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**Research** Letter



# Copper-carbon hybrid nanoparticles as antimicrobial additives

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#### Abstract

Millions of cases of hospital-acquired infections occur every year involving difficult to treat bacterial and fungal agents. In an effort to improve patient outcomes and provide better infection control, antimicrobial coatings are ideal to apply in clinical settings in addition to aseptic practices. Most efforts involving effective antimicrobial surface technologies are limited by toxicity of exposure due to the diffusion. Therefore, surface-immobilized antimicrobial agents are an ideal solution to infection control. Presented herein is a method of producing carbon-coated copper/copper oxide nanoparticles. Our findings demonstrate the potential for these particles to serve as antimicrobial additives.

#### Introduction

With the impact of COVID 19 and its variants, the threat that pathogenic microbes pose to public health has been brought to the forefront in an undeniable way. The common solution to prevent infections tends to rely on organic amine disinfectants, potentially corrosive disinfectants such as bleach or antibacterial/antifungal drugs. These solutions, however, may lead to drug resistance or can harm or corrode the immediate environment over repeated use. Since about 80% of microbes are transmitted through surface contact another approach is to prevent the spread of harmful microbes and develop surface technologies that prevent pathogenic infections, while avoiding the concerns of drug resistance with minimal environmental impact. Metal oxide nanoparticles such as zinc oxide and copper oxide exhibit antimicrobial properties at different degrees in a variety of materials, forms, and morphologies. Metallic nanoparticles have been reported to show antimicrobial activity especially towards pathogenic bacteria such as E. coli and S. *aureus* that can be very resistant to conventional antibiotics.<sup>[1]</sup> Demonstrated herein is an economical, scalable, and facile sol-gel method to synthesize copper nanoparticles embedded in a carbon matrix as well as a basic demonstration of applicability as an antimicrobial agent.

Porous carbon and porous carbon-metal materials have applications ranging from electrochemical electrode materials<sup>[2–8]</sup> to catalytic surfaces for various reactions,<sup>[9,10]</sup> to filtration and separation of oil and non-polar solvents from solution.<sup>[7,11,12]</sup> These porous carbon structures can be referred to in literature as foams and sponges, and have traditionally been made from non-renewable sources such as tar pitch. In the past few years, there has been a move away to precursors that would be more environmentally friendly in the case of a spill such as natural polymers and carbohydrates such as cellulose. starch, and chitin<sup>[13–16]</sup> as a renewable replacement to tar pitch. Sucrose in particular has been used as a precursor in many studies<sup>[3,11]</sup> to synthesize these foams and sponges as a stand-in for simple disaccharides and monosaccharides in the research of these porous bulk morphologies due to commonality as table sugar. In this work, we seek to expand the potential applications of porous carbon materials synthesized from polysaccharides based on previous work from our lab.<sup>[11]</sup> Antibacterial and antifungal properties of Ag and Cu nanoparticles<sup>[14,17-20]</sup> could potentially be impregnated into high surface area carbon structure foams allowing for simultaneous sorption<sup>[21-23]</sup> and bactericide. The antimicrobial additive described herein which contains Cu nanoparticles is made from foam variants optimized to produce high quantities of fine antimicrobial particles that may be of specific interest due to the ongoing pandemic.

#### **Results and discussion** Material characterization

In the pursuit to characterize the material after synthesis, we completed the following tests: XRD, SEM, EDS, and Raman spectroscopy. Using XRD, we are able to show what are the organized crystals that form during the final calcination step. Based on the representative peaks, we have two main crystalline phases: metallic copper and copper(I) oxide as shown in Fig. 2. Based on their relative intensities, we can infer that metallic copper is the more prevalent of the crystalline phases. Based on EDS, we can see about half of the atoms in the final powder sample are carbon. As both copper and copper(I) oxide do not



