physiochemical characteristics, to evaluate cell viability.

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APPENDIX

1. TEM & SEM VISUALIZATION FROM BACTERIA WITH GRAPHENE OXIDES

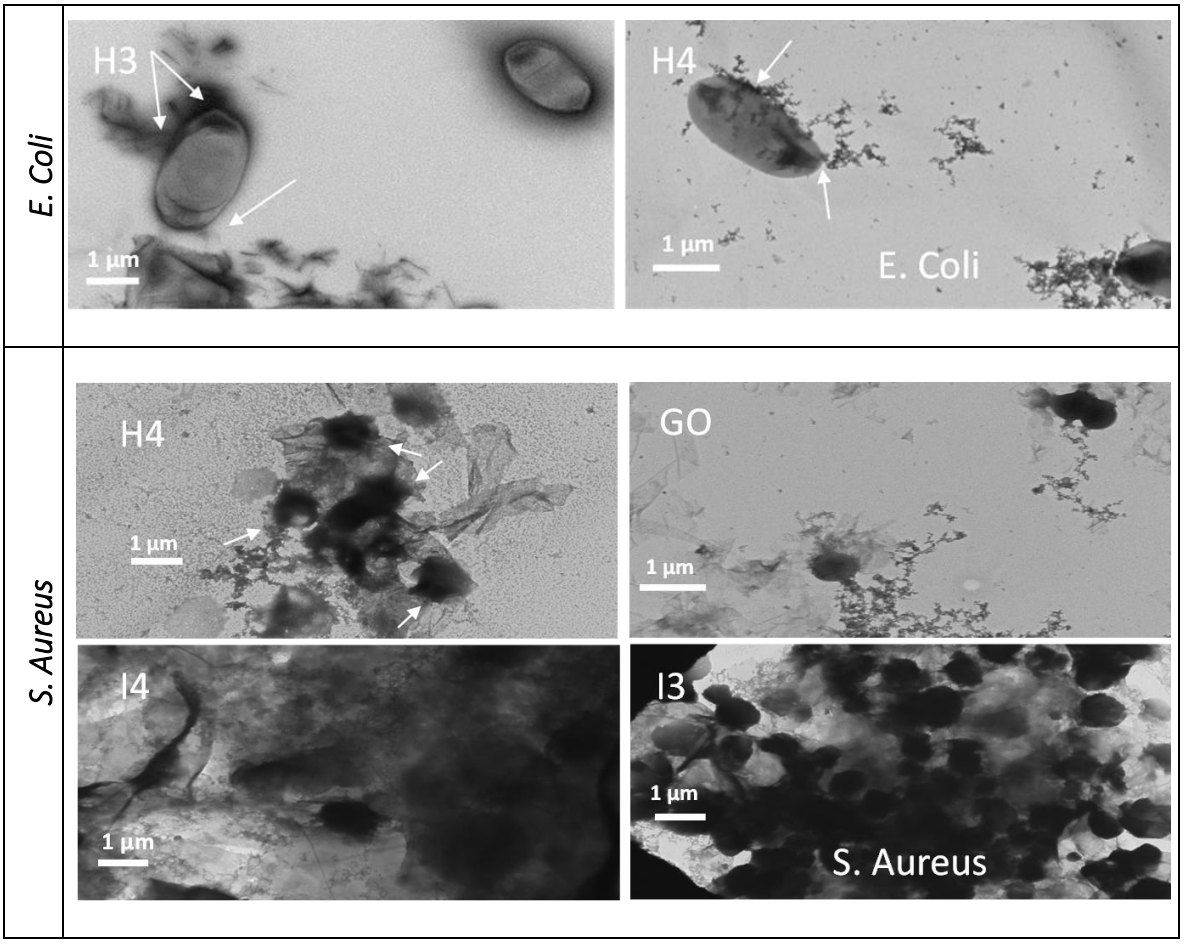

Figure S1. TEM from Bactria E. Coli & S. Aureus at concentration 200µg/mL of different graphene oxides

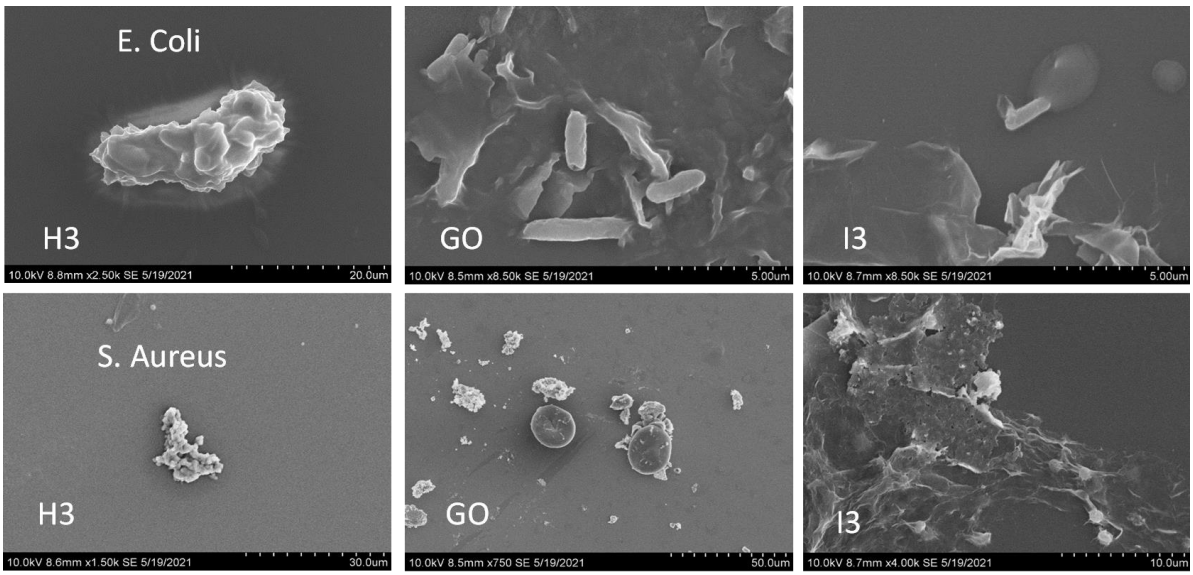

Figure S2. SEM from Bactria E. Coli & S. Aureus at concentration 200µg/mL of different graphene oxides.

VITA

Miriam Montana daughter of a math teacher, Ms. Aldaz and sister of David, was raised in El Paso Texas, from where she attended school since elementary. During her undergraduate years, she joined Dr. Chianelly's group for 3 years in the Metallurgy Department, as a research assistant under the supervision of Dr. Deemer. Following, she graduated in the winter of 2017 from her Bachelor's in Cell Molecular & Biochemistry (CBCH), from the University of Texas at El Paso (UTEP).

In the Fall of 2019, she entered the Graduate School of The University of Texas at El Paso (UTEP). While pursuing a master's degree in biomedical engineering, she interned in Ethicon, a branch from Johnson & Johnson company, working under the quality assurance (QA) and quality compliance (QC) departments. Beginning graduate school of Spring 2019, she joined the research group of Dr. Charles Spencer. As a Biomedical Engineer student in Dr. Spencer's lab, she did research on Graphene Oxides (GO) source of cytotoxicity on biological assessments. The research done on the GOs was a multidisciplinary investigation and was possible through the conjugation of both the biology and engineering departments.

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