Graphene oxide functionalized with ethylenediamine triacetic acid for heavy metal adsorption and anti-microbial applications

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ABSTRACT

The development of functionalized nanomaterials that leads to multi-functionality, such as the ability to adsorb heavy metals coupled with anti-microbial properties is very attractive for diverse applications. The present study evaluated for the first time the antimicrobial activity of graphene oxide silanized with N-(trimethoxysilylpropyl) ethylenediamine triacetic acid (GO–EDTA) against Gram-negative, *Cupriavidus metallidurans* CH4, and Gram-positive bacteria, *Bacillus subtilis*, as well as its cytotoxicity to human corneal epithelial cell line hTCEpi. The results show that GO–EDTA has improved anti-microbial properties when compared to graphene oxide (GO) alone, with 92.3 ± 10% and 99.1 ± 1.3% cell inactivation of *B. subtilis* and *C. metallidurans*, respectively. Bacterial inactivation was attributed to an oxidative stress mechanism towards the cells. No cytotoxicity was observed towards human corneal epithelial cell lines hTCEpi after 24 h exposure to GO–EDTA, suggesting that this nanomaterial has the potential for applications that have human exposure. This work also evaluated GO–EDTA’s adsorption capacity for two heavy metals, Cu^{2+} and Pb^{2+} at different concentrations, varying pH and contact time. The maximum adsorption capacity of the GO–EDTA was determined to be 454.6 mg g^{-1} and 108.7 mg g^{-1} for Pb^{2+} and Cu^{2+}, respectively, exceeding the capacity of traditional adsorbent materials, such as activated carbon.

1. Introduction

The development of novel and multifunctional nanomaterials has attracted considerable attention over the past decade [1–12]. Yet, major challenges still arise when designing nanomaterials that hold antimicrobial and metal adsorption properties needed for biomedical [13–15], catalytic [6,16,17], and environmental applications [18,19]. Materials with metal adsorption capabilities can facilitate the production of electrocatalysts capable of converting and storing energy [5,20,21], chemical sensors for medical diagnostics and food quality control [22], and adsorbents for water treatment systems [23–26]. These applications may, however, be hindered by biofouling resulting in microbial pathogenicity towards humans or inhibition of the processes performed by these materials. Thus, it is important to develop materials that hold antimicrobial properties to prevent the growth and proliferation of microbes on surfaces to maintain their efficiency and...
to protect the public health. Carbonaceous nanomaterials, such as graphene oxide (GO), have the surface chemistry to function as adsorbent of heavy metals [27–30] and antibacterial agent [2,4,31–35]. Because of this dual functionality, GO offers numerous opportunities for its application in water treatment systems [33,36,37], in the development of graphene-metal sensors [38,39], and the synthesis of non-biocorrosive materials for numerous catalytic applications [5,6,40]. Additionally, GO has huge potential for new applications because of the endless functionalization possibilities of its surface. In the present work, we functionalized GO with silanized N-(trimethoxysilylpropyl) ethylenediamine triacetic acid (GO–EDTA) and investigated its antimicrobial, human toxicity, and heavy metal adsorption capacity. EDTA is a well-known chelating agent [41], and thus was immobilized on GO surface to enhance metal adsorption. The antimicrobial property of the novel GO–EDTA material with chelating capabilities, though, has never been investigated.

Most studies on biomedical, industrial, and water treatment applications of graphene-based nanomaterials have been focused on either their antimicrobial properties [2,3,33,35,36,42] or their human cytotoxicity [2,43–46], and only one study has focused on both GO properties [35]. The antimicrobial studies have shown that GO has toxic effects to a variety of microorganisms, such as Gram-negative bacteria Escherichia coli (E. coli) [2,3,35,36,42], Cupriavidus metallidurans (C. metallidurans) [35], and Pseudomonas aeruginosa (P. aeruginosa) [3]; and Gram-positive bacteria, such as Bacillus subtilis (B. subtilis) [35] and Staphylococcus aureus (S. aureus) [2,36]. However, for nanomaterials to be safely used in biomedical and environmental applications they need to present both low cytotoxicity to human cells and high antimicrobial characteristics [35].

In the present study, we investigate for the first time the adsorption of Cu²⁺ and compare the Cu²⁺ adsorption with Pb²⁺ adsorption by GO–EDTA. We also demonstrate for the first time that GO–EDTA is non-toxic to human cells and that it can present improved anti-microbial properties against Gram-negative C. metallidurans CH4, and Gram-positive bacteria, B. subtilis when compared to GO alone. These microorganisms were selected for this study because C. metallidurans CH4 tolerates high concentrations of heavy metals [47], and B. subtilis is commonly utilized as a model organism for toxicity studies [48,49].