normally contain carbon, we can infer that the carbon must be predominantly in an amorphous form that would not appreciably show up on the XRD. We can see by overlapping the EDS and SEM images that the elements copper, oxygen, and carbon are fairly evenly distributed throughout the powder. This EDS and accompanying SEM data can be seen in Fig. 1 with a high zoom level image to show the microstructure in Fig. 2. Based on this, we infer the amorphous carbon must be a coating on the copper and copper(I) oxide particles. The Raman spectroscopy on these samples was not easy as taking a Raman scan in most locations in the powder sample does not yield a Raman response. It is our understanding that the amorphous carbon coating disperses the Raman laser sufficiently to prevent registration of any Raman signal. However, taking a Raman scan of a metallic copper particle that had its amorphous carbon coating removed during milling will give the Raman signal of graphene. This implies that the interface between the metallic copper and the amorphous

carbon grows a single layer of graphene. This single layer of graphene and amorphous carbon layers are important for many reasons. These layers help determine the final particle size as it prevents the Cu from agglomerating into an ingot during the calcining step of the synthesis. Sugar serves as reductants for the copper(II) oxide intermediates to copper(I) oxide and copper metal while the remainder becomes the carbon coating. This small amount of crystalline carbon was too little to show up on the XRD but it does show up on a Raman scan of an exposed crystallite face. The Raman spectrum and what the exposed crystallite face looks like can be observed in Fig. 2.

### Antimicrobial susceptibility: Kirby–Bauer disk diffusion results

The gold standard of measuring antimicrobial susceptibility is the Kirby–Bauer Disk Diffusion method of susceptibility. This method allows microbial susceptibility categorization



**Figure 1.** (a) Wide area SEM image showing what the microstructure looks like, (b) SEM elemental EDS mapping overlapping SEM image, C) Copper EDS spectrum map, (d) Carbon EDS spectrum map, (e) Oxygen EDS spectrum map, (f) EDS elemental spectrum.



**Figure 2.** (a) XRD spectrum indicating labeled peaks for copper oxide (CuO) and copper (Cu) with 2θ values at 36.5, 43.2, 50.4, and 74.1 degrees, respectively. (b) High zoom level SEM to show microstructure of the resulting particles, (c) Raman spectra showing Graphene peaks, G peak around 1580, 2D peak around 2700, (d) Microscope image showing the Raman spot location at ×100 zoom.

of bacterial isolates against a variety of antimicrobial agents. These data allow for further analysis and characterization of specific antimicrobial agents.<sup>[24]</sup> In order to measure the level of antimicrobial sensitivity of the Cu@pC material, the Kirby–Bauer disk diffusion method was performed. Antimicrobial activity assessment was made by measuring the diameter of the zone of inhibition which formed around each well as can be seen in Fig. 3. We tested 4 different Cu@pC mixtures and saw insignificant differences between their zones of inhibition based on the variation of Cu concentration. Based on these results, we ran the remaining tests with a mixture of moderate Cu concentration, our 1:2.5 mixture. Positive and negative controls were run with each experiment. Effects were compared to a positive control of the standard antibiotic ciprofloxacin (concentration 20 µg/mL). A clear diameter zone of inhibition

formed around ciprofloxacin antibiotic disks of 30 mm after an eighteen-hour incubation, which is considered a standard antibiotic sensitive reading. Control plates with no antibiotic showed clear expected growth covering the agar plate.

# Viability and cell proliferation assays results

Resazurin viability assays are vastly advantageous because they perform with superior sensitivity and contain a wide dynamic range. Measuring light emitted during a chemical reaction is practical because the light output is a relative quantification of the number of viable cells. Since cell growth creates a reduced environment and inhibition of growth maintains an oxidized environment, the resazurin





**Figure 3.** Experimental setup for Kirby–Bauer disk diffusion with four variants of Cu@pC as well as an antibiotic control showing growth inhibition of *E. coli*. (a) Pre-incubation image of the agar plates, (b) post-incubation showing growth inhibition around the Cu@pC and antibiotic, (c) relative fluorescence of *E. coli*, *P. aeruginosa*, or *S. aureus* resazurin assays treated with Cu@pC compared to antibiotic control and untreated control.

