Cu/graphene oxide composited coatings for preventing clinical implant bacterial infections: an antibacterial mechanism study

C. H. Zhao[a], Y. Q. Yang[a], H. L. Yang[a], J. M. Tan[a], R. H. Gong[a], Y. X. Yang[a], X. P. Zhang[b*]

[a]*School of Medicine, Hubei Polytechnic University, Huangshi 435003, China*

[b]*Division of Academic Research, Tongren Polytechnic College, Tongren 554300, China*

Recently, graphene oxide (GO) based materials have shown great potential in the treatment of implant bacterial infections due to its inherent antibacterial activity. However, the effect of GO-based materials on biological systems particularly the antibacterial mechanisms is still not clear. In this study, GO, $NaBH_4$ treated GO (GO-Y), copper decorated GO (GO-Cu, GO-Cu-GO) composited coatings were prepared on the surface of silicon dioxide (SiO_2) substrate by spin-coating and chemical in-situ formation. The growth of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) on GO-Y, GO-Cu, and GO-Cu-GO were significantly inhibited, especially on GO-Cu and GO-Cu-GO coatings. The implied antibacterial mechanism of GO-Cu-Cu coatings was further studied and discussed. The enhanced antibacterial performance of GO-Cu-GO coatings has significant potential application in preventing clinical implant bacterial infections. Moreover, the systematic study of various antibacterial effects also enriches our knowledge of the possible antibacterial mechanisms of graphene-based materials.

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1. Introduction

In clinic, bacterial infection is a major impediment to the utility of medical implants, leading to patients needing further medical care[1]. To reduce bacterial adhesion and proliferation, one method is coating implant surface with antibacterial materials. Due to its excellent adsorption, amphiphilic and surface functionalization, GO has shown remarkable promises in biomedical applications such as bioimaging, biosensors, drug delivery and antibacterial coatings[2]. Further, the strong antimicrobial properties of GO make it a good candidate for antimicrobial surface coating material[3, 4]. However, the antibacterial mechanism of GO based coating materials is still not clear.

* Corresponding author: 627316399@qq.com

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Generally, the antibacterial mechanism of GO includes sharp edge mediated cleavage, reactive oxygen species (ROS) or induced oxidative stress[5, 6]. Nanoparticles such as Au, Cu, and Ag have been employed to interact with bacteria as antibacterial agents for a long time[7]. Low doses of Cu^{2+} was reported to show a positive effect of cell behavior on biomedical scaffolds[8]. Moreover, Cu^{2+} was found to have the potential to promote bone marrow stem cell (BMSC) proliferation and differentiation into osteoblasts, which benefiting bone repair and bone tissue engineering[9]. Therefore, we developed a GO-Cu coating and verified the feasibility of using GO-Cu as an antibacterial coating. Due to the integrity of the organism, the antibacterial mechanism of the antibacterial agent is very complex, which is often observed as a synergistic effect of a variety of antibacterial mechanisms[10].

The purpose of this study was to evaluate the antibacterial properties of two different copper decorated GO coatings— GO-Cu and GO-Cu-GO, as implant coatings, and study on the antibacterial mechanisms of GO based coating materials.

2. Experimental section

2.1. Preparation of GO-based coating on SiO2 substrates

SiO_2 substrates (diameter 14 mm, thickness 1 mm) were washed by ultrasonication in acetone, methanol, and anhydrous ethanol for 10 min respectively, followed by rinsing with DI water for 10 min. After drying at 60 °C for 1 h in an air oven, the substrates were treatment to carry out salinization modification by 2% (3-amminopropyl) triethoxysilane (APTES) ethanol solution. Then the samples were left to dry again at 60 °C for 1 h in nitrogen atmosphere.

GO, GO-Y, GO-Cu, and GO-Cu-GO coatings are prepared according to the previous report[11]. Briefly, GO aqueous suspension (1mg mL^{-1}) was dropped on the SiO_2 substrate surface, then GO coating was obtained via a spin coating process.