2. Experimental

2.1. Synthesis of EDTA-functionalized graphene oxide (GO–EDTA)

The silanization of GO was conducted based on reported literature, briefly the functionalization of GO to form GO–EDTA was done by reacting N-(trimethoxysilylpropyl) ethylenediamine triacetic acid (EDTA-silane) with GO in an ethanol solution in a silylation process followed by filtration and washing with methanol and water sequentially [50]. In the present study, the following modifications were done to the published protocol: 10 mg of GO was dispersed in 50 mL H2O through ultrasonication for 60 min, then 5 mL of 5.0 wt.% of EDTA-silane was added and stirred for 12 h at 75 °C ± 5, followed by room temperature – stirring for 6 h. The product was washed with water several times until no traces of EDTA-silane could be detected. The EDTA-silane was monitored by spotting the supernatant on a thin layer chromatography (TLC) plate and placed in iodine chamber. Then GO–EDTA was finally washed with methanol, dried in a rotavap, and further dried in a vacuum oven.

2.2. Characterization of GO–EDTA and GO

Ultraviolet–Visible (UV–Vis) Spectroscopy, Attenuated Total Reflectance - Fourier Transform Infrared (ATR-FTIR) Spectroscopy, and X-ray Photoelectron Spectroscopy (XPS) were employed to determine the successful functionalization of GO and GO–EDTA. Atomic Force Microscopy (AFM) was used to ascertain the degree of exfoliation of GO sheets. Height profiles using AFM was also utilized to monitor the change in thickness after functionalization with the EDGA group. UV–Vis spectra were recorded from Agilent 8453 spectrometer. For ATR-FTIR, the spectra were collected on a Digital FTS 7000 equipped with an HgCdTe detector from 4000 cm⁻¹ to 600 cm⁻¹ wavenumbers. All spectra were taken with a nominal spectral resolution of 4 cm⁻¹ in absorbance mode. The measurements were obtained under ambient and dry conditions. For AFM studies, GO and GO–EDTA solutions in methanol were drop-casted on clean mica substrate, followed by vacuum-drying for 24 h. AFM imaging was done under ambient conditions with a piezo scanner from Agilent Technologies. Commercially available tapping mode tips (TAP300, Silicon AFM Probes, Ted Pella, Inc.) were used as cantilevers with resonance frequencies in the range of 290–410 kHz. The scanning rate was between 1 and 1.5 line/s. On the other hand, XPS measurements were conducted on a PHI 5700 X-ray photoelectron spectrometer equipped with monochromatic Al Kα X-ray source (hν = 1486.7 eV) incident at 90° relative to the axis of a hemispherical energy analyzer. Low and high resolution spectra were collected with pass energies of 23.5 and 187.85 eV, respectively, a photoelectron take off angle of 45° from the surface, and an analyzer spot diameter of 1.1 mm.

2.3. Microorganisms, human cells, and growth conditions

The bacterial strains used in the present study were B. subtilis 102 and C. metallidurans CH4. The growth medium used for both microorganisms was tryptic soy broth (TSB) (Oxoid Ltd., Basingstone, Hampshire, England). Phosphate-buffered solution (PBS) (0.01M PBS, pH = 7.4 at 25 °C, 0.0027 KCl, 0.137 NaCl, Fisher Scientific, USA) was used as a buffer solution for bacterial suspensions and dilutions. For all experiments, a single isolated colony was inoculated in 5 mL of TSB to grow overnight at 35 °C. The grown culture was centrifuged at 3000 rpm for 10 min, and the bacterial pellet was washed once and resuspended in PBS. The optical density (OD) of the suspension was adjusted to 0.5 at 600 nm, which corresponds to a concentration of 10⁷ colony forming units per milliliters (CFU mL⁻¹). The concentration was determined based on plate counts for each bacterium using trypskyte soy agar (TSA) (Oxoid Ltd.).
Human corneal epithelial cell lines hTCEpi were obtained from the College of Optometry at the University of Houston. The cells were cultured at 37 °C in 5% CO₂ humidified incubator (NuAire, USA) for 48 h with a KBM-2 complete media made from KGM-2 Bullet Kit (Lonza, USA Catalog# CC-3107). Human corneal epithelial cells of passage numbers 50 and 53 were harvested from the cell culture flask by aspirating the old media and then adding 1 mL of Tryple (Gibco by life technology, USA). The flask with Tryple and the cells were incubated for 10 min in 5% CO₂ humidified incubator at 37 °C. After incubation and centrifugation, the cells were suspended into growth medium and were quantified with a hemocytometer. A density of 3.0 × 10⁶ cells per 100 μL was seeded to a sterile 96-well plate (Falcon, USA) and incubated at 37 °C in the 5% CO₂ humidified air incubator for 24 h. After, the media was aspirated from each well, all the wells were rinsed gently three times with 1X sterile phosphate buffer saline (PBS) (pH 7.4, 10× sterile PBS, Gibco by life technology USA).