assay can be used to reproducibly detect cells. Here, bacterial strains Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa), and Staphylococcus aureus (S. aureus) are incubated in the presence of Cu@pC and their viabilities are measured by fluorescence of resazurin reactions. E. coli, P. aeruginosa, and S. aureus are inoculated in liquid Mueller-Hinton broth (Sigma) and incubated overnight at 37°C. No active growth of E. coli, P. aeruginosa, or S. aureus was detected in samples treated with Cu@pC as indicated by blanked RFU relative to control, untreated wells as seen in Fig. 3, following end-point measurements after overnight incubation. Resazurin viability assay results indicate that Cu@pC prevents the growth of E. coli, P. aeruginosa, or S. aureus in vitro. We chose to use these organisms as antimicrobial test candidates as they contribute to the cause of most hospital-acquired infections worldwide. While resazurin viability assay alone only indicates viable, metabolically active cells and does not consider inactive or dormant cells which may turn active given the right conditions it does demonstrate a growth inhibition in these major disease pathogens.



**Figure 4.** (a) Pathogen viability over 2 h of exposure to Cu@pC material, (b) snapshot showing growth of the fungus *C. parapsilosis* positive control on bottom half of agar plate and C. parapsilosis treated with Cu@pC on top half of the agar plate, (c) live/dead cytotoxicity assay, showing the spectrum of four Cu@pC variants verses the commercial antibiotic Cipro.

#### Liquid broth culture method results

The previous resazurin viability assays have indicated that Cu@pC can prevent the growth of common bacteria E. coli, P. aeruginosa, or S. aureus. An important parameter that must be analyzed is the kinetics and type of growth inhibition. Here, we test Cu@pC against gram-positive bacteria, gram-negative bacteria, as well as fungus. E. coli, P. aeruginosa, or S. aureus, C. parapsilosis, E. faecalis, and K. pneumoniae are inoculated in liquid Mueller-Hinton broth (Sigma) and incubated overnight at 37°C. Colony forming units are quantified and plotted over time. No viable cells were present an hour following incubation with Cu@pC as can be seen on the graph in Fig. 4. All positive controls had acceptable growth. Cu@pC also expressed 100% effective killing of the fungal organism, C. parapsilosis, as seen in the agar plate pictured in Fig. 4. Figure 4 shows a fungal plate split in half, with growth of C. parapsilosis positive control on the bottom half and Cu@pC-treated sample on the top half. These results suggest that the Cu@pC is bactericidal/ fungicidal rather than just biostatic.

# Modified cytotoxicity fluorescence assay results

To investigate the antimicrobial effect and activity on a surface treated with Cu@pC, a modified live/dead cytotoxicity assay was designed. Modification of this fluorescence staining technique helps to distinguish surface antimicrobial agents from solution diffusion agents. Here, an inoculation chamber is created with the test substrate and a glass coverslip. Cu@ pC is embedded in PMMA to simulate a surface coating and inoculated with fluorescently stained bacterial cells then cover slipped. Two distinct populations are probed by fluorescence microscopy for viability: cells in solution and cells adhered to the surface of the test substrate. Effective killing of cells is found only on the substrate surface of truly surface immobilized agents, whereas exclusively diffuse agents result in killing of cells only in solution.

Immediately following inoculation of PMMA-embedded Cu@pC with *E. coli* and *S. aureus*, the inoculation chambers are visualized with a fluorescence microscope. No live cells are visualized at the surface of Cu@pC as characterized by red fluorescing cells in Fig. 4, while green fluorescing cells are visible which is an indication of cell death. While the intensity of the peak is greater for green on Cu@pC samples than on Cipro this is just an indication that more cells are retained on the surface likely due to surface roughness, as all samples do not show live red fluorescing cells. In addition to PMMA-embedded Cu@pC coating providing effective killing of *E. coli* and *S. aureus*, killing was observed immediately after contact with the surface. Some killing was observed in the solution portion of the chamber, indicating that some agent diffusion is still taking place in PMMA-embedded Cu@pC coating.