A combination of spin coating and chemical in-situ formation method was used to prepare GO-Cu and GO-Cu-GO coatings. The obtained GO coating was immersed in $CuSO_4$ solution to absorb Cu^{2+}, after removing the excess $CuSO_4$ solution on the surface by spinning at 8000 rpm min^{-1}, and drying at 37°C, the coating samples were immersed in $NaBH_4$ solution for 1h, then washed in DI water, and dried at 37°C to obtain GO-Cu coating. A layer of GO was self-assembled on the surface of GO-Cu coating again by a spin coating method to obtain GO-Cu-GO coatings. The GO-Y coating was obtained by immersing the GO coating directly in NaBH4 solution for 1h.

2.2. Characterization

The surface elemental composition of the coating samples was characterized by X-ray photoelectron spectroscopy (XPS). Fourier transform infrared spectroscopy (FTIR) was also employed to characterize and analyze GO based coatings.

2.3. Antibacterial activity evaluation

The antibacterial performance of GO, GO-Y, GO-Cu, and GO-Cu-GO coatings were test against *E. coli* and *S. aureus* respectively, with the SiO_2 substrate as the control. The schematic diagram of sample placement and inoculation solution is shown in Figure 1a. The samples were sterilized by UV irradiation for 2 h, then inoculated with the bacterial suspension (concentration:

10^6 CFU mL^{-1}, density: 60 μL cm^{-2}) at 37 °C for 6 h. Then the dissociated bacterial solution was collected, diluted, and spread onto a LB medium and let to grow for 24 h at 37 °C. Colony counting method was applied to evaluate the viability of E. coli on GO-based coatings. All the tests were carried out three times. Bacterial cell viability can be calculated by formula (1):

$$\text{Bacterial cell viability}\,(\%) = \frac{N_{\text{Experiment}}}{N_{\text{Control}}} \times 100 \quad (1)$$

$N_{Experiment}$ =the number of bacteria in the experimental group (CFU mL^{-1})
$N_{Control}$= the number of bacteria in the control group (CFU mL^{-1})

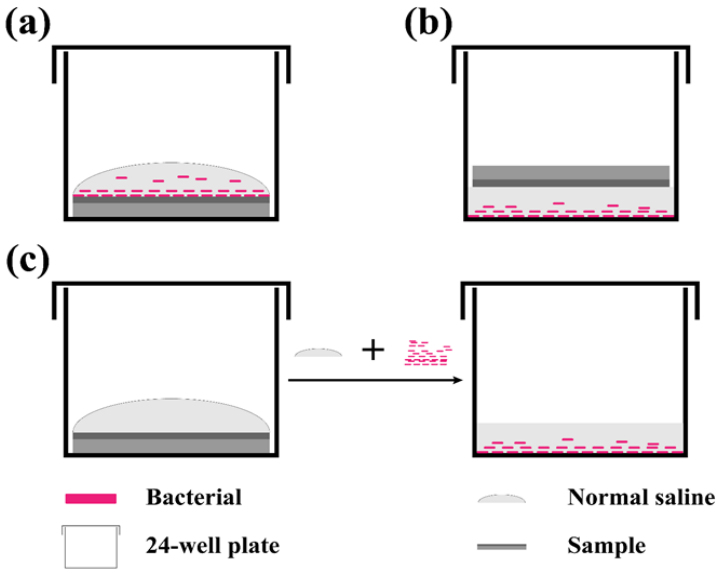

Fig. 1. Schematic diagram of sample placement and inoculation. (a) Positive culture was used to detect the antibacterial properties of the coating. (b) Inverted culture was used to detect the non-contact antibacterial of the coating. (c) The extract culture was used to detect the antibacterial properties of Cu^{2+} released by the coating.

2.4. Analysis of the antibacterial mechanism

E. coli and *S. aureus* were used to evaluate the non-contact antibacterial activity of GO, GO-Y, GO-Cu, and GO-Cu-GO coatings respectively, with SiO_2 as the control (Figure 1b). After the coating sample was exposed to UV for 2 h on a clean bench, a bacterial suspension droplet with a concentration of 10^6 CFU mL^{-1} was added under the coating, with a density of 60 μL cm^{-2}. Most bacteria are gradually deposited in the lower layer of the solution, without directly contacting the coating surface, which was inverted in the solution. After incubation at 37 °C for 6 h, the bacterial suspension under the coating was diluted with 0.9 wt% NaCl solution and coated on the LB medium. After incubation at 37 °C for 24 h, the colony count was carried out to evaluate the activity of GO-based coatings after no contact with *E. coli* and *S. aureus*, and images were taken. All tests were carried out in triplicate.

