Graphene copper-chalcogenide composite materials display antimicrobial activity against Gram-positive bacteria

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Abstract

The use of touchscreens in today's world has increased drastically, especially in healthcare. The spread of pathogenic microbes from touchscreens to people has been a cause for concern in recent years. The ability to create an antimicrobial touchscreen would help stop the spread of pathogenic bacteria not only in healthcare but anywhere touchscreens are utilized. To create a material that could be incorporated into touchscreens to make them antimicrobial, they would need to kill microbes, be optically transparent, and be able to conduct electricity. Our research tested the antimicrobial properties of graphene-copper chalcogenide composite materials, specifically graphene-copper oxide and graphene-copper sulfide. Graphene-copper sulfide was synthesized and both materials were analyzed using X-ray photoelectron spectroscopy. These materials were tested against *B. subtilis* and *E. coli* using standard agar disk diffusion. Both composite materials displayed antimicrobial activity against *B. subtilis*. Our data show a promising first step in creating an antimicrobial touchscreen.

Introduction

The use of touchscreen devices across all professional sectors has drastically increased, especially in healthcare (1). While the increase in technology has helped patients and workers alike, the spread of pathogenic microorganisms through touchscreen devices has been cause for concern (2). Ultraviolet-C light and isopropyl alcohol wipes have previously been used to sanitize touchscreen devices (2, 3). However, these methods are not foolproof, and recontamination occurs. An antimicrobial film over touchscreen surfaces would help stop the spread of pathogenic microorganisms in high contamination spaces such as hospitals. There are several obstacles to overcome when looking at how to create a material that meets the requirements to be in a touchscreen. The material must have antimicrobial properties, be able to conduct electricity, and be optically transparent.

Antimicrobial properties of copper and copper nanoparticles

Copper was one of the first metals to be confirmed as having antimicrobial properties and has been used in many settings to prevent the spread of pathogenic microorganisms (4)**.** In a hospital setting, the use of copper fixtures instead of standard materials has decreased the amount of microorganisms found on the fixtures (4). Copper/copper alloy fixtures such as door handles, bed railings, toilet seat levers, and other commonly used equipment have decreased microbial abundance (5, 6). Schmidt *et al.* found that replacing normal fixtures in hospital rooms with copper alloys allowed for an 83% reduction in microorganisms found on the fixtures (6). Additionally, copper has been seen to kill a wide range of microorganisms, including both Gram-positive and Gram-negative bacteria (4).

Copper (II) oxide (CuO) nanoparticles have a wide range of uses in multiple industries (7). Nanoparticles are typically between one and a couple hundred nanometers in length and

nanoparticles display unique properties that are absent or diminished in bulk materials (8). Additionally, CuO nanoparticles are of high interest as they are the simplest copper compound, relatively inexpensive, and have a plethora of properties that could be used in different industries (8). CuO and copper (I) oxide (Cu_2O) nanoparticles have antimicrobial properties against a wide range of bacteria. Cu_2O nanoparticles were tested against *B. subtilis* and *P. aeruginosa* using minimal inhibitory concentration (MIC) which was found to be 66.5 µg/mL (9). CuO nanoparticles were also seen to be antimicrobial when tested against several types of bacteria (*E. coli*, *S. typhi*, *M. luteus*, *P. fluorescens*, *S. flexneri*, and *V. cholera*) using standard agar disk diffusion (10). *M. luteus* was found to have the largest zone of inhibition while, *E. coli* and *P. fluorescens* had the smallest zones (10).

The mechanism of how copper oxide kills microorganisms is not well known; however, several mechanisms are proposed. The two main mechanisms discussed are initial contact killing and the use of reactive oxygen species (ROS) to damage the cell (11). It has been proposed that CuO and Cu_2O use different mechanisms to kill microorganisms, with CuO relying heavily on creating ROS to damage the cell membrane and organelles inside the cell (11, 12). It is thought Cu_2O relies mostly on copper ions to target the cell envelope, rupturing the cell membrane and disrupting cell function (11).

Copper sulfide (CuS) also has known antimicrobial properties that are currently being used in research across several industries. CuS nanoparticles when combined with carrageenan films display antimicrobial activity against several types of bacteria including, *S. aureus*, *E. coli*, and *L. monocytogenes* (13, 14). Additionally, CuS nanoparticles are effective in treating bacterial infections in *in vivo* models (15). In both rat and zebrafish models, CuS nanoparticle treatment significantly reduced bacterial colonization compared to controls (15, 16). The zebrafish models

injected with CuS nanoparticles were still viable after 10 hours while the control fish had perished due to bacterial infections (15). Research on CuS antimicrobial properties is still in the early stages but could lead to a variety of antimicrobial surfaces.

Much like copper oxide, the mechanism by which CuS kills microorganisms is not well known. It is thought that CuS disrupts cellular function through the formation of ROS similar to CuO (15). There is evidence that CuS targets the cell wall of bacteria in addition to the cell membrane (15).

Properties of graphene

Adding graphene to copper compounds opens up new avenues for these composites to be used as antimicrobial surfaces. Graphene is a single 2D layer of carbon atoms, arranged in a honeycomb-like structure (17). This material exhibits many valuable characteristics including strength, high thermal conductivity, large surface area, optical transparency, and electrical conductivity (18). Many of graphene's characteristics stem from the way the carbon atoms are arranged and the sp^2 hybridization of the atoms (18). In the electronics sector, graphene is already replacing silicon in the making of electronic chips and there have been several medical applications proposed (17, 19). Graphene-based coating of medical devices, such as catheters, has been proposed due to antimicrobial properties of graphene derivatives such as graphene oxide (20).

Rationale

Identification of antimicrobial materials to use in healthcare will be imperative in combating the spread of hospital acquired infections. While CuS nanoparticles are antimicrobial, the antimicrobial properties of CuS have not been tested in a composite material with graphene. The addition of graphene to the material would allow for it to be used in many products such as

electronics, including touch screen devices. With graphene's ability to conduct electricity and still be transparent and CuS ability to kill microbes the composite material graphene-CuS could have large implications in creating antimicrobial touchscreens.

Here, we compared the antimicrobial properties of graphene-CuS and graphene-CuO against Gram-positive and Gram-negative bacteria. By changing the anion in the compound to sulfur, the effectiveness of the copper's ability to kill microbes was tested, and insights into the mechanism copper uses to kill microbes were assessed.