## Conclusion

As has been demonstrated by various sources copper metal, copper salts, and its alloys are antimicrobial.<sup>[17-19]</sup> Herein, we have demonstrated that this antimicrobial property is retained when precursors of copper nitrate, sucrose, and nitric acid are processed into a carbon-coated copper/copper oxide nanoparticle. Copper nitrate in addition to being a common fertilizer is a waste product of precious metals refinement in particular silver refinement where it is commonly produced as a byproduct of reducing metallic silver out of a solution of silver nitrate. It can also be produced by dissolution of copper oxide or by oxidation and dissolution of metallic copper in nitric acid. Copper nitrate's decomposition vapor products also can be captured and converted back into nitric acid for reuse as nitric acid precursors or used to generate more copper nitrate from copper scrap. The demonstration of a method of converting a common copper waste/recycling byproduct into a usable antimicrobial additive cannot be understated as a strength in future low to zero waste societies. We used sucrose as a renewable source of carbon but in theory any other water-soluble organic molecule should also suffice as a carbon source for generation of these carbon-coated nanoparticles. These findings lay the groundwork for further testing into making antimicrobial additives from copper and copper alloys as well as testing with other carbon sources for their applicability to form antimicrobial powders. One advantage of using copper as an antimicrobial surface additive over traditional oral antibacterial or antifungal agents is that its use does not lead to antibiotic resistance to

these organic oral antibiotics thus helping preserve their effectiveness into the future while still having the advantages of an antimicrobial surface. By demonstrating that our Cu–carbon composites not only inhibit but kill microbes such as bacteria and fungi, we demonstrate a renewable and large-scale method to produce an antimicrobial additive that would not contribute to antibiotic resistance of traditional organic antibiotics.

## Material synthesis, characterization, and antimicrobial testing methods Synthesis method

Cu@pC was produced utilizing a four-step process. First step is to generate a copper containing foam from a solution of sucrose, copper nitrate, and nitric acid dissolved in DI water by heating on a hot plate set to 475°C. In preparation for transfer from beaker to crucible, the foam is desiccated in a vacuum oven at 150°C to embrittle the foam. After this second step, the foam is transferred from the beaker to an alumina crucible for the next step where it is calcined in a vacuum oven set to temperature in the range of 500–1000°C to produce a carbon matrix that contains our desired copper/copper oxide particles. The final step is to transfer the carbon matrix to a ball mill to mill the foam into a fine powder which is mostly made up of the copper/copper oxide particles coated in carbon.

## **Characterization method**

Crystal and molecular structure were characterized and measured using a PANalytical Empyrean Series 2 Copper  $k_a$  source X-ray diffractometer. X-ray diffraction readings were taken from 10 to 80° at a scan step size of 0.105 at 287.38 s per step.

Morphology imaging and elemental analysis were observed under a Thermo Fisher Scientific NNS450 scanning electron microscope (SEM) with an Oxford Instruments X-Max energydispersive X-ray spectroscopy (EDS) detector. All measurements and images were taken at an accelerating voltage of 20 kV and a 5 mm working distance.

Raman measurements were taken on a Horiba LabRam system at  $100 \times$  optical zoom with a 60 mw 532 nm laser filtered by a 10% neutral density filter. Raman was performed with a multi window scan between 1000 and 3000 cm<sup>-1</sup>.

## Kirby–Bauer disk diffusion

On a nutrient-rich agar petri-dish, a bacterial lawn consisting of the isolated organism is cultured in the presence of an antimicrobial agent. Minimum inhibitory concentration (MIC) values are determined based on the diameter free of microbial growth (zone of inhibition) around each disk. These data allow for further analysis and characterization of specific antimicrobial agents.<sup>[24]</sup> Mueller–Hinton agar growth media plates were brought to room temperature and 10 mm wells were punched out of the agar.<sup>[25]</sup> The plates were then inoculated with a pre-cultured solution of 0.5 Mcfarland adjustment (~150e6 CFU/mL) concentration of *E. coli* using a densitometer. Wells were filled with 0.2 g of Cu@ pC and with carbon sponge lacking copper, and incubated overnight. Plates were incubated overnight at 37°C and 5% carbon dioxide.<sup>[26–28]</sup> Plates were removed and observed the following day.