ROS produced on the sample was directly displayed by ROS fluorescence staining. 10^6 CFU mL^{-1} bacteria were inoculated on the surface of the sample at a density of 60 µL cm^{-2}. After 6 h of culture. It was stained in the dark for 30 min with the ROS Assay Kit, and then rinsed with 0.9 wt% NaCl solution and observed under a fluorescence microscope.

In addition, *E. coli* and *S. aureus* were used to evaluate the antibacterial activity of GO, GO-Y, GO-Cu, and GO-Cu-GO coating extracts, and SiO_2 substrate was used as the control (Figure 1c). The coating was immersed in 93 µL 0.9 wt% NaCl solution and placed in 37 °C incubator for 6 h. The released amount of Cu^{2+} ions in the solution was detected by atomic absorption spectrophotometry (AAS). After sterilization by 0.22 µm water filtration membrane, 10 µL *E. coli* or *S. aureus* (10^7 CFU mL^{-1}) were mixed and cultured in 37 °C constant temperature incubator. After 6 h, they were taken out, diluted and re-cultured on the agar culture plate. After incubation at 37 °C for 24 h, the colony count was carried out to evaluate the effect of the GO-based coatings extract on the activity of *E. coli* and *S. aureus*, and images were taken. All tests were carried out triplicate.

2.5. Statistical Analysis

The antibacterial studies were performed in quadruplicates for each group. The values were expressed as mean ± standard deviation. The statistical analysis was performed using the Student's T-test and $p < 0.05$ or 0.01 in the differences between groups were considered to be significant or extremely significant.

3. Results and discussion

3.1. Characterization of GO and GO-based coatings

FT-IR results (Figure 2) showed that the typical peaks of oxygen-containing groups of GO at 3422 cm^{-1}, 1728 cm^{-1}, 1398 cm^{-1}, and 1053 cm^{-1}[12], did not show any obvious changes after the GO was dried as coatings. After treatment with $NaBH_4$, the C=O groups showed an obvious decrease. Compared with GO-Y, the functional groups on GO-Cu did not show any obvious changes after cu introduced, which indicated that no chemical bonding between Cu-NPs and GO, but rather electrostatic adsorption linked each other. After treated with $NaBH_4$, the peak around 1728 $cm^{-1}$was lost in GO-Y, GO-Cu, and GO-Cu-GO coatings, indicating the reduction of GO after $NaBH_4$ treatment.

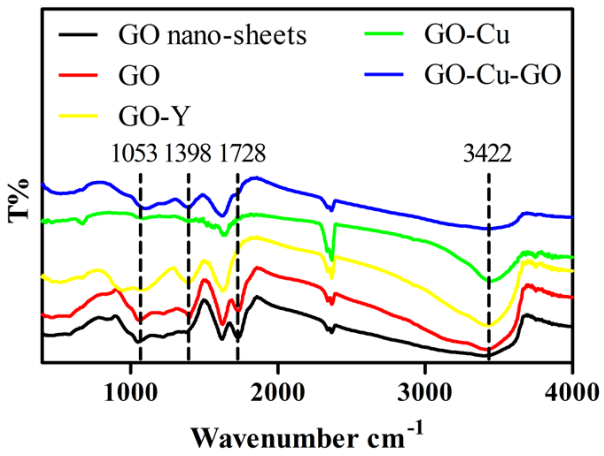

Fig. 2. FTIR analysis of GO nanoflakes, GO, GO-Y, GO-Cu, and GO-Cu-GO coatings.

The GO-Cu and GO-Cu-GO coatings were further analyzed by XPS. The peak area was calculated respectively to analyze the oxygen-containing groups (Figure 3). It was found that C=O was reduced, which was consistent with previous reports[13].

