Antibacterial rGO-CuO-Ag film with contact- and release-based inactivation properties

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Abstract

To reduce the high operational costs of water treatment because of membrane biofouling, next-generation materials are being developed to counteract microbial growth. These modern anti-biofouling strategies are based on new membrane materials or membrane surface modifications. In this study, antimicrobial films comprising rGO, rGO-CuO, rGO-Ag, and rGO-CuO-Ag were synthesized, evaluated, and tested for potential biofouling control using *Pseudomonas aeruginosa* PAO1 as the model bacterium. The combined rGO-CuO-Ag film displayed enhanced reduction (10-log reduction) in biofouling in comparison to the rGO film (control), followed by the rGO-Ag film (8-log reduction) and rGO-CuO film (0-log reduction). This demonstrated that the use of mixed antimicrobial agents is more effective in reducing biofouling than that of a single agent. The rGO-Cu-Ag film exhibited consistent, controlled, and moderate release of silver (Ag) ions. The release of Ag ions produced a long-lasting antimicrobial effect. These results underscore the potential applications of combined antimicrobial surface-based agents in practice and further research.

Keywords: Reduced graphene oxide composite; Antimicrobial; Biofilm control; Silver ion release

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43 **1. Introduction**

44 Biological fouling or biofouling of membranes owing to the proliferation of microbes and
45 subsequent biofilm development remains a critical issue in water treatment involving the use of
46 membranes (Vrouwenvelder et al., 2008). Biofilms, resulting from microbial cell deposition on
47 membrane surfaces, reduce the permeate quality, limit process efficiency, and increase
48 operational costs (Gao et al., 2011; Nagy et al., 2011). Therefore, it is imperative to develop an
49 anti-biofouling strategy to increase the efficiency of the water treatment system and considerably
50 lower the operational costs.

51 One promising approach is the use of antimicrobial membranes, which are of two types: (1)
52 membranes that prevent the adhesion of microbial cells without necessarily killing or
53 inactivating the microbial cells and (2) membranes that have the ability to kill microbial cells
54 upon contact or by releasing antimicrobial agents into the surroundings (Hong et al., 2012; Li et
55 al., 2005; Pal et al., 2007; Sambhy et al., 2006). Contact-based antimicrobial surfaces exhibit a
56 microbial inactivating ability but do not release an antimicrobial agent, whereas release-based
57 antimicrobial surfaces exert antimicrobial activity by releasing an antimicrobial agent into the
58 surroundings. Although contact-based control strategies may exhibit excellent antimicrobial
59 properties, the build-up of inactivated microbes on the surface can eventually prevent microbial
60 cells in bulk liquid from coming into contact with the antimicrobial surface, thereby limiting its
61 long-term effectiveness. Furthermore, other inorganic, particulate, and organic molecules can
62 bind to the potent antimicrobial surface, causing a reduction, and eventually, the complete loss of
63 antimicrobial effectiveness. Therefore, a release-based approach seems more promising for long-
64 lasting antimicrobial membranes. Quaternary ammonium compounds, metals and their oxides,

and nanoparticles have been investigated as the sources of membrane-released antimicrobial agents (Buffet-Bataillon et al., 2012; Laloyaux et al., 2010; Morones et al., 2005). This is done by blending, grafting, or coating these biocides on surfaces. The interactions between these biocides and membrane materials generally include adsorption, electrostatic interactions, covalent bonding, and deposition (Buffet-Bataillon et al., 2012; Laloyaux et al., 2010; Morones et al., 2005). The most important aspect of this strategy is to manage proper release rate; all biocides should not be released simultaneously. Moreover, these biocides should be released in the appropriate concentration(s) necessary to exert an antimicrobial effect and to ensure long-lasting antimicrobial membrane effects. This approach to membrane modification for biofilm control is preferable to dosing biocides into the feed water of the membrane system because the microbial population and cell number during membrane-based water treatment may vary. Typically, biocides are dosed into feed water at a concentration above the effective concentration (over-dosing); hence, a considerable amount of biocide is wasted in the outlet water of the membrane system, potentially resulting in negative effects on the environment and operational costs.