Methods

Synthesis of material

The synthesis of graphene-CuS was based on the experimental methods of Little *et al.* synthesizing zinc oxide-graphene composite material (27). The copper (II) thiourea precursor was synthesized from a precipitation reaction. 5.08g of copper (II) sulfate (student prepared) was dissolved in 20 mL of deionized water, and 3.89g of thiourea (Fisher, Certified ACS) was dissolved in 20 mL of deionized water. Once each was dissolved the solutions were slowly mixed together. A gel-like polymer started to form and the reaction was left to sit for several days. A brown precipitate was formed and dried in the drying oven overnight.

Graphene flakes (Alfa Aesar, S.A. 500 m^2/g) were used as the graphene precursor. Stoichiometric amounts of solid copper (II) thiourea and graphene flake precursors were measured using a balance and then mixed using a mortar and pestle. Samples were mixed to create a Cu:C mole ratio of 1:5. Each of the mixtures was suspended in 15 mL of deionized water and ultrasonicated at 60% power for 30 min to break up graphene clumps. The suspension was heated to 100 °C to remove the liquid water, and then to 200 °C to completely dehydrate the

powder. The sample was then heated to 300 °C for 6 h under an N_2 atmosphere for complete product formation. The graphene-CuO used was previously synthesized.

X-Ray Photoelectron Spectroscopy

The synthesized graphene-CuS was analyzed by X-ray photoelectron spectroscopy (XPS). The products were obtained with a Phi 5100 XPS system using an Al anode. XPS data were analyzed using XPSPEAK 41 software.

Media and growth conditions

E. coli (BW251143) and *B. subtilis* (3610) were the strains of bacteria used in testing. Each strain was inoculated into 5mL Luria broth (Lennox formulation) and grown overnight in 37 °C incubator with shaking.

Disk Diffusion

Graphene-CuS, graphene-CuO, and graphene control disks were prepared for disk diffusion. A suspension of each material was made. 4.9mL of N-Methylpyrrolidone (NMP) (TCI, 99%) and 111mg of graphene-CuO were added together in a vial. The graphene-CuS suspension was made using 4.7mL of NMP and 33mg of graphene-CuS (the concentrations are different due to the limited amount of graphene-CuS powder available). Each material was sonicated for 30 minutes. Immediately after sonication was completed, 10 microliters of material (either graphene-CuO or graphene) was pipetted into a glass vial, along with 50 microliters of denatured ethanol. The pipetting process was repeated 5 times, each time with a new vial. The graphene-CuS suspension had 30 microliters pipetted into each vial to account for a lower concentration of material. Once the material was in the vials, they were slowly (over 2 hours) pipetted onto filter disks. Approximately 10 microliters was pipetted onto a disk at a time, and allowed to dry before more was pipetted. Each vial corresponded to one disk. The disks were set in a petri dish prior to

pipetting, and each material had its own petri dish containing 6 disks. Once each material was fully pipetted onto its corresponding disk the disks were then sterilized in a furnace at 200 °C in the petri dishes, then were allowed to cool.

A single colony was inoculated in 5mL LB (Lennox formulation) media and grown at 37 °C with shaking. After 24 hours, saturated cultures were diluted to an optical density of OD=0.1. A sterile swab was dipped in each culture and streaked across the plate in three directions, rotating the plate after each direction.

A graphene-CuS, graphene-CuO, and a graphene control disk were placed onto each plate using sterile tweezers. Each disk was labeled appropriately on the plate. The plates were then incubated at 37 °C for 48 hours total. Plates were imaged at 24 hours and 48 hours. Three replicates of disk diffusion were performed for each species. For analysis, the plates were scanned into a computer, and zones of inhibition were then measured and analyzed using ImageJ (26). Average zone of inhibition for graphene-CuO and graphene-CuS were compared with a Student's *t*-test.

Results

X-ray Photoelectron Spectra Analysis of Composite Materials

X-ray photoelectron spectra (XPS) of both graphene-CuS and graphene-CuO were conducted to verify composition of both materials. Graphene-CuO had peaks fit for copper-2p, oxygen-1s, and carbon-1s. The adventitious carbon peak in the C1s spectrum was used to calibrate the rest of the XPS data. The carbon spectrum was fit to four peaks which was to be expected (Figure 1). The oxygen spectrum was fit to two distinct peaks, indicating the oxygen had two distinct bonding environments within the material (Figure 2). Additionally, the copper spectrum was to be expected based on literature results of CuO XPS peaks (Figure 3).

