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Article

Antibacterial Effects of Copper Microparticles/Copper Nanoparticles/Copper(II) Oxide Nanoparticles and Copper Microparticles/Copper Nanoparticles/Copper(I) Oxide Nanoparticles from Ultrasono-Electrochemical with Hydrothermal Assisted Synthesis Method

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Abstract. Copper is a versatile metal with various properties, including antibacterial effects. There are many methods to produce nano-enhanced copper. In this study, we explore the ultrasono-electrochemical with hydrothermal assisted method to produce copper/copper oxides nanoparticles by using ultrasono-electrochemical process to produce copper micro/nano particles, ultrasonication process to produce copper oxide nanoparticles and hydrothermal process to produce cuprous oxide nanoparticles. Antibacterial properties of the produced particles were tested by conventional bacterial identification test and conventional total viable count test using 4 standard bacteria: *Escherichia coli, Salmonella enteritidis, Staphylococcus epidermidis* and *Staphylococcus aureus.* The results show that ultrasono-electrochemical method can produce high purity copper micro and nano particles with zero oxidation with the average size of 575 and 118 nm, ultrasonication process can produce copper oxide nanoparticles on copper microparticle and nanoparticle surfaces with the average size of 56 nm, and hydrothermal process can produce cuprous oxide nanoparticles on copper microparticle and nanoparticle surfaces with the average size of 50 nm. All particles with concentration of 0.5 mmol/ml are highly effective as antibacterial agents against *Staphylococcus epidermidis.* Copper oxide nanoparticles are effective against *Salmonella enteritidis, Staphylococcus epidermidis* and *Staphylococcus aureus*, while cuprous oxide nanoparticles are highly effective against all 4 species of bacteria, at over 99.17%.

Keywords: Copper/copper oxides nanoparticles, antibacterial effects, ultrasono-electrochemical method, ultrasonication method, hydrothermal method.

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1. Introduction

Copper (Cu) is a versatile material due to their various characteristics, including high conductivity, recyclability, and reactivity [1]. Copper is commonly used as electrical conductors, structure supporters, molds, water purifiers, and fertilizers [2-5]. Moreover, copper oxides, including copper(II) oxide (CuO) and copper(I) oxide (Cu_2O) are applied as semiconductors, antibacterial agents and chemical catalyzers [6].

Due to the development of nanotechnology, properties of copper and copper oxides in nanomaterial forms are continuously investigated. Copper nanoparticles (CuNPs) are described as particles with the size of 100 nm or less [7-9]. The CuNPs retain their electrical conductivity, but enhance the recyclability and chemical reactivity properties. At ambient conditions, CuNPs agglomerate but still retain all their nano-enhanced properties. Enhanced properties of copper oxides nanoparticles (CuONPs and Cu_2ONPs) are accelerated chemical reactions and antibacterial properties.

The antibacterial effects and applications of copper and copper compounds have been extensively investigated over the past decade. It has been found that copper and copper compounds are oligodynamic. A very small amount of them is capable of causing harmful effects to bacterial cells, known as 'contact killing', initiated by the release of copper ions from the copper surface. Membrane degradation and toxicity of copper are the two main mechanisms that cause damage or even death to the bacterial cells.

Membrane degradation is considered the initial effect of copper and copper oxides on the bacterial cells. Pure copper and copper oxides are capable of releasing ions to the environment. The positively charged copper ions can readily bind to the negatively charged molecules on the bacterial outer membrane. This disrupts the membrane functions and causes membrane degradation of both gram negative and gram positive bacteria.

Copper is an essential trace element for most living organisms, a small amount of copper ion is required for many metabolic activities of the bacterial cells. Many bacteria species use some mechanisms to transport extracellular copper ions into the cell. In normal condition, low level of copper ions can be maintained through Cu homeostasis mechanisms. However, at high level, copper ions can cause harmful effects to the bacterial cells.

There are several pathways for copper ions to enter the bacterial cells: the ions can bind with membrane transporters and being transported into the cell via some ion transport mechanisms or nano-sized copper ions can be imported via an outer membrane porin [10, 11]. Membrane damage also provide a way for copper ions to enter the cell.

Once copper ions are in the cell, CuO produces reactive oxygen species or ROS, e.g., hydroxyl ions and superoxides, through Fenton-like reaction. ROS play a significant role in the homeostasis of the cells. In normal condition, the cells maintain ROS at low and stationary levels. However, at high level, ROS impair lipids, proteins, and nucleic acids, causing genetic materials degradation, cell circulation interruption and cell death [11, 12].

According to Fenton-like reaction, Cu(II) ions can be reduced to Cu(I) ions by hydroxyl ions and superoxides from cell components. The Cu(I) ions damage the metabolize enzyme by binding to the intracellular proteins, e.g., to the disulfide group of respiratory enzymes of cell membrane, leading to cell respiratory arrest and genetic materials degradation [10, 13, 14].