Graphene oxide (GO) has attracted widespread attention for its potential application as a next-generation material for membrane fabrication as it is usable in water and wastewater treatment systems owing to its structure and remarkable properties (Boretti et al., 2018; Hegab and Zou, 2015). The anti-biofouling properties of these membranes have been extensively studied; however, stacked GO or reduced GO (rGO) membranes have insufficient antimicrobial properties for biofouling control (Alayande et al., 2019a; Alayande et al., 2019b; Wang et al., 2019). Therefore, some biocides have been incorporated into or coated onto graphene-based membranes to improve their antimicrobial and anti-biofouling properties (Alayande et al., 2020;

Jiang et al., 2015; Pang et al., 2019; Yang et al., 2018; Zhu et al., 2017). Desirable antimicrobial membrane strategies should include both inactivation of microbial cells and prevention of the adsorption of dead microbial cells onto the membranes.

Copper (Cu) is the first antimicrobial agent to have been certified by the U.S. Environmental Protection Agency owing to its broad-spectrum antimicrobial properties (Grass et al., 2011). The contact killing of microbes by copper surfaces has been well documented (Elguindi et al., 2009; Faúndez et al., 2004). Silver (Ag) compounds have also been used as antimicrobial agents for several applications for decades, including water treatment (Haider and Kang, 2015). When Ag is incorporated into materials or placed on surfaces as decoration, one of its major antimicrobial mechanisms is the release of Ag ions (Mollahosseini and Rahimpour, 2013). Therefore, for the present study, it was hypothesized that the combination of these two biocides (Cu and Ag) would impart both contact- and release-based antimicrobial properties. The goal of this study was to prepare a novel membrane material for biofouling control. Consequently, four film types—rGO, rGO with copper oxide (CuO) (rGO–CuO), rGO with Ag (rGO–Ag), and rGO with CuO and Ag (rGO–CuO–Ag)—were developed, and their potential for biofilm control was evaluated. The antimicrobial properties of these nanocomposite films were investigated using *Pseudomonas aeruginosa* PAO1 as the model bacterium. To our knowledge, this is the first study on the combined antimicrobial properties of rGO–CuO–Ag composite films.

2. Materials and methods

2.1. Preparation of nanocomposite films

The details of our study are illustrated in Table S1.

To synthesize rGO and rGO-Ag solutions, 1 mg/mL of GO solution (Graphene Supermarket, Calverton, USA) was sonicated, put into a Teflon-lined reactor, and heated in an oven at 100°C for 24 h. rGO was pre-treated by centrifuging at 10,000 rpm for 30 min. The pellet was washed by adding deionized (DI) water and centrifuging at 10,000 rpm for 30 min. The resulting rGO pellet was dried in an oven. The rGO powder product was re-dissolved in N-methyl-2-pyrrolidone (NMP; Daejung Reagent Chemicals, South Korea), and the concentration was adjusted to 1 mg/mL. The prepared rGO suspension was centrifuged at 10,000 rpm for 30 min, and the upper portion was collected. The rGO-Ag solution was synthesized by adding 25 µL of silver nitrate (AgNO₃) to 5 mL of the rGO-CuO suspension and stirring for 20 min. The rGO-CuO solution was synthesized by sonicating 50 mL of 1 mg/mL GO, and 50 mL of 6 mg/mL copper acetate monohydrate in methanol was added and stirred using a magnetic stirrer for 2 h. The solution was added into a Teflon-lined reactor and heated in an oven at 100°C for 24 h to synthesize the rGO-CuO composite. The mixture was then pretreated by centrifuging for 30 min at 10,000 rpm. The resulting rGO-CuO pellet was collected and washed using DI water and dried in an oven. The product (rGO-CuO powder) was dissolved in NMP, and the concentration was adjusted to 1 mg/mL. To synthesize rGO-CuO-Ag film (Figure 1), 25 µL of AgNO₃ was added to 5 mL of the as-prepared rGO-CuO suspension and stirred for 20 min. The films were fabricated by filtering the solutions through a polytetrafluoroethylene filter with a pore size of 0.1 µm and diameter of 47 mm (PTFE; JHWP 04700, Merck Millipore Ltd., Ireland) under ultraviolet (UV) light to synthesize silver nanoparticles (AgNPs) (Yang et al., 2018). After fabrication, the films were cleaned using DI water and dried in a desiccator before testing.