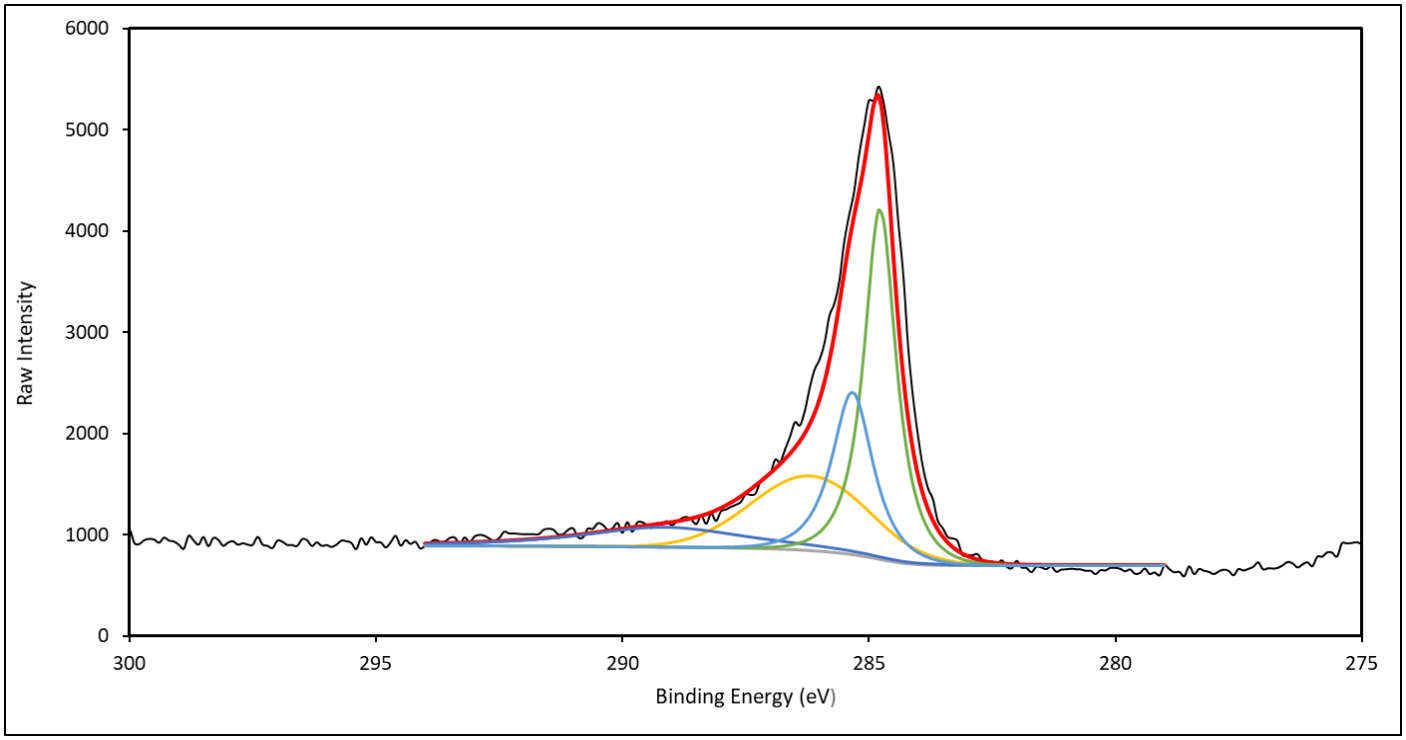

Figure 1. *C 1s spectra of graphene-CuO. Fit to 4 peaks.*

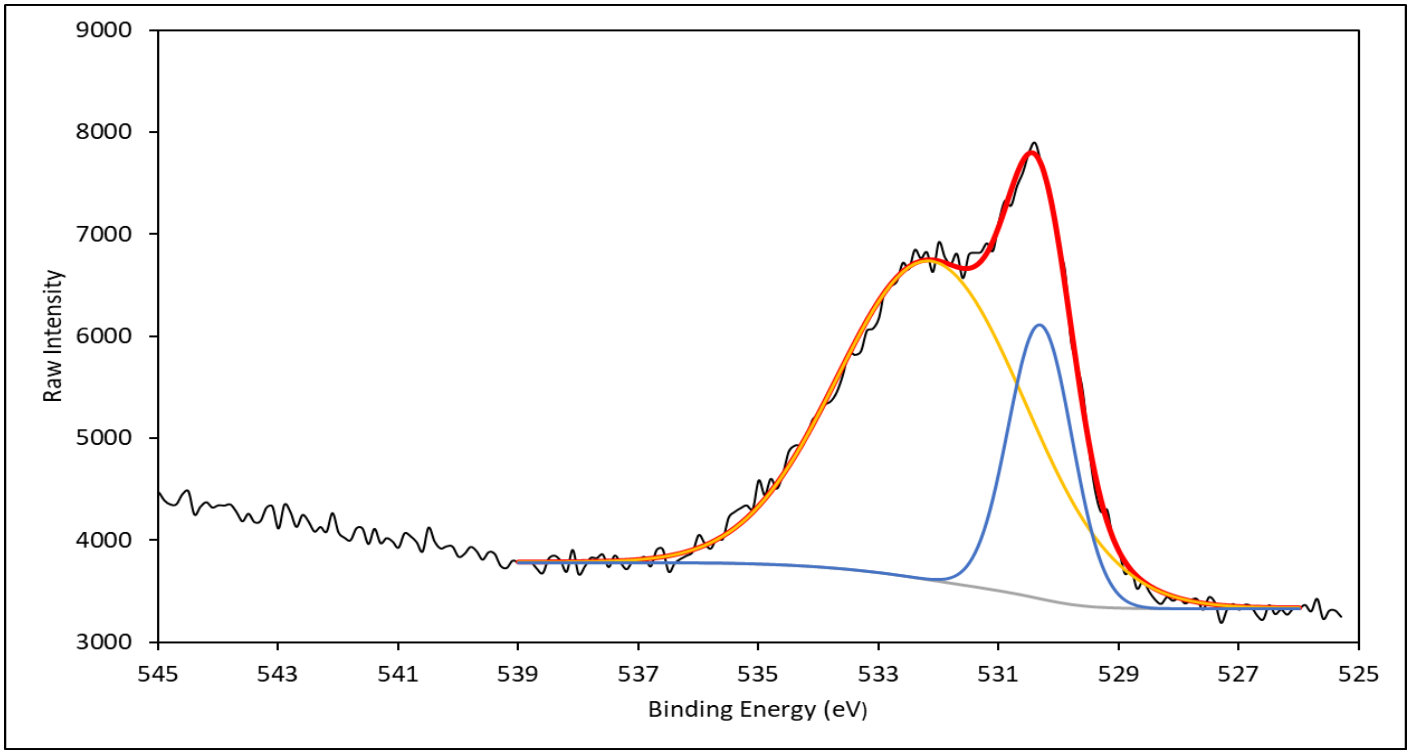

Figure 2. *O 1s spectra of graphene-CuO. Fit to 2 peaks.*

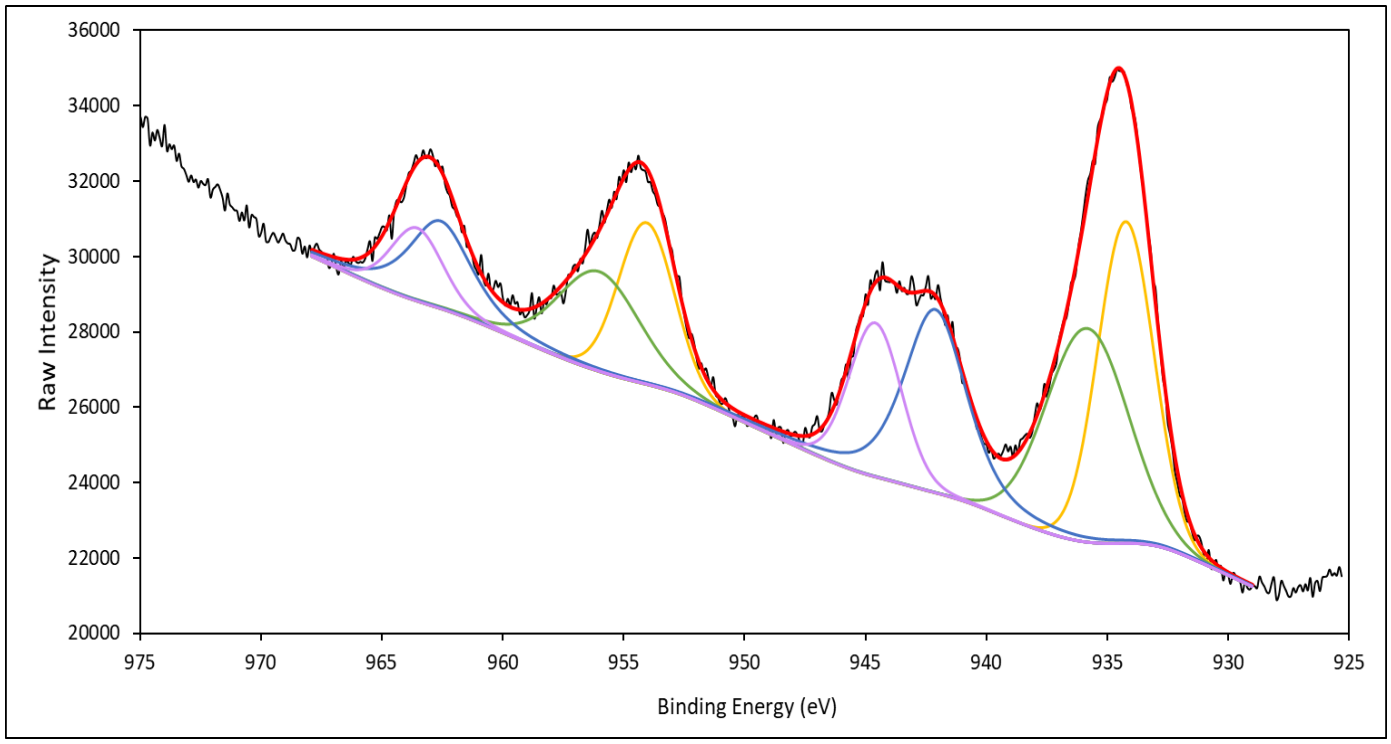

Figure 3. *Cu_2 3p spectra of graphene-CuO. Fit to 8 peaks.*

The graphene-CuS material was fit for peaks based on the copper-2p, oxygen-1s, sulfur-2p, and carbon-1s spectra. The graphene-CuS data however was not as distinct due to a malfunction with the XPS instrument. The carbon spectrum was fit to 2 peaks and was overall similar to graphene-CuO carbon fit (Figure 4). The oxygen spectrum was fit to one peak indicating only one dominant bonding environment for oxygen atoms in this material (Figure 5). The copper spectrum was the least distinct, however overall it was similar to what was expected based on literature XPS data (Figure 6).The sulfur spectrum could not be fit due to poor quality of the data from the malfunctioning instrument (Figure 7).

