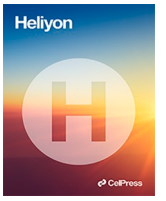
Heliyon 10 (2024) e27903

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Evaluation of antibacterial activity and influencing factors of normal and nanostructured copper-based materials

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ARTICLE INFO

Keywords:
Nanomaterials
Antibacterial performance
Influence factor

ABSTRACT

Background: Copper-based materials have garnered extensive recognition for their effective nature against microorganisms and their minimal toxicity. However, the evaluation for their antibacterial activity is still in its nascent stages, and the evaluation results based on existing criteria are not representative of real-world application.

Aim: To evaluate the antibacterial activity and primary determinants of influence of copper-based materials in order to investigate their practical antibacterial activity and potential mechanisms of such materials.

Methods: *Staphylococcus aureus* and *Escherichia coli* bacterial suspensions were applied via inoculation onto the surfaces of normal and nanostructured copper foil. Following incubation of the inoculated surfaces under diverse experimental conditions—including varying compositions of the bacterial suspension, the use of chemical neutralizers, the existence of organic interferents, and low temperature and humidity—surviving bacteria were enumerated. Using the scanning electron microscopy and X-ray photoelectron spectroscopy, the surface changes of copper-based materials were examined.

Findings: Following 1 h of exposure to 37 °C and 90% relative humidity, *Staphylococcus aureus* was reduced by 4.45 log10 on normal copper foil, while all of the bacteria were eradicated on nanostructured copper foil. In addition, it was discovered that preparing a bacterial suspension with PBS results in a significant number of *Escherichia coli* fatalities during the test, whereas using TPS promotes the bacteria's normal growth. Furthermore, the outcomes of the antibacterial activity test were diminished when chemical neutralization was employed, and the presence of organic interferents had distinct impacts on normal copper foil and nanostructured copper foil. Additionally, low temperatures and humidity diminished the antibacterial activity of copper foil, whereas normal copper foil produced significantly better results.

Conclusion: While copper-based materials exhibit robust antibacterial activity as determined by standard assays, their efficacy in real-world applications is subject to various influencing mechanisms. In order to objectively evaluate the antibacterial activity of copper-based materials and provide precise guidance for their development and practical application, it is essential to regulate test conditions with targeted.

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https://doi.org/10.1016/j.heliyon.2024.e27903
Received 15 December 2023; Received in revised form 7 March 2024; Accepted 7 March 2024
Available online 9 March 2024

1. Introduction

It is common knowledge that copper-based materials destroy microorganisms more effectively than materials like plastic, glass, steel, cotton and paper [1–4]. The advancement of nanotechnology in recent years has garnered considerable interest, particularly with regard to nanostructured copper-based materials. The antibacterial and antiviral activity of nanostructured copper and its compounds is significantly greater than that of bulk copper and its compounds. This is because surface-exposed copper ions on nanostructure surfaces exhibit much higher activity than those on bulk ones [5–12].

When evaluating the antibacterial activity of copper-based materials, ISO 22196:2011 is typically referenced [13].For 24 h, inoculated samples were subjected to 37 °C, 90% relative humidity in these experiments, which is not representative of practical use scenarios. Comparable outcomes are hindered by the influence of sample pretreatment methods, reference substances, and film materials on antibacterial performance, as evidenced by numerous studies [14]. In particular, to maintain target bacteria in a moist state and under alternative experimental conditions for a duration of 24 h, changes are made to the physical and chemical properties of copper-based materials [15–18]. The existing criteria may not be entirely suitable for evaluating antibacterial materials that possess active chemical activity, which could result in exaggerated antibacterial properties [19]. A comprehensive understanding of the actual activity and developmental direction of copper-based materials in the domain of disinfection is impeded by this circumstance. Hence, the establishment of a rigorous and appropriate scientific methodology to evaluate the antibacterial properties of copper-based materials is of critical importance.