2.4. Antibacterial activity of GO and GO–EDTA to planktonic cells by plate count method

The toxicity of GO and GO–EDTA was determined by plating the bacteria after 1 h and 3 h of exposure to all concentrations of the nanomaterials (100, 500, and 1000 μg mL⁻¹) as previously described [31]. Briefly, Aliquots of 180 μL of bacterial suspensions at 0.5 OD₆₀₀ in PBS were pipetted in a 96-well plate containing 20 μL of different concentrations of GO and GO–EDTA in DI water (i.e. 1000 μg mL⁻¹, 500 μg mL⁻¹, and 100 μg mL⁻¹). Positive controls consisted of 180 μL of bacterial suspensions with 20 μL of DI water. Negative controls were prepared with 200 μL of media with GO and GO–EDTA only. All experimental samples and controls were prepared in triplicates. After the exposure time, a serial dilution was performed with the bacteria. Non-diluted samples and all dilutions were plated in TSA media and incubated overnight at 37 °C. The antibacterial activity was quantified by counting the colony forming units (CFU) in each plate (CFU mL⁻¹). Averages and standard deviations were calculated from triplicates. The percent toxicity was expressed as the percent of the ratio of the dead cells exposed to the nanomaterial to the control cells. In order to test for toxicity differences in nanomaterials with different concentrations, we performed t-tests using the raw CFU mL⁻¹ values of the plate count analysis, and comparing each value to the control (bacteria without nanomaterials). The data was normalized using logarithm-base 10 values.

2.5. Scanning Electron Microscopy (SEM) imaging

A drop of a solution at a concentration of 1000 μg mL⁻¹ of each nanomaterial was placed directly onto the lacy carbon film supported on a 200-mesh Cu grid (SPI applies, West Chester, PA) and dried overnight. The bacteria, bacteria-GO, and bacteria-GO–EDTA samples were prepared following the procedure described by Li et al [51]. All samples were analyzed under the scanning electron microscope (SEM), JSM 6010LA (Jeol, USA). The accelerating voltage was set at 10 keV.

2.6. Reactive oxygen species assay: thiol oxidation and quantification

Ellman’s assay was used to quantify the fraction of glutathione (GSH) in reduced form, as previously described [52,53]. Nanomaterial solution of 1000 μg mL⁻¹ GO and 100 μg mL⁻¹ GO–EDTA were used in this experiment. No bacteria were used in this experiment. In a 20 mL eppendorf, 225 μL of GO or GO–EDTA (1000 μg mL⁻¹) and 225 μL of GSH (0.4 mM in 50 mM) containing bicarbonate buffer (NaHCO₃, pH = 8.6) were mixed. Positive and negative controls were prepared with 225 μL of GSH oxidized with H₂O₂ (30%) and 225 μL of GSH without nanomaterials, respectively. Further preparation of the GSH solution was done following the manufacturer’s procedure. The absorbance of the GSH solution after the interaction with the nanomaterial was measured at 412 nm using a Synergy MX Microtiter plate reader (Biotek, USA). The loss of GSH in each sample was calculated using the following formula:

\[
\% \text{ GSH loss} = \frac{\text{absorbance of negative control} - \text{absorbance of sample}}{\text{absorbance of negative control}}
\]

2.7. Cytotoxicity assay on human corneal epithelial cells

The human corneal epithelial cells and CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, USA) were used to investigate the cytotoxicity of GO and GO–EDTA, as described by the manufacturer [54], with cells exposed to 100 μL of fresh media and 100 μL of each nanomaterial at a concentration of 1000 μg mL⁻¹. As controls, 100 μL of the nanomaterial and 100 μL of media without cells were added to separate wells. The controls were used to subtract the absorbance of the media and the nanomaterials and eliminate the background. Untreated cells were used as negative controls. The positive controls contained the cells in PBS with 10% paraformaldehyde to allow complete cell inactivation. The plate was mixed gently before placing into the humidified incubator for another 24 h. After incubation, the media was aspirated from the wells, and the wells were rinsed 3× with PBS. The plate was incubated in a humidified incubator containing 5% CO₂ at 37 °C for 2 h. The absorbance of the formazan product, which is proportional to the number of living cells, was read at a wavelength 490 nm using a microplate reader FLUOstar Omega (BMG Labtech, Germany). The results were expressed in terms of percentage of living cells, which was calculated by dividing the absorbance of formazan in the samples (nanomaterials + cells) by the absorbance of the negative controls.

2.8. Batch adsorption studies

Stock solutions of Pb²⁺ (0, 5, 10, 20, 30, 40, 60, and 100 ppm) and Cu²⁺ (0, 5, 10, 20, 30, 40, 60, and 100 ppm) were prepared by dissolving lead and copper standards for AAS (1000 mg L⁻¹, Sigma Aldrich, St. Louis, MO) in de-ionized water. Adsorptions of 10 mL Pb²⁺ and Cu²⁺ ions were carried out by batch experiments with 0.25 mL aqueous solution containing 1000 μg mL⁻¹ of nanomaterial (GO–EDTA or GO), and agitated at 125 rpm and room temperature.
To determine the equilibrium contact time, a 20 ppm solution of each metal was exposed to the nanomaterial as described above, and samples were taken every 5 min. for 90 min. The samples were filtered through a 0.22 μm membrane filter, and the residual concentrations of Pb$^{2+}$ and Cu$^{2+}$ in the filtrates were determined by atomic absorption spectrometer (AAAnalyst 300 AA, PerkinElmer, USA) at a wavelength of 283.3 nm and 324.8, respectively.