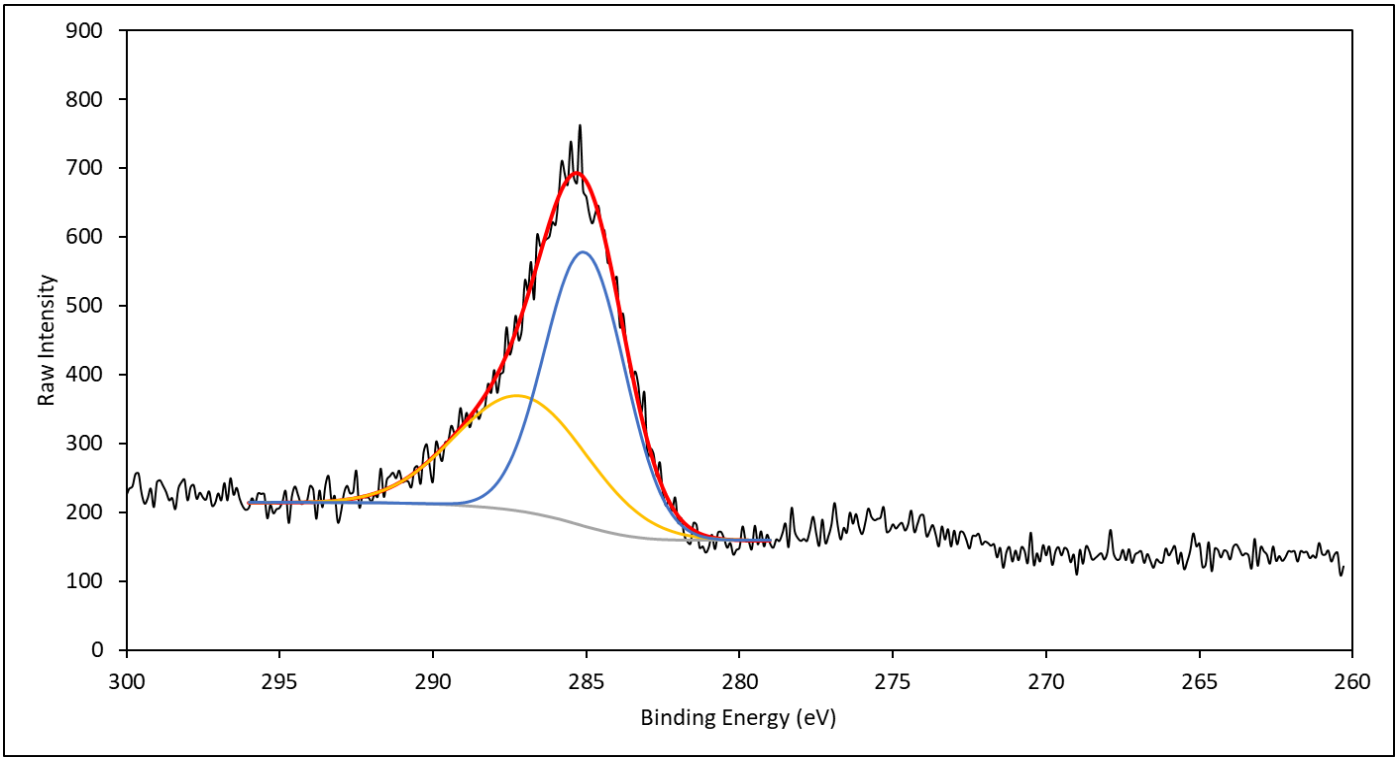

Figure 4. *C 1s spectra of graphene-CuS. Fit to 2 peaks.*

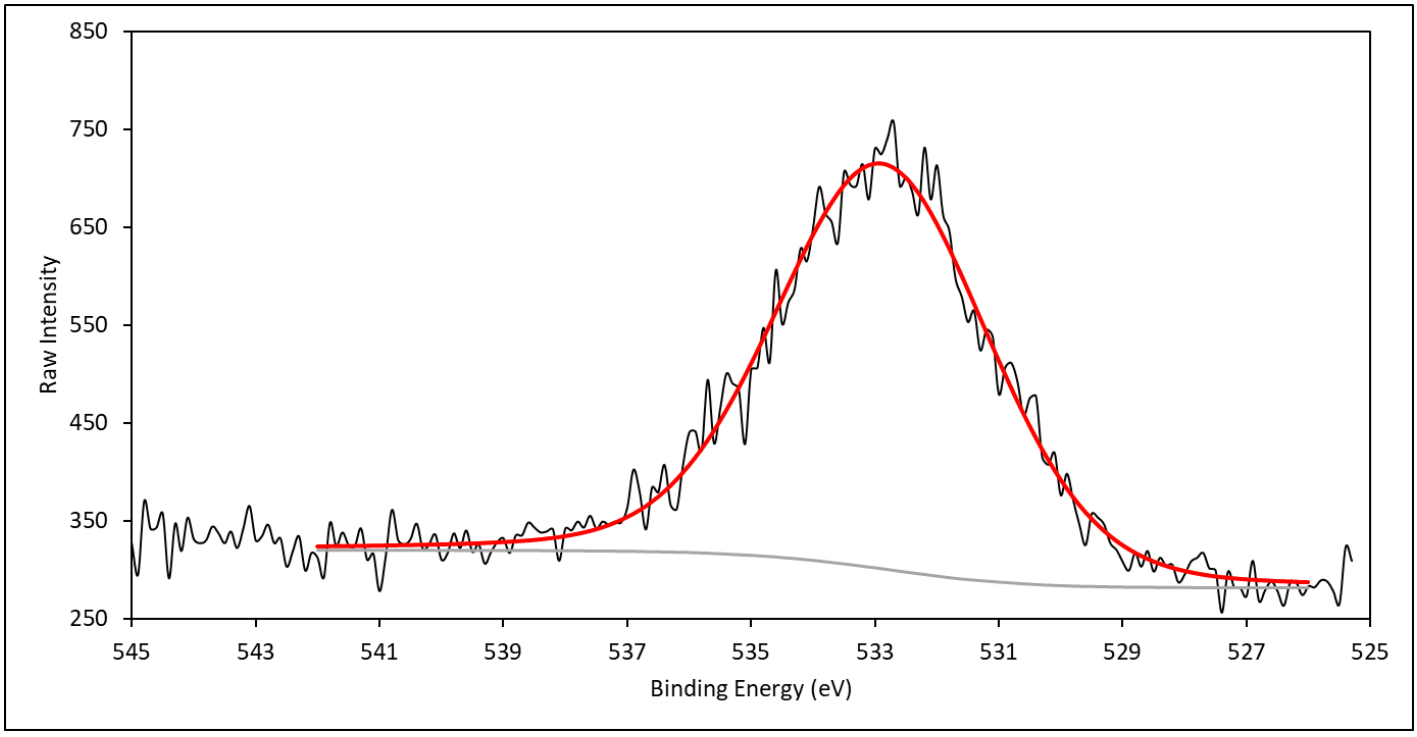

Figure 5. *O 1s spectra of graphene-CuS. Fit to 1 peak.*

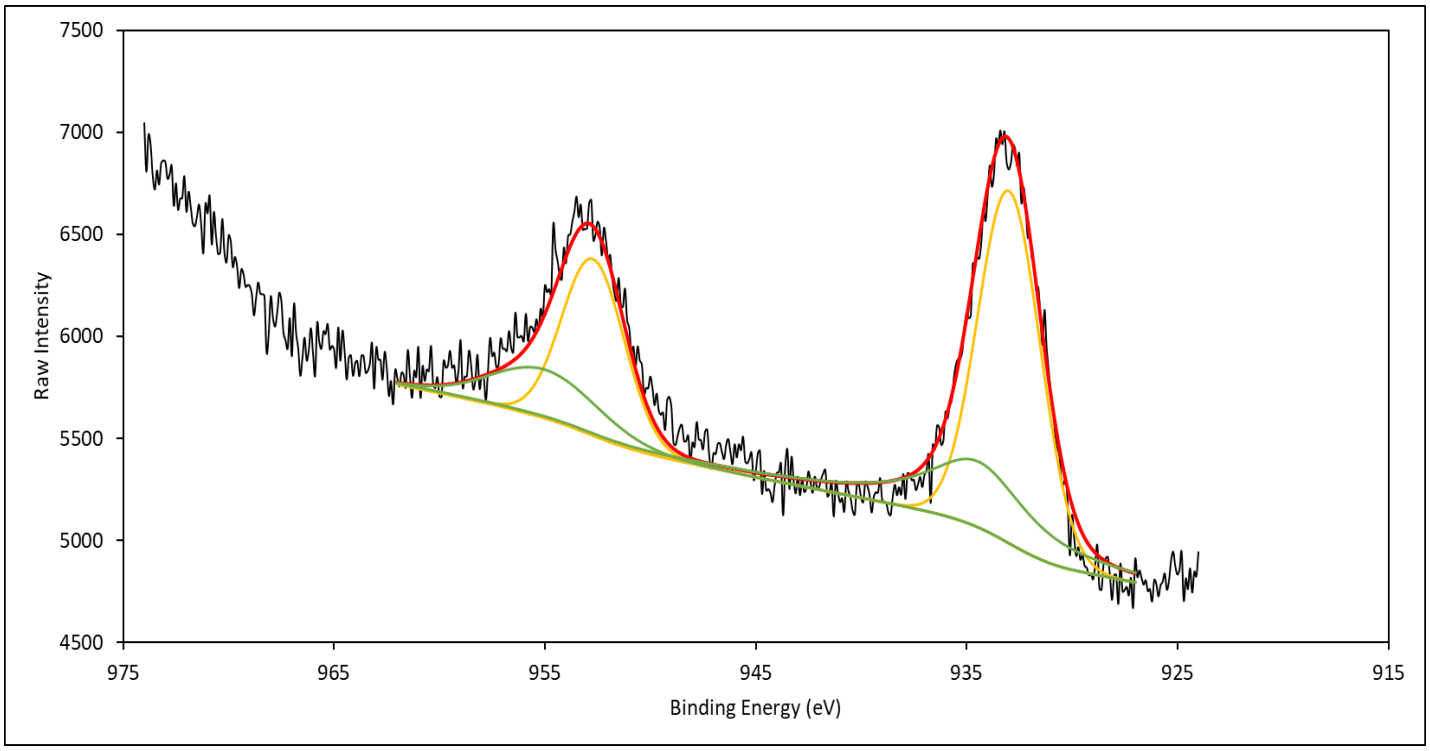

Figure 6. *Cu_2 3p spectra of graphene-CuS. Fit to 4 peaks.*

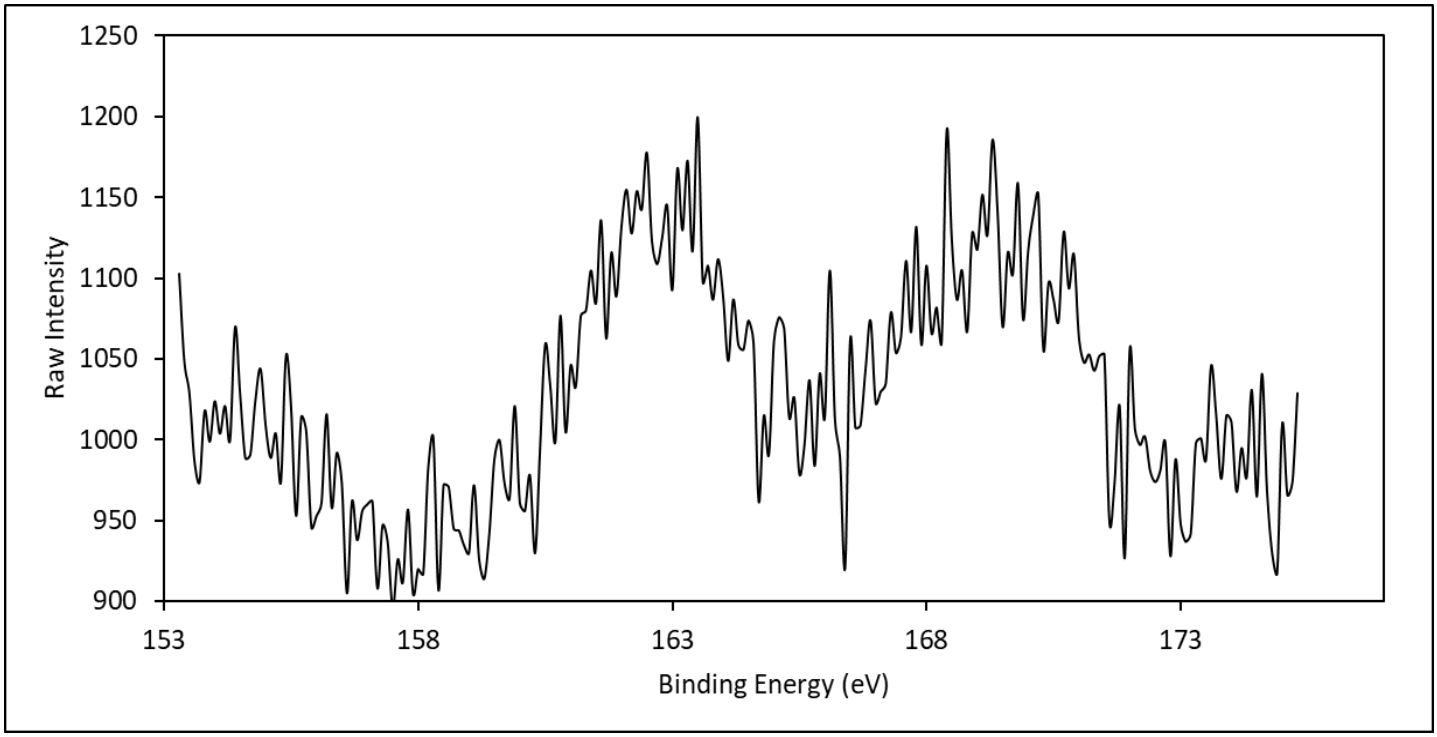

Figure 7. *S 1s spectra of graphene-CuS. No peaks fit*

CuS and CuO graphene nanoparticles display antimicrobial properties

To assess the antibacterial activity of our materials, the composites were cast on filter discs and used in a disk diffusion assay with *B. subtilis* and *E. coli*. For *B. subtilis* both graphene-CuS and graphene-CuO discs resulted in zones of inhibition after 24 hours in the incubator (Figure 8a). However, the zones for both materials were hazy, so they were left in the incubator for additional 24 hours, after which time they were more distinct (Figure 8b). The average diameter of the zone on the *B. subtilis* plate for graphene-CuS was 29.3 mm while the average zone diameter for graphene-CuO on the *B. subtilis* plate was 27.6 mm after 48 hours (Figure 8). While the average zone was larger for graphene-CuS using a Student's *t*-test the difference in diameter was found to not be statistically significant ($p > 0.05$) (Figure 9). Additionally, results from day 1 and day 2 for both materials were compared to determine if a longer duration in the incubator would create a larger zone. The difference in the zones of inhibition for both materials was insignificant as ($p > 0.05$). There were no zones of inhibition for either material found on the *E. coli* plates after two days (Figure 10).