### Viability and cell proliferation assays

Here, bacterial strains Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa), and Staphylococcus aureus (S. aureus) are incubated in the presence of Cu@pC and their viabilities are measured by fluorescence of resazurin reactions. E. coli, P. aeruginosa, and S. aureus are inoculated in liquid Mueller Hinton broth (Sigma) and incubated overnight at 37°C. Approximately 150 million CFU of each bacterial strain is measured using optical density measurements and dispensed in a 96-deep well plate containing Cu@pC to a final volume of 1 mL using fresh Mueller-Hinton nutrient broth.<sup>[29]</sup> The plate is incubated on a shaker overnight at 37°C. Following incubation, 100 µL of Resazurin (Biotium Resazurin Cell Viability Kit) is added to each well of the test samples and controls and incubated again for 1 h at 37°C. Fluorescence is measured using an Eppendorf plate reader with an excitation wavelength of 571 nm and emission wavelength of 585 nm. Background fluorescence is subtracted from each test sample.

# Liquid broth culture method

Liquid broth culture method is a way of testing the kinetics and type of growth inhibition. Measuring the growth over time can be accomplished by incubating the test sample in liquid broth and dispensing a fixed volume of the broth onto agar plates over time and counting the number of colonies that form on each time-interval agar plate.<sup>[25]</sup> Introducing the bacteria to a nutrient-rich environment and out of the stressed environment gives any viable cells an opportunity to grow and to be quantified. This method also can reveal if the antimicrobial in question is bacteriostatic or bactericidal. For the purposes of our Liquid Broth Culture test, we used gram-positive bacteria, gram-negative bacteria, as well as fungus. E. coli, P. aeruginosa, and S. aureus, C. parapsilosis, E. faecalis, and K. pneumoniae were inoculated in liquid Mueller-Hinton broth (Sigma) and incubated overnight at 37°C. Approximately 150 million CFU of each bacterial and fungal strain is measured using optical density turbidity measurements and dispensed in a 96-deep well plate containing Cu@pC to a final volume of 1 mL using fresh Mueller-Hinton nutrient broth and incubated overnight at 37°C.<sup>[29]</sup> Positive controls of each microorganism are tested in wells without the addition of Cu@pC and negative controls are run with each organism in the presence of a susceptible antibiotic.<sup>[30]</sup> The plate is incubated on a shaker. Every hour, the plate is removed and 10uL of each sample is dispensed onto agar plates and smeared evenly across the plate with a disposable plastic loop. Agar plates and deep well sample plates are returned to the incubator. A final aliquot is taken at 12 h and agar plates are incubated 18 h.

# Modified cytotoxicity fluorescence assay

Centrifuge tubes of Mueller–Hinton broth were inoculated with slants of *E. coli* and *S. aureus* and incubated overnight. The cultures were pelleted in a centrifuge and washed three times in saline. The concentration was adjusted to about 107 CFU/mL using optical density. The live/dead stain solutions were prepared from the Baclight Bacterial Viability Kit from Invitrogen. The stain stock solution was prepared by mixing 1.5  $\mu$ L of propidium iodide stock and equal volume of Invitrogen SYTO9 stock in 97  $\mu$ L saline. 100  $\mu$ L of the stock solution was then added to 900  $\mu$ L of each washed solution of *E. coli* and *S. aureus* and incubated for 15 min.<sup>[31]</sup> Hundred milliliters of the stained cells were dispensed onto the surface of Cu@pC-PMMA and cover slipped.

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# **Data availability**

All data generated and analyzed during this study are included in this article.

# **Declarations**

#### **Conflict of interest**

All authors declare that they have no conflicts of interest.

# **Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1557/s43579-022-00294-2.

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