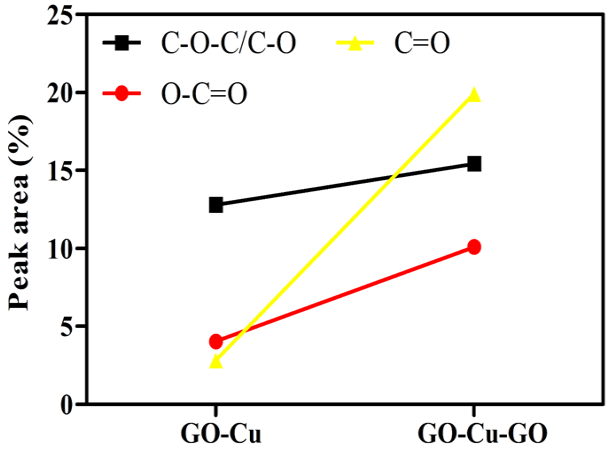

Fig. 3. Peak area comparison of oxygen groups on the surfaces of GO-Cu and GO-Cu-GO coatings. After treated with $NaBH_4$, the ratio of oxygen groups on the surface of GO coating decreased, among which the C=O group decreased the most, indicating that $NaBH_4$ mainly removed the carbonyl groups on the surface of GO.

3.2. Evaluation of antibacterial activity of GO-based coatings

Figure 4 shows the typical images and counting results of E. coli or S. aureus on the surface of the sample cultured on agar medium. As for the control and GO group, a large number of bacterial colonies was found on the agar medium, which means that the E. coli can growth well on the SiO_2 substrate and GO coating. The bacterial colonies from the GO-Y group decreased significantly, indicating that the growth of bacteria on the surface of GO-Y coating was inhibited. No bacterial colonies were found for GO-Cu and GO-Cu-GO groups, which indicates that E. coli and S. aureus could not survive on the surface of the GO-Cu and GO-Cu-GO coatings.