Figure 1. Schematic diagram of the fabrication of the rGO-CuO-Ag composite film

2.2.Characterization

The atomic compositions and surface morphologies of the fabricated composite films were analyzed using field emission scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy (FESEM/EDX; JSM-700F, JEOL Ltd., Japan) and X-ray photoelectron spectroscopy (XPS; VG Microtech MultiLab ESCA 2000, Thermo VG Scientific, United Kingdom). The functional groups of the composite films were examined employing Fourier transform infrared spectroscopy (FT-IR; Frontier FT-IR/NIR, Perkin Elmer, USA).

2.3.Bacterial cell preparation

The model bacterium (*P. aeruginosa* PAO1) was prepared by the following procedure outlined in a previous report (Alayande et al., 2018). Briefly, the bacteria were cultured overnight in Luria-Bertani (LB) broth in a shaking incubator (150 rpm) at 37°C. After overnight culture, the bacteria were washed in phosphate-buffered saline (PBS) solution by centrifuging at 8,000 rpm for 10 min. The cells were washed twice by centrifuging at 8,000 rpm for 5 min. The optical

density of the bacterial suspension was set at 0.2 at a wavelength of 600 nm. All experiments were conducted using this initial bacterial concentration.

2.4. Antibacterial tests

The impact of the concentration of Ag on the antimicrobial efficacy of rGO–Ag and rGO–CuO–Ag films was tested using different concentrations of AgNO₃ (0.1, 0.5, 1, 5, 10, and 20 mM) during the film synthesis process. The antimicrobial properties of films were evaluated using the zone of inhibition (ZOI) method. LB agar plates were prepared and inoculated with 100 µL of bacterial cell suspension (in triplicate). The films were cut into 1 cm × 1 cm sections and gently placed on inoculated LB agar plates. The plates were incubated at 37°C for 24 h and the ZOI was measured. The optimum concentration was used for comparative assessment.

To enumerate the total live cells on the films, the plate count method was used. Films measuring 1 cm × 1 cm were fixed to a 24-well plate. LB broth containing 1% bacterial cells was inserted into the well and cultured for 24 h. After culture, the medium was gently removed and discarded. The unattached bacterial cells on the films were removed by gently rinsing the films with PBS. The film was transferred into a microtube containing 1 mL of PBS. The bacterial cells attached to the film were detached by vortexing the microtubes containing the films for 2 min and sonicating for 5 min, after which the bacterial suspension was collected. The obtained bacterial solution was diluted accordingly and cultured on LB agar. The plates were allowed to grow in an incubator, and the total colony-forming units were enumerated after culturing at 37°C for 24 h.

The morphology of the bacterial cell and the structure of the biofilm attached to the fabricated films were examined using FESEM (JSM-700F, JEOL Ltd., Japan) and confocal laser scanning microscopy (CLSM); Leica CTR 6500, Germany) (Yanar et al., 2020). The films were fixed to

the wells of a 24-well plate, exposing the active side to the bacterial cells. Then, 2 mL of LB broth comprising 1% bacterial cells was put into each well and incubated at 37°C for 24 h. After growth, the medium was discarded, and the film was gently washed with PBS. For FESEM analysis, the attached bacteria on the films were fastened with 5% glutaraldehyde solution and placed in a refrigerator. The films were subsequently dehydrated using 98.8% ethanol for 10 min. The films were dried in a desiccator before FESEM analysis. The biofilm layer on the film was analyzed by staining the biofilm using the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Carlsbad, CA, USA) containing propidium iodide (PI) dye, which stains dead bacteria red, and SYTO 9 dye, which stains live bacteria green. Fluorescence images were viewed using CLSM.