As research subjects for this study, copper foil and nanostructured copper foil are selected. By conducting targeted adjustments to experimental conditions and employing X-ray photoelectron spectroscopy to examine surface changes in copper-based materials under diverse conditions, we investigate the factors that influence the detection process and potential antibacterial mechanisms. By establishing appropriate methods for evaluating the performance of disinfection, this analysis contributes to the sustainable disinfection of copper-based materials.

2. Materials and methods

2.1. Materials for preparation of copper foil surfaces

The copper foil of purity 99.9% and all other chemicals (analytical pure) were obtained from Sinopharm. The copper foil was exposed in room environment at 25 °C with 50% relative humidity (RH) for a week, allowing it to form an oxidized surface, to imitate daily used copper coating surface. The sufficiently exposed copper foil was used for antimicrobial experiment.

Nanostructure copper foil was obtained by hydrothermal treatment of copper foil. First, copper foil of purity 99.9% was washed by 1 M NaOH, 1 M HCl and deionized water. Then 1*1 cm^2 of it was transferred into autoclave with 32 mL of 5 M NaOH aqueous solution with addition of 0.15 M $(NH_4)_2S_2O_8$, 0.02 M cetyltrimethylammonium bromide (CTAB) as surfactant and 0.00385 M sodium dodecyl sulfate (SDS) as co-surfactant [20]. The Cu on the surface of copper foil is relatively stable and only dissolved at a reduced rate by NaOH. Conversely, $(NH_4)_2S_2O_8$ is capable of rapidly oxidizing the copper foil's surface [21]. Upon dissolution, Cu^{2+} promptly undergoes a reaction with OH^- to generate $Cu(OH)_2$, which is subsequently reduced to CuO_2 via hydrothermal conditions conducted at elevated temperatures. Eventually, these crystal nuclei will coalesce into nanowires as they proceed in the direction of CTAB and SDS micelles. The autoclave was sealed and heated at 170 °C for 24 h with the temperature raising speed of 1 °C/min. Then the sample was washed with deionized water and ethanol to remove any salt and preserved in ethanol for use.

Sterile stainless-steel discs with a diameter of 2 cm were used as the control group.

2.2. Evaluation of antibacterial activity

2.2.1. Growth of microbial strains

Staphylococcus aureus (*S. aureus)* ATCC 6538 and *Escherichia coli* (*E. coli*) 8099 were provided by the general microbiology center of the China Microbial Species Preservation and Management Commission. Both were cultured for 24 h at a temperature of 37 °C in tryptophan soybean broth (TSB). A phosphate buffer (PBS) containing 0.1% Tween-80 was utilized to prepare bacterial suspensions with an approximate concentration of 10^9 CFU/mL. A 1 mL volume of the suspension was subsequently diluted to the appropriate concentration. As a positive control, inoculated it onto a tryptone soy agar (TSA) plate in order to ascertain the initial titer.

2.2.2. Antibacterial activity test

A bacterial suspension containing *S. aureus* and *E. coli*, each with a volume of 10 μL, was inoculated and evenly both control stainless steel and test copper material surfaces using the tip of the pipette. Cut polyethylene film to appropriate dimensions and immerse it in 75% ethanol for disinfection purposes. Cover the surfaces of normal copper foil and nanostructured copper foil with thin films, respectively, once the thin film has dried. This will prevent the bacterial suspension on the sample surface from losing an excessive amount of water during the test. For 1 h, the samples were exposed to a constant temperature and humidity box at 37 °C and 90% RH. Following exposure, delicately and repeatedly rinse the sample and its surface-covering film with 5 ml of PBS using a pipette using a pipette. In order to prevent the sample surface from detaching onto the eluent, it is not advisable to employ mechanical elution techniques like vibrators and vortexes. Inoculate the eluent onto TSA plates after diluting it with an appropriate gradient. 24 h of

incubation at 37 °C were used to determine the bacterial activity. The $\log_{10}$ reduction in relation to the bacterial count on the control material was calculated at the end of the 1-h test period. There were three repetitions of the test.