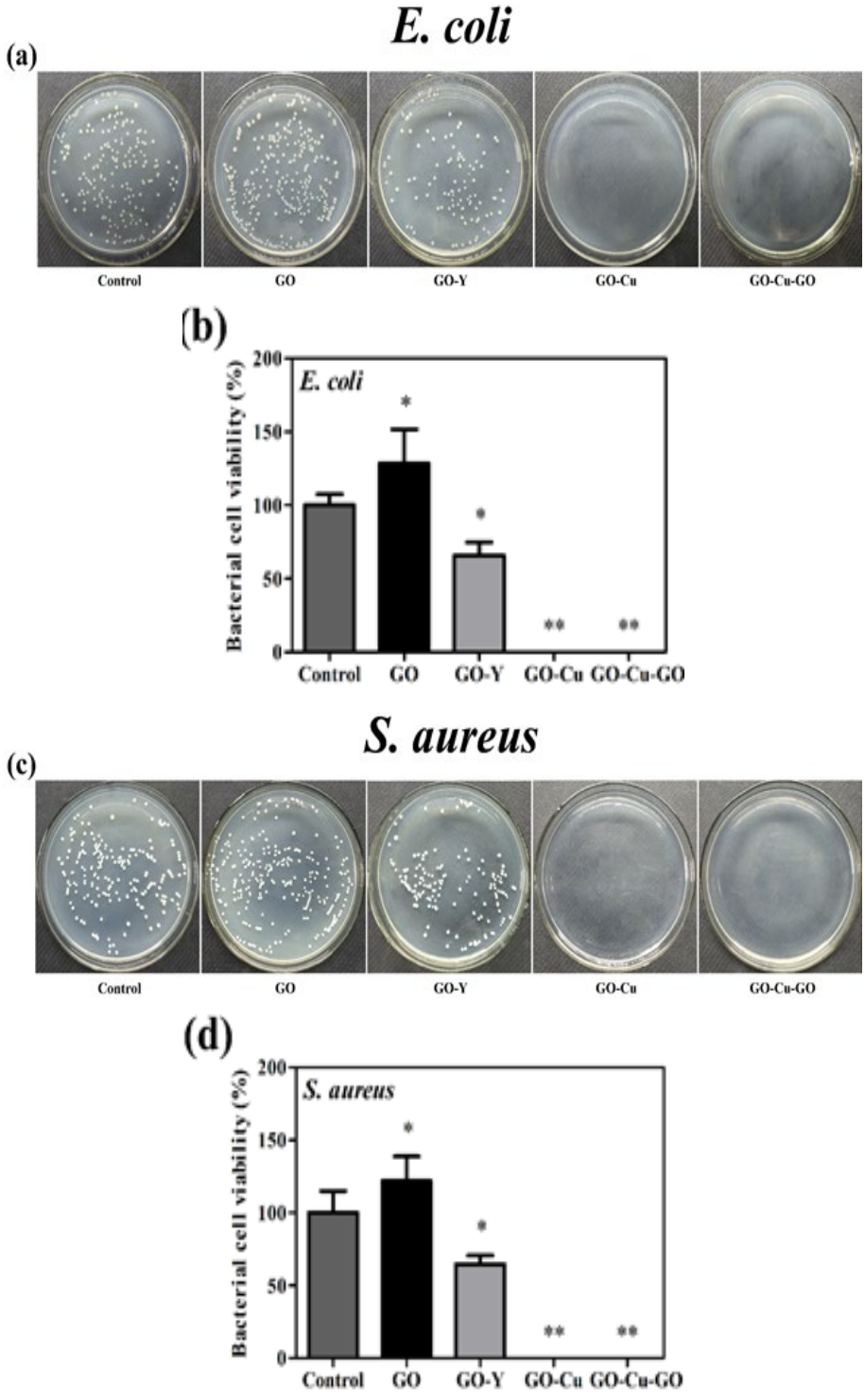

*Fig. 4. Typical photos (a, c) and counting results (b, d) of E. coli and S. aureus colonies cultured on agar culture plate after bacteria with concentration of 10^6 CFU mL^{-1} were incubated on the surfaces of SiO_2 (Control), GO, GO-Y, GO-Cu, and GO-Cu-GO for 6 h at a density of 60 μL cm^{-2}. * $P < 0.05$ relative to the control, ** $P < 0.01$ relative to the control.*

3.3. Study on the antibacterial mechanism of GO-based coatings

Although the potential mechanism of antibacterial activity is not clear, there are two reasons were reported for the toxicity induced by GO. On one hand[14-16], once contacting with

bacteria, sharp edges of GO will insert or cut the bacterial cell membrane, causing serious membrane damage and cytoplasm leakage, thus killing the bacteria; on the other hand[16, 17], GO induces the production of ROS, leading to the oxidation of proteins, lipids and nucleic acids in bacteria. According to these reports, GO-induced toxicity might arise from three factors: contact induced antibacterial activity, Cu^{2+} ion induced antibacterial activity, and ROS induced activity.

In graphene or GO aqueous dispersion, the bacterial membrane damage caused by cutting is mainly mediated by the positive contact with the sharp edge of GO nano-sheets. In contrast, GO-based coatings are mainly prepared by the spin coating method, and there is no sharp edge of GO nano-sheet exposed on the surface, while bacteria are mainly deposited on the surface of GO-based coatings by plate stacking, so there is no orthogonal contact with the edge of the GO nano-sheet. Therefore, GO-based coatings are less likely to cut the bacterial cell membrane through the sharp edge of the surface to resist bacteria. When we inverted GO-Cu, GO-Cu-GO, and SiO_2 in the bacterial suspension (as shown in Figure 1b), the coating is no longer contacting with bacteria with the bacteria deposited on the bottom of the 24-well plate, and the distance between the coating and the bacteria is sufficiently far. However, after re-culturing on the agar plate (Figure 5), it was found that GO-Cu and GO-Cu-GO did not produce obvious colonies which means these coatings still showed strong antibacterial activity. The results indicate that the contact-induced physical membrane damage is not the main antibacterial factor in the highly effective antibacterial activity of GO-Cu and GO-Cu-GO coatings.

ROS is a series of atomic groups or molecules, including $\cdot O_2^-$, H_2O_2, $\cdot OH$, etc., which are produced by the gradual single-electron reduction of molecular oxygen[18]. They have unpaired electrons and have active chemical properties. A small amount of ROS can promote cell growth, while excessive ROS can cause oxidative stress, and induce the oxidation of proteins, lipids, and nucleic acids in cells, and eventually lead to bacterial death[19]. The observation of fluorescence results for ROS (Figure 6) showed that the fluorescence intensities increased in the order of GO, GO-Y, and GO-Cu coatings, indicating higher ROS level in the same order. These results are consistent with the antibacterial performance described above (Figure 4), indicating that ROS induced activity plays an important role in the antibacterial activity of GO based coatings.