The influence of pH on Pb$^{2+}$ and Cu$^{2+}$ removal by GO–EDTA and GO was examined at pH values varying from 2 to 9. The pHs of these solutions were adjusted by adding HNO$_3$ or NH$_4$OH to the metal stock solutions. The pH was measured using a pH meter (HORIBA Model D-21). The mixture of 1000 μg mL$^{-1}$ GO–EDTA or GO and 20 ppm lead Pb$^{2+}$ or Cu$^{2+}$ in different pH values were agitated at 240 rpm and room temperature for 90 min, which was the equilibrium contact time. The effects of Pb$^{2+}$ and Cu$^{2+}$ concentrations on the adsorption capacity of the nanomaterial was investigated by varying the amount of metal in the solution (0, 5, 10, 20, 30, 40, 60 and 100 ppm) for 90 min under optimum pH conditions for each metal. The Langmuir adsorption isotherm was used to model the experimental isotherm data:

$$\frac{C_e}{q} = \frac{1}{bq_s} + \frac{C_e}{q_s}$$

where $C_e$ is the equilibrium concentration of the aqueous Pb$^{2+}$ or Cu$^{2+}$ ions in mg L$^{-1}$, $q$ is the amount of Pb$^{2+}$ or Cu$^{2+}$ ions adsorbed per unit weight of GO–EDTA or GO at equilibrium concentration in mg g$^{-1}$, $q_s$ is the maximum uptake capacity per unit volume of GO–EDTA or GO in mg g$^{-1}$, and $b$ is the Langmuir equilibrium constant related to the affinity of Pb$^{2+}$ or Cu$^{2+}$ ions to the binding sites, in L mg$^{-1}$.

3. Results and discussion

3.1. Characterization of GO–EDTA

To populate the surface of GO with Si-EDTA, we performed a reaction based on previously reported literature [50], and carefully characterized the GO–EDTA surface with UV–Vis, ATR-FTIR, and XPS. GO contains numerous hydroxyl functional groups, which is also evidenced by FT-IR and XPS analyses (Figs. 1 and 2). In the GO–EDTA synthesis, the hydroxyl moiety reacted with Si-EDTA, which possessed a hydrolysically sensitive center, to form a covalent bond with GO. The dehydration–condensation reaction resulted in the GO–EDTA product as shown in Fig. S9. The UV–Vis spectrum of GO exhibited two characteristic peaks that can be utilized for identification. Fig. 1a presents the UV–Vis profile of the as-synthesized GO solution showing a maximum peak at 231 nm and a shoulder feature at 300 nm which corresponds to the π-π* transitions of aromatic C=C bonds and the π-π* transitions of C=O moieties, respectively [55].

The linkage of EDTA group to GO was attributed from the hydrolysis of the trialkoxy groups of silane-EDTA, which generates –Si–OH moieties that further reacts with the C–OH groups of graphene oxide, forming a Si–O–C bond [50]. Compared with GO, the absorption peak of GO–EDTA (Fig. 1a) at 231 nm was red-shifted to 262 nm. The same is true for the shoulder band. The observed shift in the absorption band is consistent with the reported literature [50]. We also prepared a stable dispersion of GO–EDTA in water (Fig. 1b), owing to the additional hydrophilic nature of EDTA. The identity of the as-synthesized GO was further confirmed by ATR-FTIR

![Fig. 1](image-url)
spectroscopy (Fig. 1c) revealing characteristic bands at 1051 cm\(^{-1}\) (C–O stretching vibrations), 1239 cm\(^{-1}\) (C–OH stretching vibrations), 1608 cm\(^{-1}\) (skeletal vibrations of the unoxidized graphitic domains), 1723 cm\(^{-1}\) (C=O stretching from carbonyl groups) and a broad band centered at 3368 cm\(^{-1}\) (O–H stretching vibrations)[55]. The presence of a new band at 2975 cm\(^{-1}\) in GO–EDTA corresponds to the stretching of the methylene groups from the silane-EDTA molecules while the new band at 1396 cm\(^{-1}\) is attributed to the C\(_2\)H\(_2\) group of EDTA[50].

Additional evidence of the attachment of GO–EDTA groups was analyzed by XPS. The presence of the silicon, nitrogen, and sodium signals on the GO–EDTA sample confirms the successful synthesis of GO–EDTA.

In Fig. 2b, the Si–OH and siloxane (Si–O–Si–) bonds are shown by the peak with binding energy of \(\sim105\) eV, resulting from the partial hydrolysis of the silane molecules during the silylation reaction [50]. The peak of Na1s at \(\sim1072\) eV from GO–EDTA corresponds to the Na ions that serve as counter ions for the EDTA group. The N1s peak of GO–EDTA at \(\sim402\) eV, represents the amine moiety introduced to the GO surface.

Meanwhile, a graphene oxide sheet that appears to be 1 nm thick in AFM is considered a fully exfoliated graphene oxide [56]. Fig. 2c shows fully exfoliated graphene oxide and this is further evidenced by the line profile (Fig. 2e) showing approximately 1 nm thickness. On the other hand, the average thickness of GO–EDTA is roughly 1.5 nm. The increase in thickness is due to the grafted silane-EDTA molecules onto the surface of GO, further confirming the successfully tethered silane-EDTA group.