2.3. Effects of different factors on the antibacterial activity test

2.3.1. The composition of bacterial suspension

The bacterial suspension was prepared by eluting the 24-h culture of *E. coli* with tryptone-normal saline (TPS), with *E. coli* serving as the indicator bacterium. All other conditions remained constant throughout the three repetitions of the test.

2.3.2. Use of chemical neutralizers

For copper-based materials, select the broad-spectrum neutralizer D/E neutralizer (D/E), which is composed of the following components: 0.1% sodium thioglycolate, 0.6% sodium thiosulfate, 0.25% sodium bisulfite, 0.7% lecithin, and 0.5% Tween-80. In lieu of PBS, wash the treated sample and film with 5 mL/E. Dilute the eluent with appropriate gradients using D/E before inoculating it onto TSA plates. Repeat three times, conduct the antibacterial activity test under the aforementioned conditions.

2.3.3. The existence of organic interferents

Bovine serum albumin (BSA) was employed to simulate organic matter interference by adding 3% BSA to the *S. aureus* suspension. All other conditions remained constant throughout the three repetitions of the test.

2.3.4. Low temperature and humidity

The following intentions were set: temperature reduction (20 °C, 90% RH) and humidity reduction (37 °C, 30% RH).Under the specified conditions, the antibacterial activity test was performed, employing S. aureus as the indicator bacterium. The test was replicated three times.

2.4. Scanning electron microscopy and X-ray photoelectron spectroscopy

Before subjecting copper foil and nanostructured copper foil to the antibacterial test, a scanning electron microscope (SEM) was utilized to characterize their surfaces. Copper foil and nanostructured copper foil samples were tested using an X-ray photoelectron spectrometer (SHIMADZU, AXIS SUPRA+) under standard conditions, organic interference conditions and low temperature conditions.

2.5. Statistical analysis

The data were presented in the form of the mean and standard deviation. The data underwent transformation through decimal logarithm conversion. Additionally, statistical analysis was performed utilizing the software application known as the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Antibacterial activity test results

Both normal copper foil and nanostructured copper foil exhibited remarkable efficacy in eliminating *S. aureus* and E. coli under the standard conditions of 37 °C and 90% RH for 1 h. The mean log10 reduction of *S. aureus* on the surface of normal copper foil was 4.45, whereas the bacteria were entirely eradicated by the nanostructured copper foil (Table 1). Notably, *E. coli* not only failed to proliferate on either surface, but also exhibited impaired growth on the control material, which consisted of stainless-steel discs.

3.2. Effects of different factors on antibacterial activity test results

3.2.1. Preparing a bacterial suspension with TPS facilitated the regular growth of E. coli

The application of TPS for bacterial suspension preparation resulted in the regular growth of *E. coli* on the control material, a stainless-steel disc, by 6.67 logarithmic values. This indicates that TPS has the capability to stimulate the regular growth of *E. coli*. As shown in Table 1, copper foil and nanostructured copper foil eliminate all *E. coli* on their surfaces after 1 h of exposure to 37 °C and 90% relative humidity using the aforementioned method. Consequently, the log10 reduction for *E. coli* on the surfaces of both materials exceeded 6.67.

3.2.2. Chemical neutralization diminished the antibacterial activity test results

The average $\log_{10}$ reductions of copper foil and nanostructured copper foil against *S. Aureus* decreased to 2.63 and 4.15, respectively, in an antibacterial activity test in which D/E was used as a neutralizer in place of PBS for elution and dilution (Table 1). This indicates that the addition of D/E partially eliminates the sustained antibacterial effect of such materials.

3.2.3. The impact of organic interference differed between normal copper foil and nanostructured copper foil

The average $\log_{10}$ reductions of copper foil against *S. aureus* increased to 6.47 in the antibacterial activity test utilizing 3% BSA to simulate interference of organic matter; conversely, the average $\log_{10}$ reductions of nanostructured copper foil against *S. aureus* decreased to 5.87 (Table 1). The inclusion of BSA resulted in contrasting outcomes for both materials.