2.5. Release of Ag ions

The release of Ag ions from the film was monitored using inductively coupled plasma mass spectrometry (ICP-MS: 7500ce, Agilent, Santa Clara, CA, USA). In this process, 1 cm² films were fixed in the wells of a 24-well plate, and 1 mL of DI water was added to each well. The plate was continuously shaken at 100 rpm. The amount of Ag ions released was monitored every 12 h by withdrawing the entire solution for analysis and replacing the solution with new DI water.

3. Results and discussion

3.1. Film characterization

Figure S1 shows the color change of the solution during composite synthesis before and after AgNO₃ was added to the rGO–CuO composite solution prior to film fabrication. An apparent

bleaching of the dark color of the rGO–CuO solution was observed after adding AgNO₃ and stirring for 20 min.

FT-IR spectra of films are presented in Figure 2(a). The pristine rGO film exhibited a broad absorption at 3100 cm⁻¹ owing to the O-H stretching vibration due to partial removal of oxygen functional groups. Usually, in a fully reduced GO, the absorption at this wave number vanishes. Thus, the appearance of this peak indicates a partial reduction of GO (Andrikopoulos et al., 2014). The C=O stretching vibration of the carboxylic group was observed at 1715 cm⁻¹. The peak seen at 1614 cm⁻¹ is characteristic of C=C bonds. Phenolic C-O-H stretching, epoxy C-O-C deformation vibration, and C-O stretching were observed at 1363 cm⁻¹, 1210 cm⁻¹, and 1044 cm⁻¹, respectively (Zou et al., 2011). The incorporation of CuO into rGO caused the peaks at 1715 cm⁻¹, 1363 cm⁻¹, and 1044 cm⁻¹ to disappear. The addition of Ag to rGO resulted in no significant changes in rGO functional groups in the areas analyzed. This could mean that no covalent bonds were formed between Ag ions and rGO nanosheets. The rGO–CuO–Ag film exhibited characteristic changes in functional groups in comparison to the rGO–CuO film. Two new peaks were observed at 2365 cm⁻¹ and 2163 cm⁻¹, and the broad absorbance at around 3100 cm⁻¹ disappeared. This could indicate that Ag in the rGO–CuO–Ag film was attracted to CuO (Saleh et al., 2018).

The surface structure and composition of the elements of the films were analyzed using FESEM/EDX (Figure 2(c)). The FESEM image of rGO revealed a surface morphology with a characteristic wrinkled structure. The rGO–CuO film displayed a looser structure because the adsorption of CuO to rGO nanosheets increases the interlayer spacing and prevents the perfect restacking of rGO nanosheets (Nathan and Bobby, 2017). The EDX of rGO–CuO film exhibited a considerable amount of Cu. The morphology of rGO–Ag film revealed the presence of AgNPs,

which were formed as a result of solution filtration under UV light (Yang et al., 2018). These nanoparticles were adsorbed onto the surface of the rGO nanosheet. A higher magnification of the FESEM image is presented in Figure S2. An EDX spectrum ascertained the presence of Ag. Unlike the rGO-Ag film, where AgNPs could be visualized, the rGO-CuO-Ag film exhibited the incorporation of AgNPs into the film matrix. This was because the attachment of CuO to rGO nanosheets increased interlayer spacing and hindered restacking of rGO nanosheets, thereby providing a large surface area (Nathan and Bobby, 2017). The large surface area increased the number of adsorption sites for AgNPs, as shown in Figure 2(c). EDX analysis of rGO-CuO-Ag film confirmed the presence of both Cu and Ag. The elemental composition of Ag in rGO-CuO-Ag film was significantly higher than that in rGO-Ag film although the same concentration of AgNO_3 was added to both rGO and rGO-CuO solutions owing to the larger surface area for adsorption of AgNPs.