The antibacterial properties of copper microparticles (CuMPs), copper nanoparticles (CuNPs), copper(II) oxide microparticles (CuOMPs) and copper(II) oxide nanoparticles (CuONPs) are similar to those of silver nanoparticles (AgNPs), but copper is more abundant and less expensive. There are many methods to synthesize copper and copper(II) oxide particles. Chemical reaction methods are the most studied as there are various factors (e.g., the temperature or the pH of the solution) to control the shape and the size of the particles [7, 8]. Most chemical reaction methods use at least one type of copper compound ($CuSO_4$, $CuCl_2$ or $Cu(NO_3)_2$) combine with a reducing agent (ascorbic acid, oleic acid or $NaBH_4$) and a stabilizing agent (starch, PVP, tree resins, or chitosan) or a buffer (H_3BO_3 or $C_4H_4KNaO_6$) [8, 9, 15-21]. The size of the obtained copper particles ranges from 37 nm to 150 nm. However, these methods always use toxic precursors or produce toxic wastes such as hydrochloric acid or sulfuric acid [9, 19, 21].

There are alternative methods for the synthesis of copper nanoparticles including wire explosion technique, photochemical method and ultrasono-chemical method. The wire explosion technique uses high energy pulse electrical discharge to explode copper wires. Although this technique uses no chemical reaction, it requires high voltage electricity and complicated synthesis equipment [22, 23]. The photochemical method uses YAG laser on copper pieces dipping in a solvent (a mixture of water, ethanol and/or acetone). This method can produce high purity CuNPs [24-26]. However, a high energy light source and complicated equipment are required. The ultrasono-chemical method applies the ultrasonication to assist chemical reactions in the solution of a copper salt and a reducing agent. This accelerates the chemical reactions, hence increases the production of CuNPs. This technique requires low-cost materials and simple experimental equipment, but the CuONPs are also produced from the soluble oxygen in the solution which lowers the purity of the obtained CuNPs [27, 28]. However this CuONPs can be used to produced Cu_2ONPs using the hydrothermal process [29, 30].

In this study, we investigate the antibacterial properties of the copper micro/nano particles and the copper oxides nanoparticles we synthesized using ultrasono-electrochemical method. The CuONPs in this study were produced by the ultrasonication process and the Cu_2ONPs in this study were produced by the hydrothermal process. The advantage of these methods is that no toxic substances are used or produced. The properties of the copper and the copper oxides particles

were determined using scanning electron microscopy (SEM), and energy dispersive x-ray spectrometry (EDS). The antibacterial properties were tested by conventional bacterial identification test and conventional bacterial viable count test using 4 ATCC standard bacteria: *Escherichia coli, Salmonella enteritidis, Staphylococcus epidermidis* and *Staphylococcus aureus.*

2. Materials and Methods

2.1. Synthesis of CuMPs and CuMPs/CuNPs

The electrochemical system for synthesizing CuMPs and CuMPs/CuNPs is composed of two electrodes partly submerged into the electrolyte. The electrolyte was prepared using a mixture of 10 mmol of ascorbic acid, 2 mg of gum arabic and 200 ml of deionized water (type II DI water). The solution was thoroughly stirred for 5 minutes and then put in a 250 ml airtight beaker and maintained under ambient conditions for 1 hour. All the chemical reagents in this experiment are analytical grade without further purification. A copper rod (as the anode) and a tungsten rod (as the cathode) were used as the electrodes in this electrochemical system.

The synthesis of CuMPs was performed by applying a DC voltage of 0.75 V to the electrodes for 6 hours under ambient conditions. For CuMPs/CuNPs, the synthesis was performed under ambient conditions by applying a DC voltage of 0.75 V to the electrodes for two hours and 20 minutes during which ultrasonication (35W and 42 kHz) was also applied for 30 minutes, rested for 5 minutes and was repeated 4 times during the synthesis.

2.2. CuMPs/CuNPs/CuONPs and CuMPs/CuNPs /Cu_2ONPs

To synthesize CuMPs/CuNPs/CuONPs, the suspended CuMPs/CuNPs were separated from the electrolyte by sedimentation and 50 ml of DI water was added into a 250 ml beaker. The beaker of CuMPs/CuNPs were ultrasonicated for 1 hours 45 minutes until the color of the solution changed from pink to dark brown as shown in Fig. 1(c). To synthesize CuMPs/CuNPs/ Cu_2ONPs, the suspended CuMPs/CuNPs/CuONPs were heated at 100ºC for two hours until the color of the solution changed from dark brown to yellow (CuMPs/CuNPs/Cu_2ONPs), as shown in Fig. 1(d).

(a) (b) (c) (d)

Fig. 1. From the left to right (a) CuMPs (b) CuMPs/CuNPs (c) CuMPs/CuNPs/CuONPs and (d) CuMPs/CuNPs/Cu_2ONPs.