### 3.2. Time dependent viability of planktonic cells exposed to GO–EDTA at different concentrations

The antimicrobial activity of GO–EDTA on bacteria was investigated in planktonic phase to assess the effect of the nanomaterial’s concentration and exposure time to the microbial cells. Similarly, the GO inactivation was measured for comparison to evaluate if the functionalization enhanced the disinfection efficiency of the nanomaterial. First, the bacterial cells were exposed to different concentrations of both nanomaterials (100 µg mL\(^{-1}\), 500 µg mL\(^{-1}\), and 1000 µg mL\(^{-1}\)) and the inactivation was evaluated by the plate count (Fig. 3) and the optical absorbance methods (Fig. S1). Fig. 3 shows that, as the nanomaterials’ concentration increases the mean toxic effect towards the bacteria increases for both types of bacteria. *C. metallidurans* exposed for 1 h (Fig. 3a) to
the lowest concentration of GO–EDTA (100 μg mL⁻¹) resulted in a 64.4 ± 23.5% inactivation of the total cells, whereas the highest concentration (1000 μg mL⁻¹) inactivated 71.7 ± 30.8% of the total cells. This trend was also observed for GO (Fig. 3a), which caused 72.9 ± 26.4% and 81.5 ± 25.2% cell death of the total C. metallidurans cells exposed to the lowest and highest concentrations, respectively. Likewise, B. subtilis cell death (Fig. 3c and d) increased with increasing nanomaterials’ concentration.

Similar findings were shown for the Gram-negative bacteria, E. coli and C. metallidurans and the Gram-positive bacteria, B. subtilis and Rhodococcus opacus, exposed to GO, graphene, poly-N-vinyl carbazole (PVK)-GO, and PVK-G for 1 h and 3 h, in our previous studies [35,57]. Cell death of all microorganisms was greater with increasing nanomaterial concentration, with 1000 μg mL⁻¹ of PVK-GO achieving 100% cell inactivation. Other studies presented GO microbial inactivation values against E. coli of 49.1% (1 h-exposure), 98.5% (2 h-exposure), and 100% (3 h-exposure) using GO concentrations of 80 μg mL⁻¹ [42], 85 μg mL⁻¹ [4], and 1000 μg mL⁻¹ [35], respectively. Although these studies were performed by different research groups, it seems that these studies also show a similar trend to our study, since their toxicity values seem to escalate with the increasing concentration of GO. Furthermore, these studies also suggest that the increasing exposure times increased the microbial inactivation. To confirm this trend, we performed plate count analyses after exposing the bacterial cells for 1 h and 3 h to GO and GO–EDTA (Fig. 3).

For B. subtilis, a longer exposure time did not increase significantly the cell inactivation, as 83.3 ± 18.1% of the cells were killed in 1 h and 91.0 ± 7.2% of the cells were killed in 3 h. For C. metallidurans, exposure for 1 h to the highest concentration of GO–EDTA (1000 μg mL⁻¹) achieved toxicity values of 71.8 ± 30.8% while this same concentration killed 99.7 ± 0.5% after 3 h of exposure. In this case, GO–EDTA presented a 28% higher loss of cell viability with a longer exposure time than GO. In the same context, the functionalization of GO increased the inactivation of B. subtilis by 10.1% during the 3 h exposure, while for C. metallidurans cells this difference was 7.1%. The difference of cell inactivation between Gram-positive and Gram-negative microorganisms may be attributed to their different thickness of the peptidoglycan layer, their different ability to adapt to environmental stresses, and the protection conferred by the membrane surface properties [35,36]. Our results indicate that the cell inactivation is also time dependent and that GO–EDTA becomes more efficient at inactivating the cells with longer cell exposures. The time dependency of GO and GO–EDTA inactivation on bacterial cells was also observed with other carbon based nanomaterials [31,32,58]. The bacterial resistance to carbon-based nanomaterials at shorter exposure times is still a topic of debate. The two most hypothesized mechanisms of toxicity for graphene-based materials are physical disruption of the cell membrane and oxidative stress [2,32,42]. Potentially, not all bacterial cells were in contact with the nanomaterial for enough time to be completely inactivated. Thus, they could still grow. Yet, more studies are needed for a full understanding of inactivation mechanisms since the toxicity depends on concentration, nanomaterial size, exposure time, and cell type [58].

3.3. Cell membrane damage of bacteria exposed to GO–EDTA

To further understand the interaction of the nanomaterials with the microorganisms, we analyzed the changes in cell
morphology with Scanning Electron Microscopy (SEM) before and after exposure to 1000 μg mL⁻¹ of the nanomaterials. The SEM images (Fig. 4) showed that while the cells exposed to GO maintained their rod shape and cell integrity (Fig. 4b), the cells exposed to GO–EDTA had their membranes deformed (Fig. 4c and f). The pronounced membrane damage agrees with the Live/Dead Assay discussed in the supporting information. Other studies also suggested that bacterial cells can become trapped within graphene sheets while maintaining their cellular integrity [59], as observed with B. subtilis exposed to GO (Fig. 4b). In such cases, the cells cannot proliferate in the media nor consume the nutrients in their surrounding environment [59]. This type of mechanism that inhibits cellular growth was also suggested for PVK-GO in our previous study [35]. However, the deformity observed for the cells exposed to GO–EDTA in the present study suggests that GO and GO–EDTA might have different mechanisms of toxicity. The mechanism of toxicity to the cell may be directly related to membrane damage, as it was also observed with other carbon-based materials, such as fullerenes [60], single-walled carbon nanotubes (SWNTs) [61,62] and graphene nanosheets [2]. Alvarez and co-workers suggested that a lower membrane potential for B. subtilis was associated with its membrane damage after exposure to fullerenes, potentially preventing a membrane proton gradient and subsequent electron transport needed for oxidative phosphorylation [60].