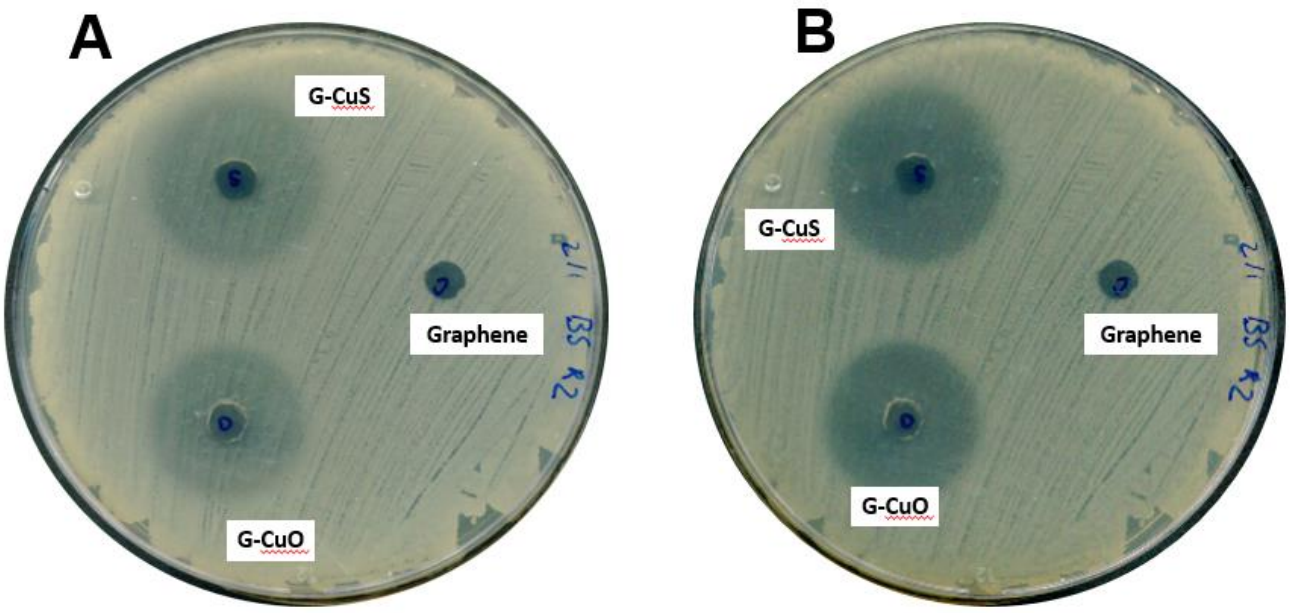

Figure 8. *Zone of Inhibition for graphene-CuS and graphene- CuO on B. subtilis after 24 hours (A) and 48 hours (B) in 37°C incubator. No zones were present for control disks.*

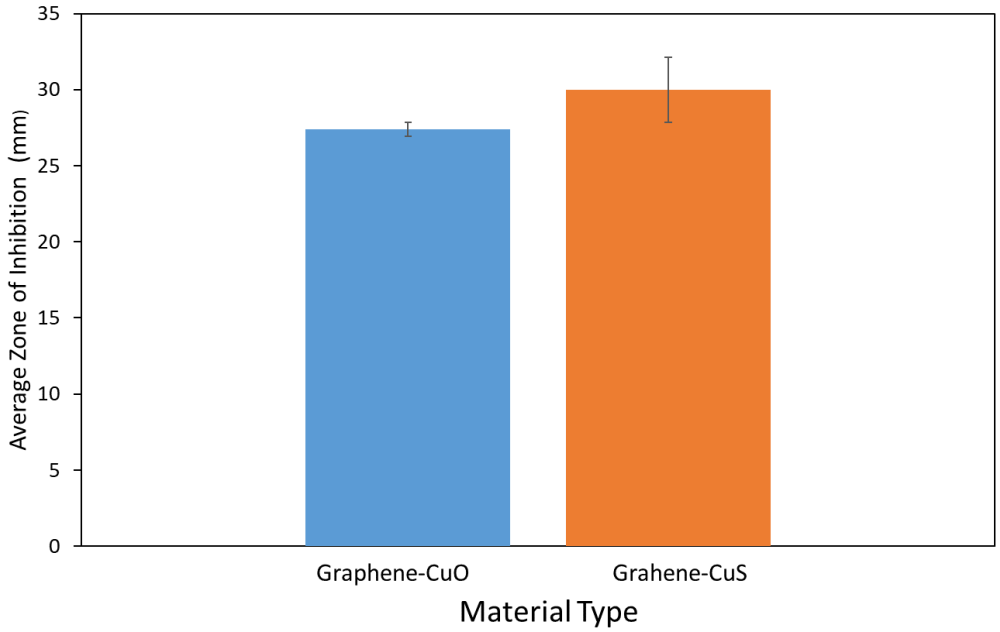

Figure 9. *The average diameter of zone of inhibition for each material at 48 hours. Error bars are shown from standard deviation. Using Student's t-test, no significance between zones was found ($p>0.05$).*

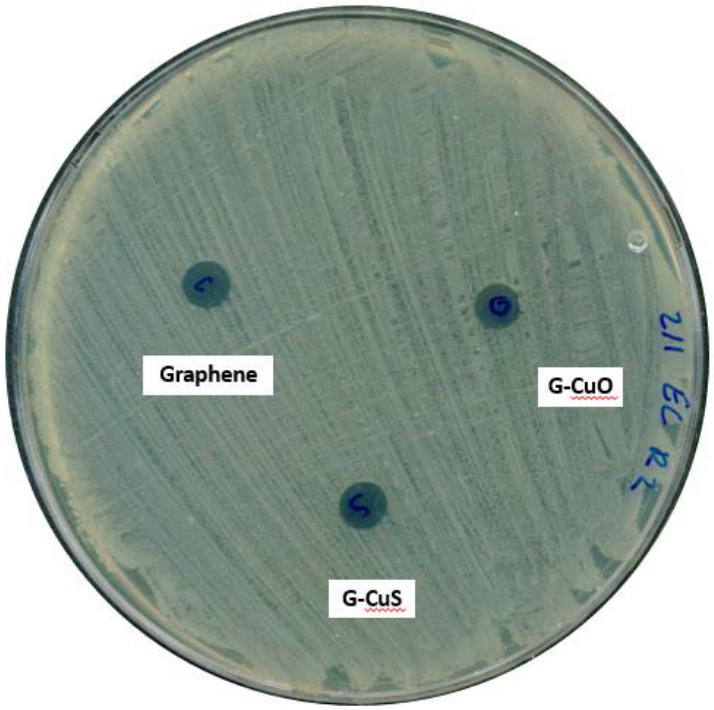

Figure 10. *No Zones of Inhibition present for any material on E.coli plates after 24 hours at37°C.*