3.2.4. Low temperatures and humidity diminished antibacterial activity, whereas normal copper foil produced a significant increase

The average $\log_{10}$ reductions of copper foil and nanostructured copper foil against *S. aureus* decreased to 2.57 and 2.78, respectively, when subjected to reduced environmental temperatures. An observed decline in environmental humidity resulted in a decrease of 3.59 in the average $\log_{10}$ reductions of nanostructured copper foil. However, in dry conditions, copper foil eradicated all *S. aureus* on its surface, as evidenced by average $\log_{10}$ reductions >5.52 (Table 1).

3.3. Scanning electron microscopy and X-ray photoelectron spectroscopy results

Fig. 1-A and Fig. 1-B present scanning electron microscopic images of copper foil and nanostructured copper foil before exposure to water in air. It can be seen that normal copper foil surface has micrometer size dense packed crystals morphology (Fig. 1-A) which is caused by oxidation of it in room environment. For comparison, the nanostructured copper foil synthesized via hydrothermal treatment has nanowires of around one hundred nanometers diameter (Fig. 1-B). Upon immersion in PBS for 1 h, it is found that the normal copper foil surface changes little (Fig. 1-C) while that of nanostructured copper foil changes much (Fig. 1-D) for the later one is chemically more active.

X-ray photoelectron energy spectrum (XPS) is highly effective in probing surface state of materials with chemical state of elements in 10 nm deep of film surface. The $Cu2p_{3/2}$ XPS of fresh normal and nanostructured copper films have been shown in Fig. 2. It can be seen from Fig. 2-A and Fig. 2-Bthat $Cu2p_{3/2}$ spectrum of fresh normal and nanostructured copper films have typical peak of metallic copper and Cu_2O at 932.2 eV and CuO at 934.3 eV respectively with extra peak of 934.6 eV for $Cu_2(OH)_2CO_3$ in compared with that of PBS treated normal and nanostructured copper films has no peaks of $Cu_2(OH)_2CO_3$ as can be seen in Fig. 2-A1 and Fig. 2-B1. The formation of $Cu_2(OH)_2CO_3$ is caused by exposure of films in air with the existence of oxygen and carbon dioxide. This indicates that the existence of PBS benefit dissolution of Cu^{2+} from $Cu_2(OH)_2CO_3$ surface. For comparison, that of BSA treated normal and nanostructured copper films have only peak of $Cu_2(OH)_2CO_3$ at 934.6 eV with no peaks of metallic copper and Cu_2O as can be seen from Fig. 2-A2 and Fig. 2-B2, indicating the presence of BSA enhanced oxidation of both normal and nanostructured copper films' surface. As shown in Fig. 2-A3 and Fig. 2-B3, compared with the samples treated with 37 °C PBS, there is still Cu_2 (OH) $_2CO_3$ on the surface of the samples treated with 20 °C PBS, indicating that Cu^{2+} ions have not been fully dissolved by PBS.

4. Discussion

Copper has been extensively demonstrated to possess potent antibacterial ability [22]. The precise mechanism by which copper and nanostructured copper sterilization occur remains unknown; however, it is widely postulated that these three mechanisms operate concurrently, with the relative significance of each mechanism contingent upon the microbial species present and the environmental conditions [23]. In the first mechanism, reactive oxygen species (ROS) are produced by copper during a "Fenton-like reaction," which causes enzymatic and non-enzymatic oxidative damage. The second mechanism is predicated on the release of copper ions, Cu^{+} and Cu^{2+}, which infiltrate cells, and cause oxidative stress involving endogenous ROS. The third mechanism involves the physical interaction between copper nanoparticles and the cell membranes, of bacteria, which leads to the destruction of the membranes and increases the vulnerability of the bacteria to copper ion damage.

As a consequence of the nanowires on its surface, the antibacterial activity test indicates that the nanostructured copper foil utilized in this study has a more potent effect on *S. aureus* than the normal one. It is challenging for pure copper and copper alloys containing different components to attain this level within a 1-h timeframe, according to other research [24]. Meanwhile, the E. coli suspension

Table 1
The average calculated titers and Log10 reduction after 1h in the antibacterial activity test and influencing factors test.