Further, XPS measurements were performed to additionally ascertain the elemental composition of rGO, rGO-CuO, rGO-Ag, and rGO-CuO-Ag films. Figures 2(d) and (e) show the survey spectra and elemental composition of films measured using XPS. Characteristic peaks were observed at 284.42 eV for C (1s), 531.15 eV for O (1s), 934.35 eV for Cu (2p), and 368.05 eV for Ag (3d₅). The elemental composition analyzed using XPS was consistent with the EDX measurements because more Ag was detected in the rGO-CuO-Ag film than in the rGO-Ag film.

(a)

(b)

253

Figure 2. (a) FT-IR spectra, (b) FESEM pictures (i) rGO film, (ii) rGO-CuO film, (iii) rGO-Ag film, and (iv) rGO-CuO-Ag film, (c) XPS survey spectra, and (d) elemental composition obtained from XPS of the films.

3.2. Antimicrobial performance analysis

3.2.1. Enhanced antimicrobial properties of the rGO-CuO-Ag film

The effect of AgNO_3 concentration on the antibacterial activity of rGO–Ag film was investigated using the rGO film without AgNO_3 as a control. The ZOI method was employed to assess the antimicrobial properties of the films. Figure S3 shows a photographic image of the ZOI after incubation with *P. aeruginosa* PAO1 for 24 h. No visible ZOI was observed in the rGO film, meaning that the rGO film did not inhibit bacterial growth. As the AgNO_3 concentration increased from 0 to 5 mM, the antimicrobial effect was proportionally enhanced, but peaked at 5 mM because the additional concentration effect was insignificant above 5 mM AgNO_3 .

The possible augmented antimicrobial effect of the rGO–CuO–Ag composite was tested using a rGO–CuO solution containing different AgNO_3 concentrations (0 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM, 10 mM, and 20 mM). Figure S4 shows ZOI images around the films. Similar to rGO–Ag films with different concentrations, the higher the concentration, the larger the diameter of the zone. For rGO–CuO–Ag films, film with 5 mM AgNO_3 exhibited the largest inhibition zone, whereas above this concentration, a slight reduction in the diameter of the zone was observed. This might have been due to the agglomeration of nanocomposites when >5 mM AgNO_3 was added. This agglomeration could have led to a decrease in the active surface area of AgNPs, meaning that less Ag was in contact with the bacteria. This possibility is consistent with the work of Choi et al. (Choi et al., 2008) who analyzed the impact of the size of AgNPs on their antimicrobial properties and reported that AgNPs larger than 20 nm exhibited less antimicrobial properties, whereas AgNPs of approximately 5 nm exhibited more favorable antimicrobial properties (Choi et al., 2008). Figure 3 shows a comparative assessment of rGO and rGO–CuO films with different AgNO_3 concentrations. Films containing a combination of CuO and Ag displayed enhanced antimicrobial properties.

Figure 3. The antimicrobial zone of inhibition (ZOI) as a function of AgNO_3 concentration in both rGO and rGO–CuO films (ZOI in mm diameter).

The antimicrobial properties of these films were further evaluated using the standard plate count method, confocal laser scanning microscope (CLSM), and through morphological observation of the attached bacteria on the films. Both rGO and rGO–CuO containing 5 mM AgNO_3 were compared using rGO and rGO–CuO films as controls. The films (1 cm^2) were cultured with *P. aeruginosa* PAO1 for 24 h at 37°C . The unattached bacteria were removed by gently rinsing the film with PBS.

The total colony-forming units (CFUs) were enumerated by detaching the attached bacterial cells from films and diluting them as necessary. The bacterial cell suspension was then spread on LB

agar plates. The plates were incubated for 24 h and the total colonies were enumerated. A higher number of bacterial cells were observed on both rGO and rGO-CuO films compared to rGO-Ag and rGO-CuO-Ag (Figure 4). Although fewer bacteria were observed on rGO-Ag film than on the rGO and rGO-CuO films (8-log reduction), even fewer bacterial cells were observed on the rGO-CuO-Ag films (10-log reduction).