Discussion

The XPS results indicate that the graphene-CuO composite is truly graphene-CuO, and the graphene-CuS composite is most likely truly graphene-CuS. Due to the unreliability of the instrument when analyzing the graphene-CuS composite it cannot be said with certainty we made the correct composite material, however, it is known sulfur is in the material, and it is not simply graphene-CuO. The oxygen spectra are the most telling analysis. The graphene-CuO O-1s spectrum shows 2 peaks indicating two different oxygen bonding environments. This would be oxygen bonded to carbon on the outer edge of the material and also oxygen bonded to copper. In the graphene-CuS O-1s spectrun there is only one peak, indicating the oxygen in the material is only bonded in one type of environment. It is assumed the oxygen here is bonded to the carbon on the outer edge of the material due to the similar binding energy the peak is at compared to O-

1s graphene-CuO spectra. There is no evidence of CuO impurity from this O-1s XPS spectrum. Additionally simply having the S-2p XPS spectrum at all shows there is indeed sulfur in the graphene-CuS material. For more certainty, the graphene-CuS material should be analyzed through XPS again to try to obtain more clear results.

Our disk diffusion results indicate that both graphene-CuS and graphene-CuO display comparable antimicrobial properties against *B. subtilis*. However, neither composite displayed antimicrobial activity against *E. coli*. These findings indicate that the anion of the graphene-copper composite does not significantly influence the way the composite disrupts cell function, ultimately leading to cell death.

For both composites on the *B. subtilis* plates, the zones of inhibition became more defined after a total of 48 hours in the incubator. While zones of inhibition were present at 24 hours (Figure 8a), the zones became more distinct after an additional 24 hours (Figure 8b), indicating the composites were more effective with more time. Recent literature has also seen with increased time the survival rate of bacteria decreases. In a study investigating the antimicrobial properties of CuO embedded in fabrics, the researchers found that the bacteria survivability decreased as the exposure time of CuO to the bacteria increased (22). They found that total viable counts of bacterial colonies reached 0% after 10 hours for *S.aureus* and 0% for *E.coli* after 12 hours (22). This is a faster killing time than seen in our study, and may have to do with the Cu:C ratio used or structure of the nanoparticles in the composite materials. Using a higher Cu:C ratio would most likely create a shorter killing time as there would be a higher percentage of copper in the material.

While both composites had zones of inhibition on the *B. subtilis* plates, no zones of inhibition were observed on the *E. coli* plates. Similar conclusions were drawn from Xie et al. in

their study investigating the antibacterial activity of cellulose/graphene copper oxide nanocomposite films (23). Their study tested the antibacterial properties of cellulose/graphene copper oxide films using disk diffusion, on *S. aureus, E. coli, B. subtilis, and P. aeruginosa* plates. They found both strains of Gram-positive bacteria (*S. aureus and B. subtilis*) had significantly larger zones of inhibition than the Gram-negative bacteria (23). While these researchers still had zones of inhibition against Gram-negative bacteria, it supports the data found in this study, copper composites are more effective against Gram-positive bacteria than Gram-negative bacteria. The differing ability of copper composites to kill Gram-positive and Gram-negative bacteria is most likely due to the structure of each bacteria's cell envelope. Gram-positive bacteria do not have an outer membrane. Instead, they have a thick wall of peptidoglycan protecting the cytoplasmic membrane, while Gram-negative bacteria have an outer membrane and a thin wall of peptidoglycan protecting the inner membrane (24). Gram-negative bacteria's outer membrane outer leaflet is composed of glycolipids, mainly lipopolysaccharides which are not found in Gram-positive bacteria (24). The lipopolysaccharides help create another barrier between the outside environment and the inside of the cell, keeping out the entry of harmful or toxic molecules such as copper ions (24). The multiple membranes and added lipopolysaccharides found in Gram-negative bacteria are most likely why no zones of inhibition were found in *E. coli*.

The mechanism by which copper sulfide disrupts normal cell function leading to cell death is being researched. Since both the graphene-CuS and graphene-CuO exhibited the same zones of inhibition on each plate, the mechanism in which each material kills microbes is most likely very similar, if not the same. More research has been done concerning copper oxide rather than copper sulfide, and the two mechanisms that have been observed are contact killing and the

production of reactive oxygen species (ROS) (11, 12 25). Contact killing is the release of copper ions which initially cause damage to the bacterial cell envelope through electrostatic interactions between the ions and the cell membrane (11). The ions damage the membrane through these interactions, causing increased permeability allowing for more copper ions to enter the cell. Intracellular copper ions can induce DNA and protein damage, inhibit enzymes through competition for metal-binding sites, and allow for cytoplasmic leaking leading to cell death (12, 25). The other mechanism by which copper ions can kill microbes is through the production of reactive oxygen species (ROS). Copper ions can synthesize ROS through Fenton-type and Haber-Weiss reactions, most often creating the highly reactive molecule H_2O_2 (25). ROS is made both internally and externally and can cause cellular damage through macromolecule oxidation (11). As our material was only effective in killing Gram-positive species, it is likely it employed the contact killing method of the copper ions puncturing the cell membrane, rather the use of ROS. Our results indicate the anion of a copper composite does not play a role in enhancing or inhibiting the proposed mechanisms in which copper kills microbes.

Our results add to the existing literature investigating copper composites' antimicrobial activity, by demonstrating graphene-CuS to be antimicrobial. This property opens avenues to investigate how this material could coat touchscreen surfaces to in turn make them antimicrobial. Antimicrobial surfaces are an area of high research due to the increase in highly virulent pathogens, and the high use of touchscreens used not only in daily life but also in medical practices (1). Further studies should be conducted testing different Cu:C ratios graphene-CuS to better understand the needed amount to effectively kill Gram-negative bacteria. These data are a preliminary step in creating antimicrobial touchscreens.

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