Additionally, chelating agents, such as chitosan and EDTA, have also been described to have anti-microbial properties due to sequestration of metal ions present in the cell wall molecules, which are crucial for cell wall stability and integrity [63–65]. It is possible that the distinct anti-microbial properties observed between GO and GO–EDTA (Fig. 4) could be caused by the synergistic anti-microbial properties of GO and EDTA. More research, however, is needed to determine the anti-microbial role of EDTA, if any, in the GO–EDTA nanoparticle.

3.4. Oxidative stress induced by GO–EDTA

Oxidative stress has been indicated as a potential antimicrobial mechanism for carbon based nanomaterials including graphite (Gt), graphite oxide (GtO), GO, reduced GO (rGO) [2,32,34,36,42,66] fullerenes [60], and CNTs [46,67,68]. Graphene-based nanomaterials have been described to generate reactive oxygen species (ROS), such as superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH⁻) [34,42,69]. In the present study, we analyzed the production of ROS, such as hydrogen peroxide, by measuring the glutathione (gamma-glutamyl-cysteinyl-glycine, GSH) reduction after exposure to GO and GO–EDTA. The ROS production has been shown to alter microbial processes by oxidizing key cellular components and hindering their functionality. GSH is a tripeptide with a sulfhydryl group produced by cells that serves as an electron donor capable of reducing reactive oxygen species [70]. Because GSH acts as a cellular antioxidant, it can be used as an indicator of possible oxidative stress induced by the nanomaterials. The assay consists in exposing pure GSH, without bacterial cells, to the nanomaterial (Fig. 5).

As shown in Fig. 5, the maximum GSH loss is given by the positive control, or the oxidation caused by hydrogen peroxide. Graphene oxide presented a slightly higher mean value for the GSH loss than GO–EDTA, however this difference is not statistically significant. A previous study demonstrated that 80 μg mL⁻¹ of GO achieved a GSH oxidation of 22 ± 0.1%, where the GSH loss increased with incubation time and the concentration of GO in the sample to up to 37 ± 1.5%. Our findings show a GSH loss by GO and GO–EDTA greater than...
The metal binding capacity of GO is dictated by the oxygen-containing functional groups, such as hydroxyl, epoxide, carboxyl, and carboxylic groups, present in the nanomaterial. In this study, we functionalized GO with EDTA, a strong chelating hexadentate ligand that can bind to most metals, to increase the number of oxygen-containing functional groups in GO and therefore, increase the metal adsorption capacity of GO–EDTA.

3.5. Cytotoxicity assay on human cells

The antimicrobial properties of graphene-based nanomaterials have created a window of opportunities for biomedical, catalytic, and environmental engineering applications [33]. It is critical, however, to evaluate the adverse effects of these nanomaterials on human health in order to determine their suitability for applications that involve human contact. For instance, antimicrobial nanomaterials may potentially serve for decentralized or point-of-use water treatment and reuse systems [33]. Some nanomaterials, such as polyethylene glycol-GO, have also been shown to facilitate chemo-photothermal therapy [71]. Both of these may include direct exposure of the nanomaterials to human skin and eyes. In humans, it has been shown that GO can generate reactive oxygen species (ROS) [46] and, depending on the human cell line, GO can be slightly toxic at concentrations varying from 50 mg mL\(^{-1}\) to 100 mg mL\(^{-1}\) [45]. However, when these nanomaterials are combined with other biologically compatible polymers (e.g. PEGylation), they exhibit negligible in vitro toxicity to many cell lines and animals, [35] even at high concentrations up to 100 mg mL\(^{-1}\) [72,73]. To investigate the cytotoxicity effects of the GO and GO–EDTA against eukaryotic cells, human corneal epithelial cells were exposed to the most toxic concentrations of GO and GO–EDTA (1000 µg mL\(^{-1}\)) for 24 h. As shown in Fig. 6, neither GO nor GO–EDTA were toxic towards the epithelial cells, as 99% of the cell culture was still alive after 24 h exposure to the nanomaterials. Previous research also confirmed a very small toxic effect of GO and PVK-GO towards NIH 3T3 fibroblast cells, with values of 9.9% and 7.4%, respectively [35].

85% at a concentration of 1000 µg mL\(^{-1}\) of each nanomaterial, suggesting that both nanomaterials can induce oxidative stress towards the cells.

3.6. Copper and lead adsorption

The metal binding capacity of GO is dictated by the oxygen-containing functional groups, such as hydroxyl, epoxide, carboxyl, and carboxylic groups, present in the nanomaterial. In this study, we functionalized GO with EDTA, a strong chelating hexadentate ligand that can bind to most metals, to increase the number of oxygen-containing functional groups in GO and therefore, increase the metal adsorption capacity of GO–EDTA.