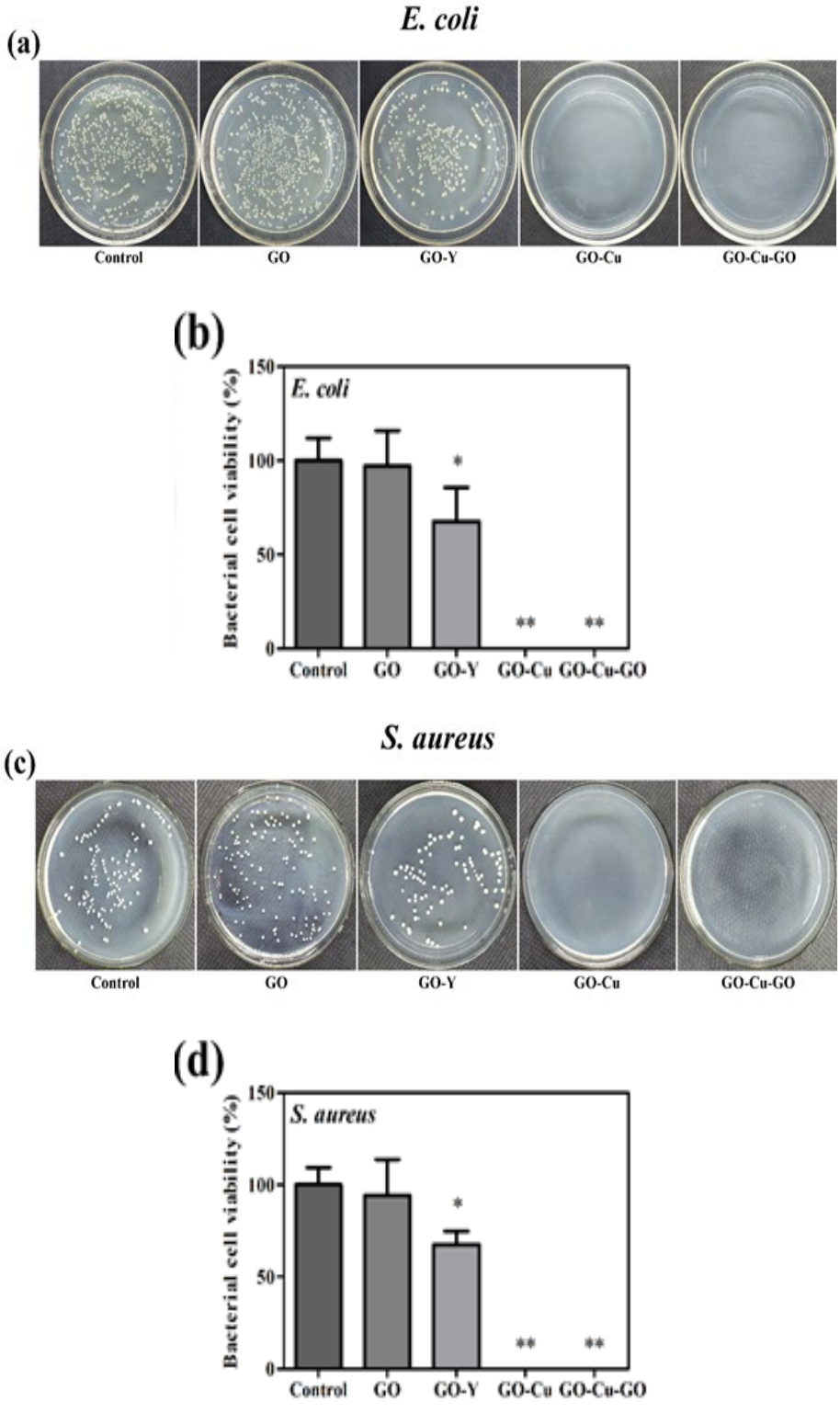

*Fig. 5. Typical photos (a, c) and counting results (b, d) of E. coli and S. aureus colonies cultured on agar culture plate after incubating bacteria with 10^6 CFU mL^{-1} in inverted SiO_2 (Control), GO, GO-Y, GO-Cu, and GO-Cu-GO for 6 h at a density of 60 μL cm^{-2}. * $P < 0.05$ relative to the control, ** $P < 0.01$ relative to the control.*