Bacteria	Positive control (CFU/mL)	Antibacterial Activity Test and Influencing Factors Test	The average calculated titers (CFU/ml)			Log10 reduction relative to the stainless-steel control (mean ± standard deviation)	
			Stainless steel	Ordinary copper foil	Nano- structured copper foil	Ordinary copper foil	Nano-structured copper foil
S. aureus	1.82×10^9	Antibacterial activity test	5.21×10^6	252	0	4.45 ± 0.34	>6.71 ± 0.08
		Use of chemical neutralizers	5.40×10^6	11686	521	2.63 ± 0.14	4.15 ± 0.52
		The existence of organic interferents	1.01×10^7	3	40	6.47 ± 0.25	5.87 ± 0.86
		Low temperature	2.22×10^6	4949	3392	2.57 ± 0.31	2.78 ± 0.31
		Low humidity	4.29×10^5	0	125	>5.52 ± 0.32	3.59 ± 0.12
E. coli	1.77×10^9	Antibacterial activity test	0	0	0	–	–
		The composition of bacterial suspension	4.68×10^6	0	0	>6.67 ± 0.07	>6.67 ± 0.07

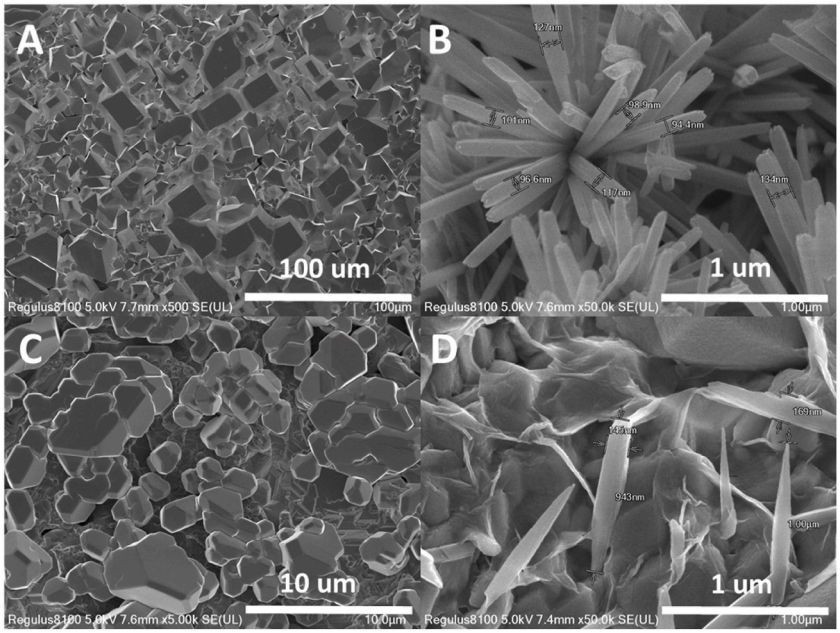

Fig. 1. Scanning electron microscopic images of (A) fresh normal copper foil, (B) fresh nanostructured copper foil; (C) normal copper foil and (D) nanostructured copper foil immersed in PBS for 1 h.

prepared with PBS was observed to be incapable of surviving on the stainless-steel disc utilized in the control group. Conversely, E. coli grown in the presence of TPS showed normal growth, suggesting that the exponential rise in phosphate concentration hindered or dehydrated the osmotic pressure-sensitive *E. coli* during the test [25]. Furthermore, results from scanning electron microscopy revealed that immersion in PBS facilitated the dissolution of $Cu^{2}+$ ions from the surfaces of the samples, a critical factor in the eradication of bacteria. Hence, it is more prudent to thoroughly contemplate the characteristics of the bacteria and the potential impact of bacterial suspension composition when employing diverse test bacteria for antibacterial performance tests.