3.6.1. Effect of contact time

The effect of contact-time was investigated to find the time required for the reaction to reach equilibrium. From Fig. 5, it appears that the metal binding to the GO–EDTA sheets occurred within the first 5 min for both metals, since after that time, the removal remained relatively constant until the end of the experiment (90 min). Previous study showed 30 min as the equilibrium time for the adsorption of Pb\(^{2+}\) into GO–EDTA.[74] We attribute the rapid adsorption equilibrium (5 min) to the increased amount of EDTA in the GO–EDTA produced in this study. Other carbon-based materials, such as multi-walled carbon nanotubes [75], Mn oxide-coated carbon nanotubes (MnO\(_2\)/CNTs) [76], and activated carbon [77] required 40 min, 2 h, and ~3 days respectively, to attain equilibrium. Thus, the equilibrium time for Pb\(^{2+}\) and Cu\(^{2+}\) adsorption onto GO–EDTA was shown to be exceptionally fast when compared to other adsorbents. This rapid adsorption has been attributed to the 2D structure of graphene sheets, in which EDTA is easily accessible by the metals [74], making the novel GO–EDTA a strong candidate for heavy metal removal. Yet, the effect of pH and initial metal concentration may also influence the adsorption efficiency of the nanomaterial.

3.6.2. Effect of pH

The adsorption of metal ions by chelation is dependent on the pH of the solution since it affects the adsorbent surface charge, and the degree of protonation of the functional groups [78]. Although a previous preliminary study has investigated the adsorption of Pb\(^{2+}\) ions onto GO–EDTA [74], the effect of pH in the adsorption process has not been evaluated for metal concentrations lower than 100 mg L\(^{-1}\). Additionally, the adsorption capacity of this material for other metals, such as copper, has not been previously analyzed. Since pH can vary in different conditions [79], it is critical to evaluate the adsorption process within a wide range of pH, at concentrations below 100 mg L\(^{-1}\) with short contact times. The metal adsorption depends on the extent of protonation of the
carboxylic and hydroxyl groups in the graphene sheets and carboxyl and carbonyl groups of EDTA, given that an increase in pH reduces the competition between $H^+$ and Pb$^{2+}$ ions [80]. Fig. 7 shows that the removal efficiency is lower in acidic media for both metals, increasing as the pH becomes basic. Although, at pH values higher than 4, the lead percent removal is higher, up to 96%, this higher removal is not only due to the adsorption of lead to the nanomaterial, but also due to precipitation of lead hydroxide formed at pH values above 4 [81,82]. Similarly, copper starts to precipitate above pH = 6 [83], so sorption at lower pH values indicate that the nanomaterial is responsible for adsorption of the metal. This study aims to investigate the influence of adsorption on the metal removal process, therefore the pH = 3 was selected for lead and the pH = 5 was selected for copper for further investigation of metal removal to avoid heavy metal precipitation conditions.

3.6.3. Adsorption isotherm

The GO–EDTA adsorption data was fitted into the Langmuir isotherm to evaluate the adsorption equilibrium between Cu$^{2+}$ and Pb$^{2+}$ ions and the GO–EDTA surface adsorption sites, assuming monolayer adsorption, as a first order reaction (Fig. 8) [80].

The Langmuir parameters for adsorption of Pb$^{2+}$ onto GO–EDTA, $b$ and $q_s$, were determined to be 0.12 L mg$^{-1}$ and 454.6 mg g$^{-1}$, respectively. The same parameters for adsorption of Cu$^{2+}$ onto GO–EDTA, $b$ and $q_s$, were 0.07 L mg$^{-1}$ and 108.7 mg g$^{-1}$, respectively. The GO nanomaterial was also modeled with the Langmuir adsorption isotherm, with calculated $b$ and $q_s$ parameters of 1.06 L mg$^{-1}$ and 303.0 mg g$^{-1}$ for lead and 0.05 L mg$^{-1}$ and 166.7 mg g$^{-1}$ for copper, respectively. The adsorption capacity found for lead in this study exceeded values obtained by other materials previously studied for lead adsorption, such as activated carbon (16.61 mg g$^{-1}$) [84], chitosan beads (32.9 mg g$^{-1}$) [85], and others [86,87]. Similarly, the GO–EDTA copper adsorption capacity exceeded values of materials previously tested for copper adsorption, such as fly ash (69.93 mg g$^{-1}$) [23] and activated carbon above 40 °C (mg g$^{-1}$) [23]. The higher adsorption capacity of GO–EDTA for lead ions (454.6 mg g$^{-1}$), when compared to its capacity for copper ions (108.7 mg g$^{-1}$) can be attributed to a higher affinity for lead ions, which is also confirmed by the Langmuir constant, $b$. Previous research showed a maximum Pb$^{2+}$ adsorption capacity of GO–EDTA of up to 479 mg g$^{-1}$, based on the Langmuir model [74]. However, our study shows
removal efficiencies above 90% with shorter contact time (5 min) than a previous study. [88] This behavior can be attributed to the increased EDTA content (5%) in the GO–EDTA synthesis. The difference in adsorption capacities between both metals onto GO–EDTA depend on the thermodynamic parameters of each metal adsorption process, such as the enthalpies, entropies, and Gibbs free energy values of each reaction [89]. These parameters may be influenced by the formation of other soluble species in water, such as PbOH\(^+\), Pb(OH)\(^3\)\(^-\), CuOH\(^+\), Cu(OH)\(^3\)\(^-\), and would need to be assessed in future research.