2.3. Antibacterial Test

Antibacterial properties of 0.5 mmol CuMPs, 0.5 mmol CuMPs/CuNPs, 0.5 mmol CuMPs/CuNPs/ CuONPs and 0.5 mmol CuMPs/CuNPs/Cu_2ONPs were tested using conventional bacterial identification test and conventional total viable count test [31] using 4 standard bacteria (McFarland Standard No. 0.5 of 1.5×10^8 colonies forming unit per ml (CFU/mL) for each standard bacterium). *Escherichia coli* (*E. coli*, ATCC 22922), *Salmonella enteritidis* (*S. enteritidis*, ATCC 24213) *Staphylococcus epidermidis* (*S. epidermidis,* ATCC 12228), and *Staphylococcus aureus* (*S. aureus,* ATCC 25293) were used as two gram negative and two gram positive bacteria representatives, respectively.

In this research, related equipment was sterilized before testing in order to prevent any contamination. The broth was prepared using a mixture of 1 g of peptone water, 1 g of sodium chloride (NaCl) and 1 l of deionized water. The agar was prepared using a mixture of 17.54 g of tryptone glucose yeast agar (OXOID™) and 1 l of deionized water.

Conventional bacterial identification test

The bacterial solutions were prepare using the mixture of 1 ml of standard bacteria, 0.5 mmol of antibacterial agent (in 1 ml solution) and 1 ml of deionized water. The 1 ml of bacterial solutions were mixed into the 9-ml broth.

This specimen was then poured into a prepared agar petri-dish for bacterial culture. The sample was incubated at 37°C for 24 hrs. After incubation, the specimen was observed for the growth of the bacterial colony. The purpose of this test is to screen the potential bactericidal activity of the agent before continuing the test for quantitative result in the next procedure.

Conventional total viable count test

For the conventional total viable count test, the control sample was prepared by mixing 1-ml of each specimen with 9-ml broth. The samples were prepared at different concentrations by the ten-fold serial dilution. After dilution, the 0.1 ml of each specimen were cultured onto the agar surface. The samples were incubated at 37°C for 24 hrs. After the incubation, the samples with the bacterial colonies ranged from 25–250 colonies were set as the final dilution. The ten-fold serial dilution is presented in Fig. 2.

The number of bacterial colonies were counted and determined as the total viable cells following Eq. (1).

$$v = n \times 10^{(1+dt)} \quad (1)$$

where

v = the total viable cells
n = the number of colonies
dt = dilution time(s)

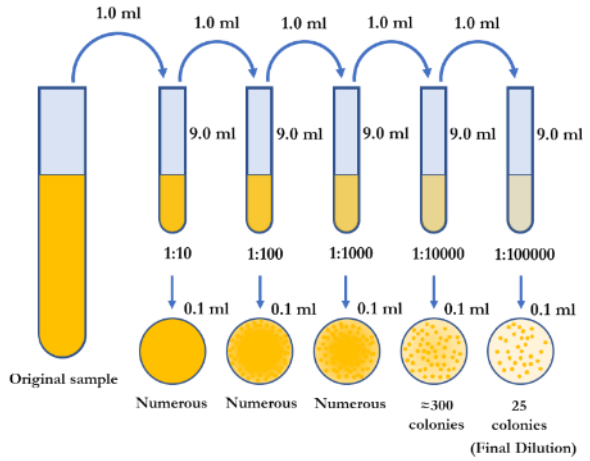

Fig. 2. The ten-fold serial dilution for conventional total viable count.

The value of the total viable cells is used to determine the percent reduction of bacteria following Eq. (2)

$$\% \text{ red} = \frac{\text{v}}{1.5 \times 10^8} \times 100\% \quad (2)$$

where

% red = the percent reduction of bacteria
v = the total viable cells

2.4. Scanning Electron Microscopy (SEM) and Energy Dispersive Spectrometry (EDS)

The obtained copper and copper oxides particles were analyzed using SEM/EDS system (Scanning Electron Microscopes SU3900, Hitachi™). The samples were prepared by spreading the obtained particles on the surface of pure graphite substrates. SEM with the magnification of ×10,000 to ×50,000 using the applied voltage of 5–20 keV was used to analyze the shape and the size of the obtained copper particles. EDS was used to analyze the elemental composition of the surface of the sample.

3. Result and Discussion

3.1. Characteristic of CuMPs, CuMPs/CuNPs, CuMPs/CuNPs/CuONPs and CuMPs/CuNPs /Cu_2ONPs

Figure 3 shows the scanning electron micrographs (SEM) and the energy dispersive spectrograms (EDS) of CuMPs, CuMPs/CuNPs, CuMPs/CuNPs/CuONPs and CuMPs/CuNPs/Cu_2ONPs. The CuMPs were formed into complete microscale crystals as shown in Fig. 3(a). From Fig. 4(a), the overall histogram reported the size of copper particles ranges from of 300–1,500 nm with normal distribution. The average particle size of CuMPs was 1,022 nm. This process produced no copper oxides because the ascorbic acid acted as an antioxidant agent, preventing the oxidation reaction. The EDS in Fig. 3(a) shows that this copper particle synthesis method yielded high purity copper with zero oxidation.