Figure 4. Total viable *P. aeruginosa* PAO1 cells attached to the composite films

CFU counts represent only the number of live cells; therefore, a fluorescence test was used to enumerate the number of intact and damaged bacterial cells. A bacterial cell was considered live if the membrane was intact, whereas if the bacterial cell membrane was damaged, it was considered dead. In this assay, the SYTO 9 dye stained both intact and damaged bacterial cells green, whereas the PI dye stained only the dead cells red. When both dyes were used together, the PI dye reduced the green fluorescence of SYTO 9 if there were dead cells. Figure 5 shows CLSM fluorescence images of bacteria attached to pristine rGO, rGO-CuO, rGO-Ag, and rGO-

CuO–Ag films after incubation with LB broth for 24 h at 37°C in a shaking incubator at 90 rpm. The green fluorescence that predominated on the rGO film revealed that the attached bacterial cells were mostly intact. Similar characteristics were seen on the rGO–CuO film, except that more red fluorescence was obtained from bacteria initially in contact with the film. Overall, a higher green fluorescence signal was observed, revealing that most bacterial cells were intact. The rGO–Ag film exhibited a significant decrease in the number of both attached and live bacterial cells. The proliferation of bacterial cells was notably hindered by AgNPs.

Figure 5. (i) Two-dimensional live/dead fluorescent pictures of *P. aeruginosa* PAO1 on (a) rGO film, (b) rGO–CuO film, (c) rGO–Ag film, (d) rGO–CuO–Ag film, and (ii) three-dimensional live/dead fluorescent pictures of *P. aeruginosa* PAO1 on (a) rGO film, (b) rGO–CuO film, (c) rGO–Ag film, (d) rGO–CuO–Ag film (at least six areas were evaluated for each sample).

Improved antimicrobial properties of rGO-CuO-Ag can be attributed to the larger surface area of Ag caused by adsorption of CuO to rGO. This provided more surface area for the attachment of AgNPs. As a result of increased adsorption sites and attachment of smaller AgNPs, rGO-CuO-Ag film displayed better antimicrobial properties than rGO-Ag film without CuO.

3.2.2. Morphological assessment of the attached bacteria

The impact of composite films on bacterial cell morphology was investigated by FESEM analysis. Figure 6 shows representative FESEM images of *P. aeruginosa* PAO1 on composite films. The results were consistent with the fluorescence test because the bacteria attached to the rGO and rGO-CuO films remained relatively intact without any severe bacterial membrane damage. Although the number of bacteria on both the films was similar, the FESEM image of rGO film showed a higher propensity for biofilm formation. The rGO-Ag film displayed severe bacterial membrane damage indicative of the loss of cellular integrity of *P. aeruginosa* PAO1 cells (severe cell rupture), whereas no bacteria were seen on the rGO-CuO-Ag film. This was indicative of growth inhibition and complete lysis of bacterial cells.

Figure 6. FESEM pictures of *P. aeruginosa* PAO1 on (a) rGO film, (b) rGO-CuO film, (c) rGO-Ag film, and (d) rGO-CuO-Ag film.

3.3. Ag ion release

The main antimicrobial mechanism of composite films was linked to the release of Ag ions; therefore, the release rate of Ag ions from composite films was measured using ICP-MS. The test was conducted in batch by replacing the solution after 12 h. Figure 7 shows the release rate after every 12-h period. It can be seen from Figure 7 that the rGO-Ag film release rate after 12 h was 908.5 ppb/cm², which decreased to 319 ppb/cm² in the next 12 h. In contrast, for rGO-CuO-Ag film, 352.8 ppm/cm² was released in the first 12 h and 231.2 ppm/cm² in the following 12 h. The rGO-CuO-Ag film was able to exhibit a controlled, consistent, lower release of Ag ions while maintaining a sufficient reserve for long-term antimicrobial efficacy. Figure 7 (a) presents trends over time in the release of Ag ions.