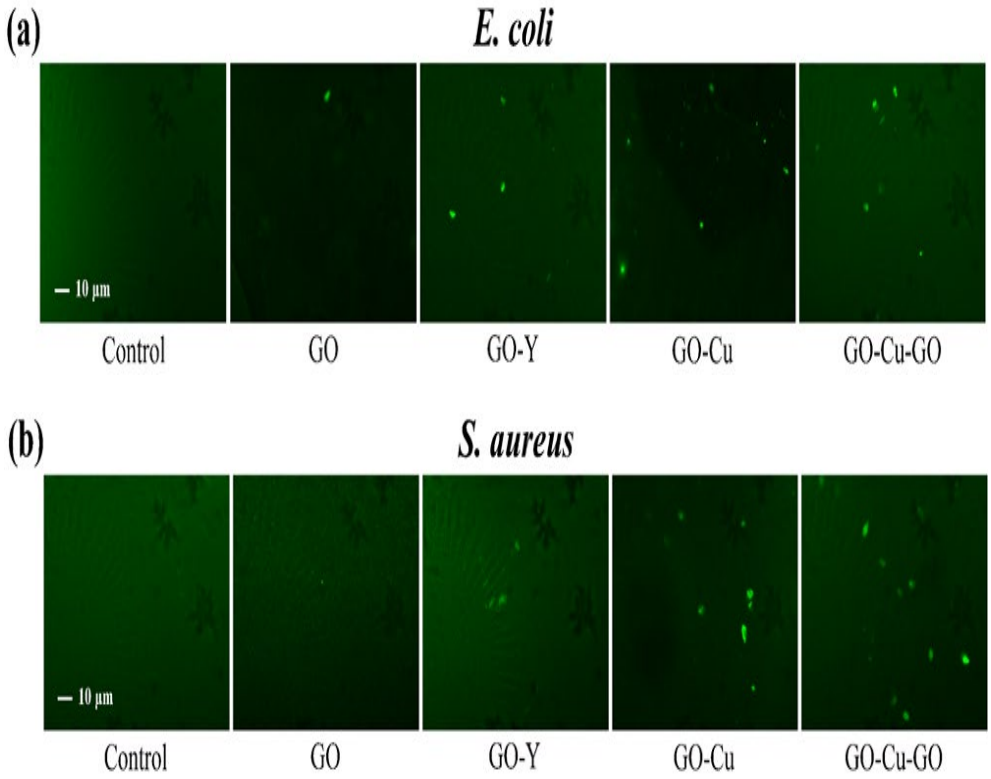

Fig. 6. E. coli and S. aureus of bacteria with concentration of 10^6 CFU mL^{-1} incubated on the surface of SiO_2 (Control), GO, GO-Y, GO-Cu, and GO-Cu-GO for 6 h produce fluorescence staining image of ROS.

To further explore the role of Cu^{2+} ions, the Cu^{2+} ion content in the coating extraction solution was detected by AAS. The results showed that (Table 1), the content of Cu^{2+} released by GO-Cu and GO-Cu-GO coatings were lower than the lowest antibacterial concentration (Sterilization rate is more than 90%) reported in the literature[10], which was 5×10^{-6} mol L^{-1}.

Table 1. Cu^{2+} release of coating detected by AAS.

Group	Concentration
SiO_2	0
GO	0
GO-Y	0
GO-Cu	2.57×10^{-7} mol L^{-1}
GO-Cu-GO	1.77×10^{-7} mol L^{-1}

Compared with the previous results (Figure 5), the antibacterial results of the coating extract showed that (Figure 7) the cell viability of *E. coli* and *S. aureus* on the surface of GO-Cu increased to 41.200 ± 5.313% and 55.528 ± 3.823% respectively. The cell viability of *E. coli* and *S. aureus* on the surface of GO-Cu-GO increased to 46.786 ± 3.706% and 60.357 ± 2.820%,

respectively. The results indicated that Cu^{2+} released from GO-Cu and GO-Cu-GO coatings contributed to but was not the main factor for the antibacterial performance of the coatings.