For CuMPs/CuNPs, the CuMPs were formed into complete crystals and the CuNPs were formed as clusters separated from CuMPs as shown in Fig. 3(b). The overall histogram reported that the particle size ranges from 50–1,000 nm with double-normal distribution. The average particle sizes of CuMPs and CuNPs were 575 and 118 nm, respectively. The result suggested that the ultrasonication process in this synthesis method caused a mechanical disturbance to the solution, which reduced the size of the CuMPs and initiated the formation of the CuNPs. From Fig. 3(b), the EDS shows that this synthesis method also yielded high purity copper with zero oxidation.

For the CuMPs/CuNPs/CuONPs, the scanning electron micrographs showed that the copper particles were formed as defected face-centered cubic and CuONPs were formed as particle clusters on the surface of CuMPs and CuNPs. The average particle sizes of CuMPs, CuNPs and CuONPs were 575, 92 and 56 nm, respectively. The carbon peak in Fig. 3(a), (b), (c) and (d) represented the graphite substrate that the particles were attached to as the method of using SEM. By neglecting the carbon weight ratio, the copper weight ratio and the oxygen weight ratio of CuMPs/CuNPs/CuONPs were 91.8% and 8.2%, respectively. In this process, the ultrasonication increased the rate of the oxidization of the suspended CuMPs and CuNPs at the CuMPs and CuNPs surfaces. Because of the high reactivity of the CuNPs, the CuONPs were formed at a rapid rate on the surface of CuMPs and CuNPs clusters.

For the CuMPs/CuNPs/Cu_2ONPs, the scanning electron micrographs showed that the copper particles were formed as defected face-centered cubic and Cu_2ONPs were formed as particle clusters on the surface of CuMPs/CuNPs. The average particle sizes of CuMPs, CuNPs and Cu_2ONPs are 568, 81 and 50 nm, respectively. By neglecting the carbon weight ratio, the copper weight ratio and oxygen weight ratio of CuMPs/CuNPs /Cu_2ONPs were 87.3% and 12.7%, respectively. During the hydrothermal process, more CuONPs are produced and transformed into CuO_2NPs. Some of the CuNPs/Cu_2ONPs cluster were observed as they were broken off from the surface of the CuMPs.

In this research, we synthesized CuMPs/CuNPs under ambient conditions (25°C, 1 atm) using electrochemical and ultrasonication method. A pure copper electrode (anode) was used to supply copper ions (Cu^{2+}) and a pure tungsten electrode was used as a cathode. Ascorbic acid ($C_6H_8O_6$) was used as a reducing agent and gum arabic was used as a stabilizer. The chemical reaction is presented in Eq. (3)

$$Cu^{2+} + C_6H_8O_6 \rightarrow Cu_{(s)} + C_6H_6O_6 + H_2 \quad (3)$$

From Eq. (3), this chemical reaction produces CuMPs/CuNPs in the solution. Gum arabic was added to reduce the mobility of the copper ions and the electrons in the solution, hence more suspended nanoparticles were maintained. Ultrasonication was used to accelerate the rate of reaction, causing rapid formation of CuMPs/CuNPs. The remaining ascorbic acid in the solution prevented the pure form of the suspended copper particles in the solution from becoming copper oxides (CuO and Cu_2O)

[32-37]. The production of copper oxides is presented in Eq. (4) and (5).

$$2Cu_{(s)} + O_2 \rightarrow 2CuO \quad (4)$$

$$4Cu_{(s)} + O_2 \rightarrow 2Cu_2O \quad (5)$$

According to Eq. (4), CuO formed in the solution at room temperature came from the copper particles and the soluble oxygen. However, this research showed that the ultrasonication greatly increased the oxidation reaction by inducing mechanical vibration, causing a large amount of CuONPs to form at the surface of CuMPs and CuNPs within a relatively short period of time (1 hours 45 minutes). CuMPs/CuNPs/CuONPs contained more surface area hence they can induce more chemical reactions.

In a low oxygen environment, Cu_2O tends to form instead of CuO as shown in Eq. (5). This study showed that by increasing the temperature of the solution, Cu_2ONPs can be formed. This suggested that, in the beginning of the hydrothermal process, the heat from the process increased the oxidation reaction, thus more CuO was formed. However, as the temperature increased higher, the oxygen solubility of the solution decreased, causing the CuO in the solution to be transformed into Cu_2O.