To terminate disinfection effects, international standards, including ISO 22196-2011 and JIS Z 2801-2010, employ soya casein digest lecithin polysorbate broth (SCDLP) medium or alternative neutralizers. The omission of neutralizers from the Chinese Standard GB/T 21510-2008 is worth noting. In order to counteract the continuous antibacterial effects caused by oxidized substances or exfoliated nanomaterials that may infiltrate the eluent during the elution process of copper foils, particularly when mechanical vortex is used, this study introduced D/E Neutralizing Broth, a broad-spectrum chemical neutralizer. The utilization of a neutralizer diminishes the Log10 reduction, as demonstrated by the test results, which suggests the presence of the effect and its potential for partial elimination. Studies have indicated that metal chelators, including ethylenediamine tetraacetic acid (EDTA), may provide bacterial protection during copper-based material testing [23]. In order to obtain results that are reasonably accurate when evaluating the antibacterial activity of copper-based materials, it is crucial to employ chemical neutralizers in a reasonable manner.

The addition of BSA, which is commonly used as an organic interferent-reducing disinfectant [26], unexpectedly increased the Log10 reduction of normal copper foil in the context of this study. Research findings indicate that copper's antibacterial properties diminish following anti-oxidation treatment [27]. This phenomenon could potentially be due to the oxidation of copper's surface, which generates CuO and Cu_2O, both of which possess antibacterial effects. BSA enhances oxidation of normal and nanostructured copper foil surfaces, but reduces the dissolution of $Cu_2(OH)_2CO_3$, according to the XPS spectra of the samples. The observed variations in response to organic interference may not be adequately explained by copper ions in isolation. The antibacterial efficacy of materials can be influenced by surface hydrophobicity, according to another study [28]. Therefore, the observed discrepancy could potentially be attributed, at least in part, to the rough surface structure of the nanostructured copper foil. Hence, it is critical to comprehend antibacterial mechanisms in order to evaluate the determinants in assessments of antibacterial materials.

Standard methods for testing antibacterial activity entail subjecting samples to 24-h exposure to comparatively high temperatures and humidity conditions, which may not simulate practical conditions for metal surfaces, especially those with high-activity materials like copper [22]. The practical conditions encompass ambient temperature, desiccation, and additional organic pollutant exposure. The chemical reaction rate of antibacterial materials is normally decreased by low temperatures and humidity; nevertheless, in this study, normal copper foil antibacterial activity increased under dry conditions. This could be caused by the phenomenon known as "contact sterilization" of copper-based materials, which states that a dry copper surface has a faster and more potent antibacterial effect than a wet one [29]. However, the absence of this phenomenon in nanostructured copper foil further demonstrated that the antibacterial mechanism of nanostructured copper foil is not completely the same as that of normal copper foil. Therefore, practical conditions should be taken into account when evaluating antibacterial activity; various temperature and humidity scenarios should be incorporated so as to better comprehend how antibacterial activity changes in practical applications.