The effects of Ag ion release on the antimicrobial properties of the film were evaluated 72 h after ion release [Figure 7 (b)]. The films were removed from the solution and dried in a desiccator. The ZOI test was performed against *P. aeruginosa* PAO1 as described in the Methods section. The rGO-Ag and rGO-CuO-Ag films displayed narrower ZOI. The percentage loss of antimicrobial properties, as quantified by measuring ZOI after soaking in DI water, was 23.3% and 14.8% for rGO-Ag and rGO-CuO-Ag films, respectively [Figure 7 (b)]. Compared to the rGO-Ag film, the loss of antimicrobial properties was lower in the rGO-CuO-Ag film owing to

the controlled and slower Ag ion

release over time [Figure

Ag

7(a)] compared to the faster

ion release observed for the rGO-Ag film.

(a)

(b)

Figure 7 (a) Ag ion release from the rGO–Ag and rGO–CuO–Ag films, and (b) loss of antimicrobial property after Ag ion release at 72 h

Ion release has been reported as a major mode of antimicrobial action in Ag and AgNPs (Mei et al., 2014; Nagy et al., 2011). The antimicrobial mechanism of Ag and AgNPs is not fully known, but several mechanisms have been postulated in the literature (Li et al., 2013; Prabhu and Poulouse, 2012; Reidy et al., 2013; Shi et al., 2014; Sondi and Salopek-Sondi, 2004). First, AgNPs generate reactive oxygen species that can damage the bacterial cell wall. Furthermore, Ag ions can disrupt the cell wall of bacteria by binding with electron donors such as sulfur and oxygen present in bacterial cell wall proteins owing to their special affinity for sulfhydryl groups. Ag ions can also form complexes with RNA and DNA, inhibiting bacterial replication. Additionally, Ag ions can block the respiratory chain of bacteria without triggering resistance to Ag ions (with some exceptions). These properties make Ag an effective agent against a broad range of microorganisms.

Conclusions

In this study, a novel rGO–CuO–Ag composite film was synthesized by simple preparatory steps. Antimicrobial and antibacterial adhesion of composite films was tested against *P. aeruginosa* PAO1. For comparison, pristine rGO, rGO–CuO and rGO–Ag films were also prepared. The ZOI

results revealed that the rGO and rGO-CuO films had no ZOI against *P. aeruginosa* PAO, whereas the rGO-Ag and rGO-CuO-Ag films both showed significant ZOIs against the model bacterium. However, the rGO-CuO-Ag film showed enhanced antimicrobial properties in terms of both ZOI and CFU count methods. These findings were supported by FESEM and CLSM analyses. In addition, an analysis of Ag ion release revealed rapid ion release by rGO-Ag film in the first 12 h of soaking in DI water. In contrast, a controlled and consistent, but slower, rate of Ag ion release was obtained with rGO-CuO-Ag film, indicating its potential for long-term antimicrobial action.

Further research should focus on the long-term antimicrobial and anti-biofouling potential of this film as a semipermeable membrane in a laboratory-scale filtration system. Furthermore, spacer coating is used in membrane-based systems to control biofouling (Araújo et al., 2012; Yanar et al., 2018); therefore, the effect of coating the spacers used in membrane-based water treatment with these nanocomposites on biofouling control should be investigated. In the meantime, this modified film (rGO-CuO-Ag) could be used to coat lead membranes in full-scale installations for biofouling control. Additionally, the rGO-CuO-Ag coating could be used in cooling towers to control biofouling. Moreover, these combined antimicrobial agents could also be used in biomedical fields to coat medical devices, such as catheters, for biofilm inhibition and control.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgment

This work was supported by the Korea Environmental Industry & Technology Institute (KEITI) through the Industrial Facilities & Infrastructure Research Program, funded by the Korea Ministry of Environment (MOE) (1485016165) and GIST Research Institute (GRI) grant funded by GIST in 2020.

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Highlights

- A combined surface-based antibacterial film (rGO–CuO–Ag) was successfully developed.
- The rGO–CuO–Ag film exhibited excellent antibacterial and antibiofilm activities against *Pseudomonas aeruginosa* PAO1.
- The film displayed both contact- and release-based antibacterial properties.
- The film has potential for use in both membrane-based water treatment and biomedicine.