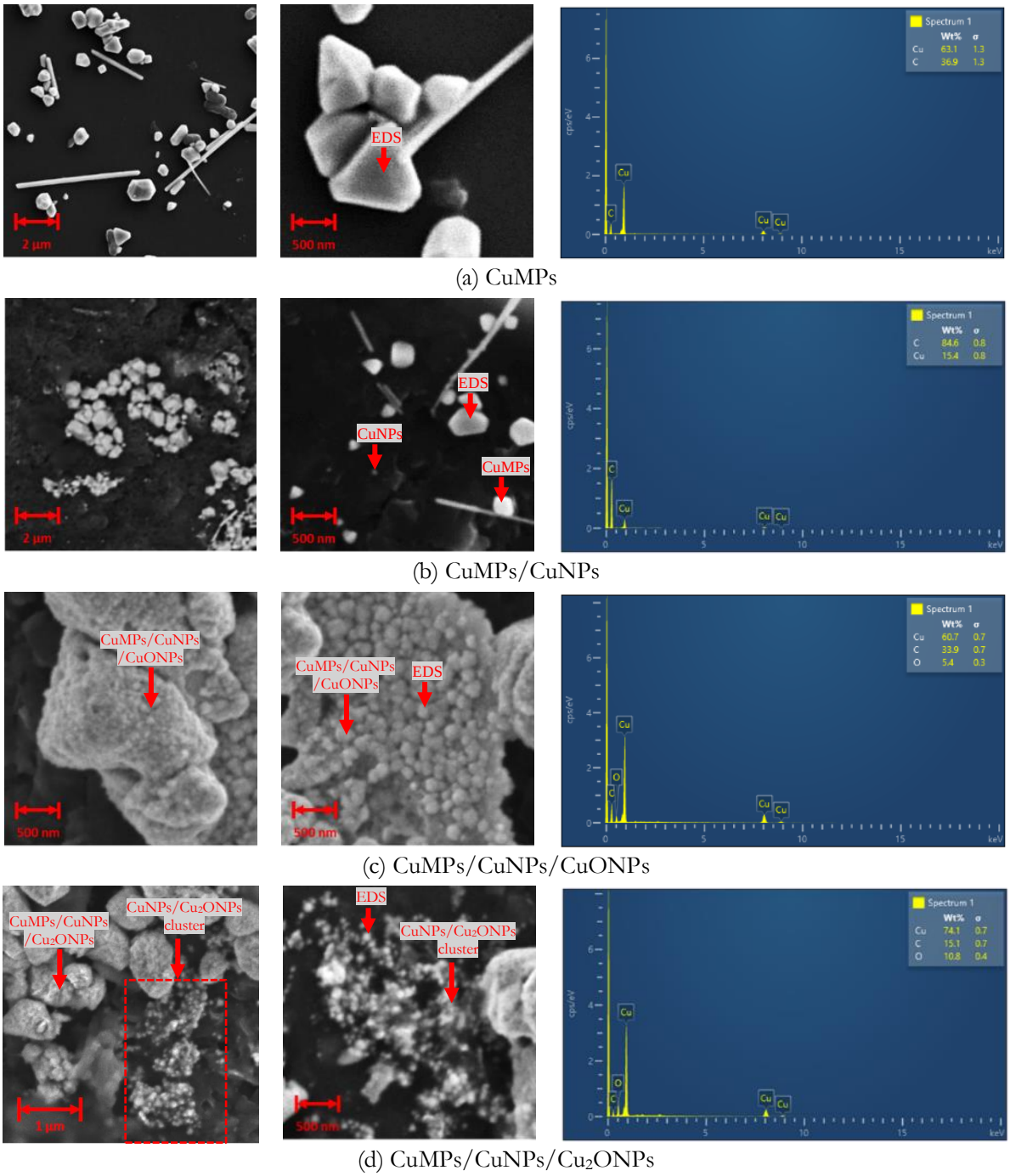

Fig. 3. The SEM and EDS of (a) CuMPs, (b) CuMPs/CuNPs,(c) CuMPs/CuNPs/CuONPs, and (d) CuMPs/CuNPs/Cu_2ONPs.