3.6.4. Binding sites for the adsorbed metals to the nanomaterial GO–EDTA

The metal adsorption to the nanomaterials GO and GO–EDTA was confirmed by EDS and FT-IR analysis of the nanomaterial-metal sample. Comparing the spectra of GO–EDTA and GO in Fig. 9, we observed the presence of a peak at 1066 cm\(^{-1}\) in the GO–EDTA spectra, which corresponds to the Si–O–C stretching vibrations. Such vibration corresponds to the terminal Si-O present in the silanized EDTA that binds to the GO [90]. Furthermore, the C–O stretching vibration at 1280 cm\(^{-1}\) was much stronger for the GO–EDTA than for the GO spectra, owed to the increased number of carboxyl groups of GO–EDTA. This peak confirms the functionalization of GO–EDTA. This peak was also lowered after both metals (copper and lead) adsorbed to GO–EDTA, as seen for the Pb\(^{2+}\)-GO–EDTA and Cu\(^{2+}\)-GO–EDTA spectra. Similarly, the 1640–1750 cm\(^{-1}\) region does not show a strong C=O band for GO, as it does for the GO–EDTA spectra. The peak at 1647 cm\(^{-1}\) was lowered after the metals adsorbed to the GO and GO–EDTA, as seen for the Pb\(^{2+}\)-GO, Pb\(^{2+}\)-GO–EDTA, Cu\(^{2+}\)-GO, and Cu\(^{2+}\)-GO–EDTA spectra. This band suggests that GO–EDTA contains more carboxylic and carbonyl groups and that these were not reduced to C–OH. The strong peak at 842 cm\(^{-1}\) in the GO–EDTA spectra corresponds to the Si–O vibration present in the silanized EDTA, suggesting the presence of the silanized functional group. In the same way, metal

![Fig. 10 – (a) X-ray Photoelectron Spectra (XPS) of survey scan and (b) high resolution scan of GO–EDTA with Cu\(^{2+}\) ions adsorbed, and (c) survey scan and (d) high resolution scan of GO–EDTA with Pb\(^{2+}\) ions adsorbed.](attachment:image)
Graphene-based nanomaterials, such as GO, have excellent lead and copper removal capabilities, due in part to their large specific surface area, and also because of the endless options to modify its surface chemistry. This study demonstrated that the novel GO–EDTA can effectively adsorb Cu\(^{2+}\) and Pb\(^{2+}\) ions, confirmed by FT-IR and EDS measurements. FT-IR analyses revealed that carboxyl and carbonyl functional groups were responsible for the metal binding. The 5% increase in metal adsorption was confirmed through XPS analysis as shown in Fig. 10. In Fig. 10a and b, the presence of Cu\(^{2+}\) signals on the GO–EDTA sample, at approximately 935 eV, confirms the successful adsorption of the metal. Similarly, the adsorption of Pb\(^{2+}\) ions was supported by the presence of the two peaks at 139 eV and 144 eV (Fig. 10c and d). XPS analysis was also done to prove the metal adsorption onto GO, and it is found in the Supporting information (Fig. S8).

### 4. Conclusions

Graphene-based nanomaterials, such as GO, have excellent heavy metal removal capabilities, due in part to their large specific surface area, and also because of the endless options to modify its surface chemistry. This study demonstrated that the novel GO–EDTA can effectively adsorb Cu\(^{2+}\) and Pb\(^{2+}\) ions, confirmed by FT-IR and EDS measurements. FT-IR analyses revealed that carboxyl and carbonyl functional groups were responsible for the metal binding. The 5% increase in metal content in the GO–EDTA made possible to achieve removal with a shorter contact time (5 min) than previous studies for both lead and copper.

Additionally, this study demonstrates, for the first time, that GO–EDTA has the capability to act as a multi-functional material. In our study, the highest microbial inactivation by GO–EDTA was 92.3 ± 10% and 99.1 ± 1.3% for B. subtilis and C. metallidurans for a 3 h exposure time with 1000 µg mL\(^{-1}\) nanomaterial concentration. The effect of GO and GO–EDTA on the bacterial cells was analyzed by GSH loss and found to be greater than 85% at 1000 µg mL\(^{-1}\). We demonstrate that both nanomaterials may induce oxidative stress towards the cells. Most importantly, GO–EDTA did not present any cytotoxicity to human corneal epithelial cells as more than 99% of the cells were still alive after exposure to the nanomaterials for 24 h. Thus, the dual functionality of the nanomaterials GO and GO–EDTA, as well as its safety towards human cells, offers enormous potential for biomedical, water treatment, and catalytic applications to attend the growing demand for materials that provide metal binding and microbial control capabilities.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbon.2014.05.032.

### References

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