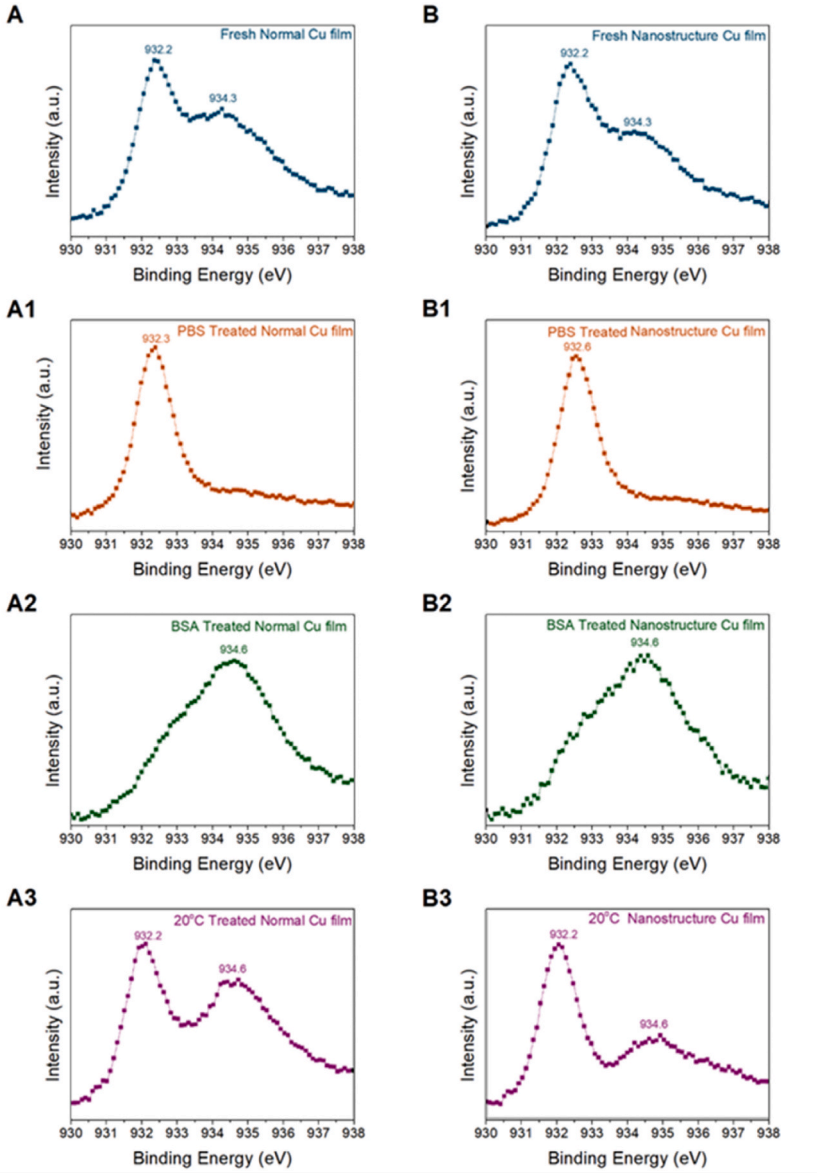

Fig. 2. Cu2p$_{3/2}$ XPS spectrums of normal and nanostructured copper foils treated at different condition: (A)fresh normal copper foil, (B)fresh nanostructured copper foil; normal copper foil treated with (A1)PBS, (A2)BSA, (A3)20 °C; nanostructured copper foil treated with (B1)PBS, (B2) BSA, (B3)20 °C.

This study employs two kinds of copper foils to evaluate their antibacterial activity and influencing variables. In contrast to normal copper foil, which represented extensively researched bulk copper materials, nanostructured copper foil represented nanostructured copper materials. Strong antibacterial activity has been observed in both normal and nanostructured copper foils; however, the test results are directly influenced by the composition of the bacterial suspension utilized in evaluation and the application of a neutralizer, if any. Its antibacterial effect is also affected by changes in environmental temperature and humidity, as well as the interference of organic substances. Furthermore, research has demonstrated the importance of considering the impact of the initial titer of dispersion utilized for inoculating the test sample [30]. This titer signifies the extent of antibacterial material contamination that would occur in practical situations. Therefore, for evaluation of antibacterial activity of antibacterial materials, it is imperative to not only consult the methods outlined in the standard but also contemplate practical applications of the materials. This will enable targeted adjustments to test conditions to be made, thereby facilitating a genuine and objective evaluation of their antibacterial activity.

Funding statement

This work was supported by the Disinfection research project of Chinese Preventive Medicine Association, XD2022-Z-03; and the

Technologies Program of Tianjin Health Commission, TJWJ2021MS016.

Data availability statement

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Jiahao Li: Writing – original draft, Methodology, Formal analysis. **Luhua Lu:** Writing – review & editing, Resources. **Yongzhong Jiang:** Resources, Funding acquisition. **Fei Tang:** Resources, Investigation. **Qiao Wu:** Visualization, Investigation. **He Liu:** Resources, Investigation. **Qili Zeng:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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