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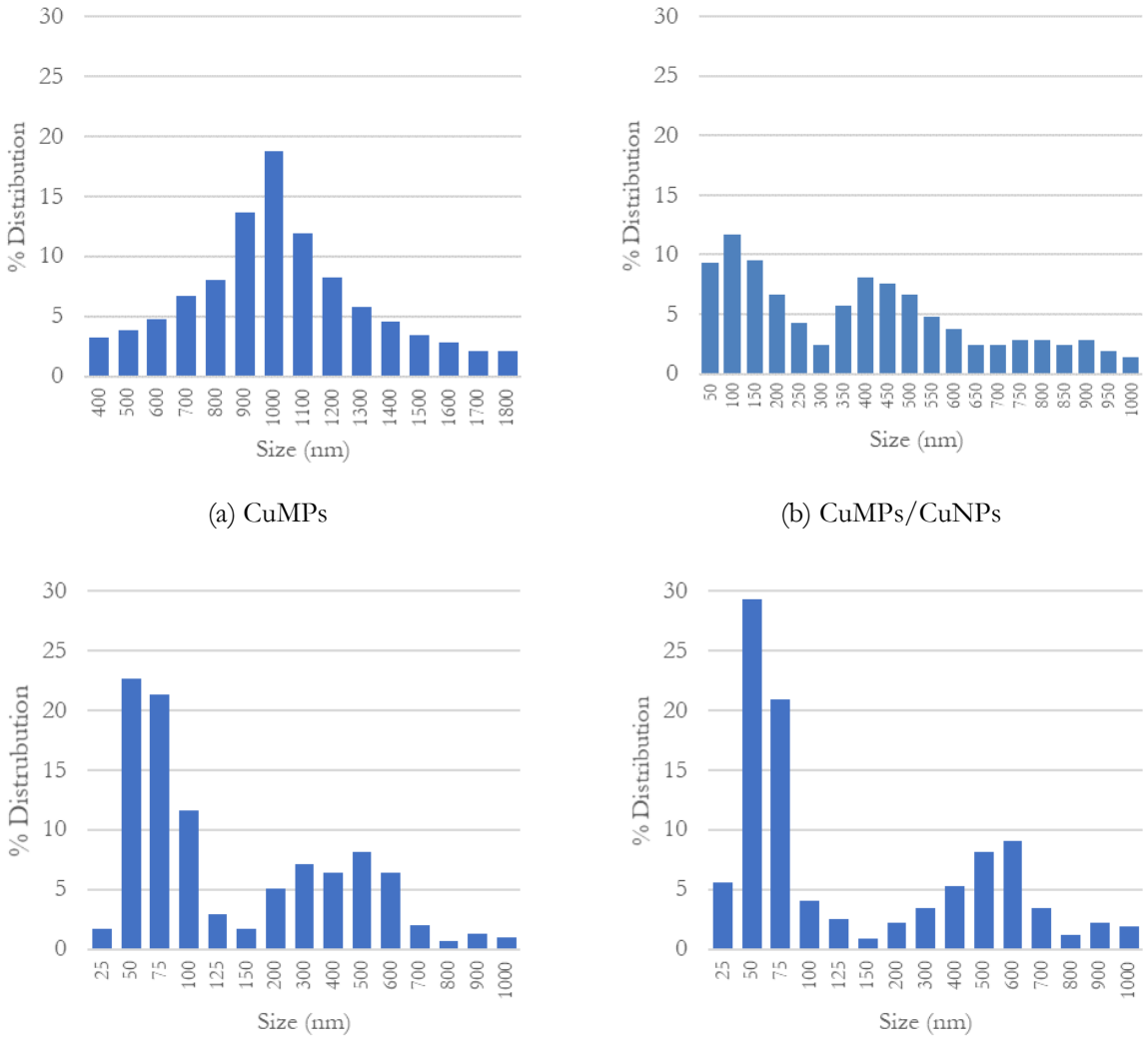


Fig. 4. The particle size distribution of (a) CuMPs, (b) CuMPs/CuNPs, (c) CuMPs/CuNPs/CuONPs, and (d) CuMPs/CuNPs/Cu_2ONP.

3.2. Antibacterial Efficacy

The results from the conventional bacterial identification tests are shown in Table 1.

According to Table 1, bacterial growth was found in all specimen with varying numbers. Uncountable colonies were found on all bacterial samples in DI water, CuMPs, and CuMPs/CuNPs.

In DI water, the bacterial colonies of *E. coli, S. enteritidis, S. epidermidis* and *S. aureus* were formed on the agar surface as the opaque biofilm. In CuMPs/CuNPs/CuONPs, the remaining bacterial colonies of *E. coli, S. enteritidis, S. epidermidis* and *S. aureus* decreased to a moderate number. In CuMPs/CuNPs/Cu_2ONPs, the remaining bacterial colonies of *E. coli, S. enteritidis, S. epidermidis* and *S. aureus* decreased to a smaller number than that of the CuMPs/CuNPs/CuONPs.

This study showed that the copper oxides particles tended to be more effective antibacterial agents compared to the pure form of copper particles. However, the information obtained from this study was not quantitatively precise enough to indicate the effectiveness. To obtain quantitative data, conventional viable count tests were performed. The results from the conventional viable count tests are shown in Table 2.