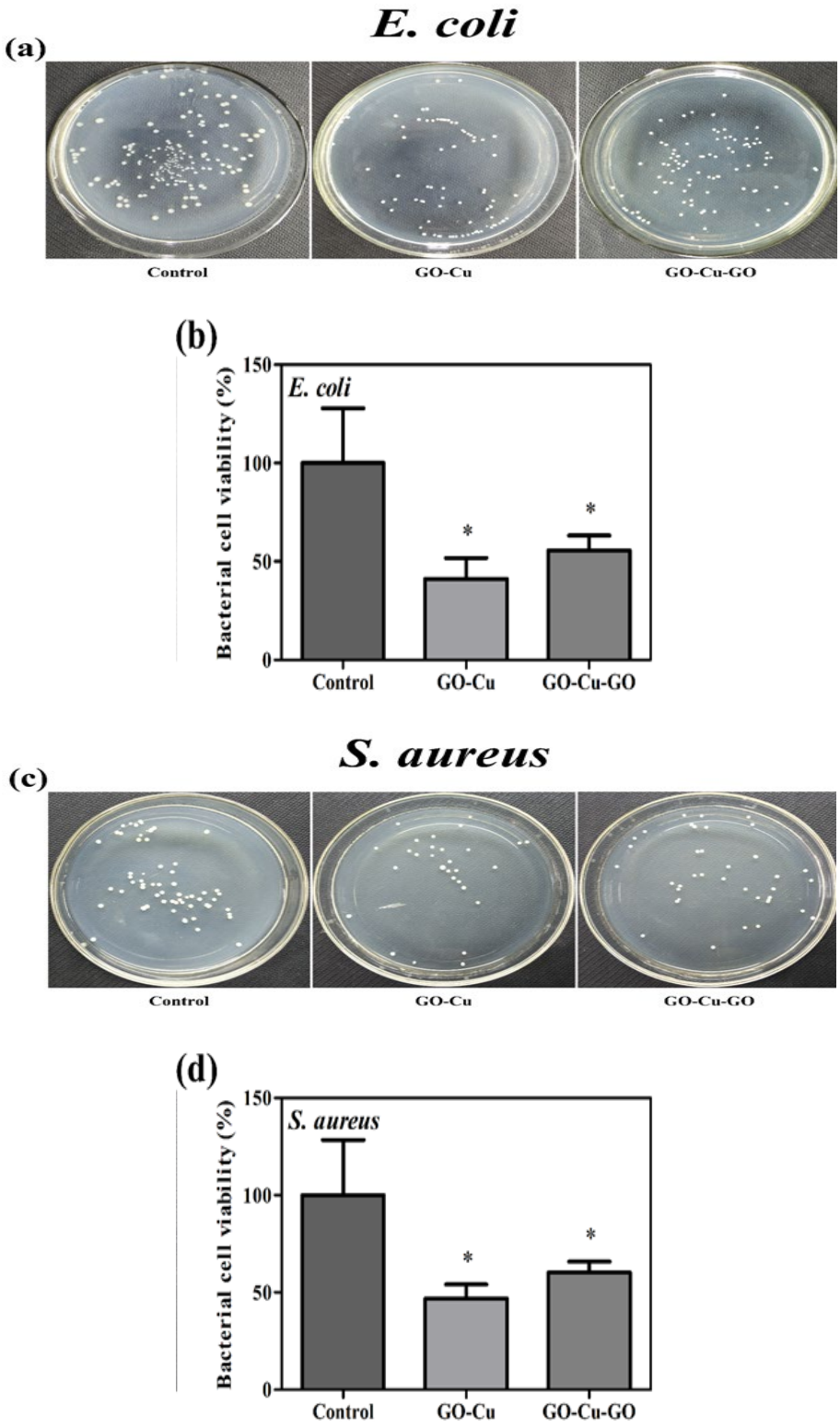

*Fig. 7. Typical photos (a, c) and counting results (b, d) of E. coli and S. aureus colonies cultured on agar culture plate after bacteria with concentration of 10^7 CFU mL^{-1} were incubated in SiO_2 (Control), GO-Cu, and GO-Cu-GO for 6 h. * $P < 0.05$ relative to the control, ** $P < 0.01$ relative to the control.*

4. Conclusions

Go-Cu and GO-Cu-GO coatings with enhanced antibacterial activity were synthesized on the surface of SiO_2 by a simple route. GO-Cu and GO-Cu-GO coatings displayed high toxicity to *E. coli* and *S. aureus*. The antibacterial mechanism of the prepared GO based coatings were studied. It was found that the antibacterial effect of GO based coatings were realized through two possible factors, Cu^{2+} ion induced antibacterial activity, and ROS induced activity. ROS induced activity plays a key role in the antibacterial performance, while Cu^{2+} ions contributed to but is not the main factor for the antibacterial performance of the coatings.

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