According to Table 2, the antibacterial effect of the 0.5 mmol/ml CuMPs, shown as the percent reduction of bacteria (%reduction), from the highest to the lowest were *S. epidermidis* (97.33%), *S. enteritidis* (92.00%), *E. coli* (87.78%) and *S. aureus* (73.33%). The antibacterial effect of the 0.5 mmol/ml CuMPs/CuNPs from the highest to the lowest were *S. epidermidis* (98.67%), *S. enteritidis* (96.00%), *E. coli* (94.00%) and *S. aureus* (46.67%). The antibacterial effect of the 0.5 mmol/ml CuMPs/CuNPs/CuONPs from the highest to the lowest were *S. enteritidis* (99.55%), *S. epidermidis* (98.14%), *S. aureus* (96.00%) and *E. coli* (94.00%). The antibacterial effect of the 0.5 mmol/ml CuMPs/CuNPs/Cu_2ONPs from the highest to the lowest were *S. enteritidis* (99.99%), *S. epidermidis* (99.60%), *E. coli* (99.40%) and *S. aureus* (99.17%). The results suggested that CuMPs and CuMPs/CuNPs are very effective antibacterial agents

against *S. epidermidis* and *S. enteritidis*, but they are less effective against *E. coli* and *S. aureus*. CuMPs/CuNPs /Cu_2ONPs are the most effective antibacterial agents in this study, since the %reduction from all 4 bacteria are higher than 99.17%

Table 1. Result from the conventional bacterial identification test.

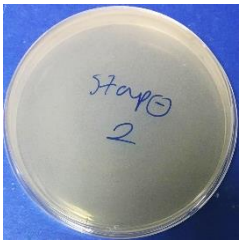
Specimen (0.5 mmol /ml)	Bacteria remaining order			
	Gram negative		Gram positive	
	Escherichia coli (ATCC 22922)	*Salmonella enteritidis* (ATCC 24213)	*Staphylococcus epidermidis* (ATCC 12228)	*Staphylococcus aureus* (ATCC 25293)
DI water (control)				
CuMPs				
CuMPs/CuNPs				
CuMPs/CuNPs/ CuONPs				
CuMPs/CuNPs/ Cu_2ONPs				

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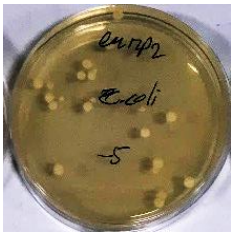
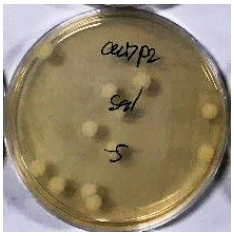
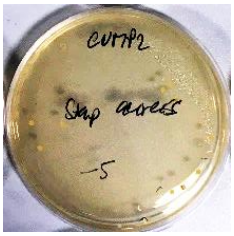
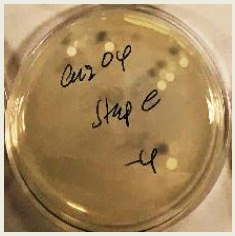
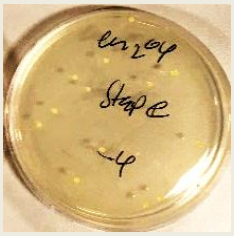
Table 2. result from the conventional viable count test.

Specimen (0.5 mmol /ml)	Total count (CFU/ml)			
	Gram negative		Gram positive	
	Escherichia coli (ATCC 22922)	*Salmonella enteritidis* (ATCC 24213)	*Staphylococcus epidermidis* (ATCC 12228)	*Staphylococcus aureus* (ATCC 25293)
CuMPs	2.0×10^7  %red = 87.78	1.2×10^7  %red = 92.00	4.0×10^6  %red = 97.33	4.0×10^7  %red = 73.33
CuMPs/CuNPs	9.0×10^6  %red = 94.00	6.0×10^6  %red = 96.00	2.0×10^6  %red = 98.67	8.0×10^7  %red = 46.67
CuMPs/CuNPs/ CuONPs	9.0×10^6  %red = 94.00	6.7×10^5  %red = 99.55	2.8×10^6  %red = 98.14	6.0×10^6  %red = 96.00
CuMPs/CuNPs/ Cu_2ONPs	9.0×10^5  %red = 99.40	2.0×10^3  %red = 99.99	6.0×10^5  %red = 99.60	1.4×10^6  %red = 99.17

By comparing the pure form of copper, CuMPs/CuNPs exhibit higher antibacterial effects than CuMPs. The nano-sized copper formed on the surface of CuMPs provide more surface area thus enhanced the ion activities of the particles.

The bacterial identification tests and the viable count tests showed that CuMPs/CuNPs/CuONPs and CuMPs/CuNPs/Cu_2ONPs are more effective against bacteria than CuMPs and CuMPs/CuNPs. Comparing the pure form coppers to the oxide forms, the result showed that both oxide forms are more effective against all 4 species of bacteria. This could be caused by the different ability to release the copper ions from the different oxidation-states of copper. In order for the pure form coppers to release ions, they need to be oxidized first. Furthermore, the average sizes of both oxide forms in our study are much smaller than the pure form coppers as shown in Fig. 3 and Fig. 4.

According to Fig. 3(c), the CuONPs was formed on the surface of CuMPs/CuNPs, which increased the surface area of the particles. The increased surface area of the particles enhanced their antibacterial effects by increasing of redox chemical activity and increased their ability to released copper ions. The proper concentration level of copper ions and the susceptibility of the bacteria to the ions can cause the cell membrane degradation and allow more dissolved copper ions to enter the cell. In this study, all bacteria we used are aerobic bacteria. The

increase amount of Cu^{2+} and CuO that enter the cell, in aerobic conditions, produces high concentration of ROS. This results in higher oxidative stress causing an increase in the number of dead cells [10, 13, 14, 38].

Several study suggests that the Cu_2ONPs and the CuONPs can release Cu^{+} and Cu^{2+} [14]. The Cu^{2+} significantly increased oxidative stress by producing more ROS and the Cu^{+} can bind to the intracellular proteins much better than Cu^{2+} do. This study shows that, the aerobic bacteria are highly vulnerable to CuONPs and Cu_2ONPs because of the high level of the released copper ions and the high level of ROS in the bacterial cells. As such, we speculated that copper oxide particles are more effective as bactericide compare to pure copper particles.

In this study, CuMPs/CuNPs/Cu_2ONPs were shown to be more effective antibacterial agent than CuMPs/CuNPs/CuONPs against all 4 bacteria species. According to Fig. 3(d), the CuNPs/Cu_2ONPs that detached from CuMPs/CuNPs/Cu_2ONPs cluster increased the contact surface area and cuprous ionization. Cuprous ions are unstable, highly diffusible, and capable of penetrating through the bacterial cell membrane. Some studies suggested that cuprous ions deactivate bacterial cell membrane by denaturing cell membrane protein, and by destabilizing the environment [6, 13, 14].

In our experiment, The CuONPs were transformed into Cu_2ONPs on surface CuMPs/CuNPs, the process for synthesizing Cu_2ONPs is an additional process to the synthesis of CuONPs. The oxidizing period for the synthesis of CuMPs/CuNPs/Cu_2ONPs was longer than the synthesis of CuMPs/CuNPs/CuONPs therefore the proportion of oxygen in Cu_2ONPs was much higher than that of CuONPs.

Some study suggests that the different antibacterial effects against gram positive and gram negative bacteria were speculated as they have different cell structures and ion transport mechanisms. In order to survive, the bacteria must be able to adapt to defend themselves using the innate immune system mechanisms. Some bacteria could adapt the copper resistance by the control of homeostasis. There are three copper resistance mechanisms: (i) sending copper outside of the cell using copper-ATPase pumps and resistance-nodulation-cell division (RND) pumps (ii) reducing the effects of copper in the cell using copper-binding metal chaperone (iii) transforming more toxic form of copper (Cu^{+}) into less toxic form (Cu^{2+}) by oxidation. The copper-ATPase pumps and copper-binding metal chaperone are found in both gram positive and gram negative. The RND mechanism primarily found in gram negative bacteria but less common in gram positive bacteria. The reducing copper toxicity mechanisms are found only in gram positive bacteria [10, 14].

Antibacterial mechanisms of both copper oxides and pure copper (CuMPs and CuMPs/CuNPs) are similar as they consist of membrane degradation and toxicity of copper from the copper ions (Cu(I) ions and Cu(II) ions). Copper oxides can readily release copper ions in negatively charged environment (e.g., bacterial proteins) but pure copper need to be oxidized first in order to released copper ions. This oxidation process required either oxidizing agents or energy, thus pure copper may require longer time to provide antibacterial effects compare to copper oxides.

The antibacterial tests we used were performed within 24 hrs. During this period of time, the pure coppers may release less copper ions than the copper oxides do. Therefore, in this study, the pure coppers appeared to be less effective in killing bacteria than the copper oxides.

Different species of bacteria may use different copper-uptake mechanisms, contain different biomolecules and have different copper resistance mechanisms. Thus, they respond differently to copper and its compounds which in turn reflect on the differences in the effectiveness of the antibacterial agents.

In this study, CuONPs and Cu_2ONPs were shown to be highly effective against both gram negative and gram positive bacteria. However, our test results show no difference in the effectiveness against gram negative and gram positive specimens. To date, there is no conclusive reports on the copper resistance of each bacterial group, and the exact effect of Cu^{+} and Cu^{2+} on cell proteins and intracellular genetic materials. In our study, there is no difference between gram positive and gram negative bacteria in their resistance to copper. This suggests that copper in its nano enhanced form is more effective antibacterial agent because of its size, shape, and its oxidation state. The CuMPs/CuNPs/Cu_2ONPs are shown to be more effective than CuMPs/CuNPs /CuONPs, which suggested that the smaller size and the higher chemical activity of the Cu_2ONPs may play a very important role in increasing the antibacterial potency of the material.

4. Conclusions

Ultrasono-electrochemical with hydrothermal assisted method is a green and effective method for the production of nano enhanced copper and copper oxides particles for antibacterial applications since this method neither requires nor produces toxic substances and can be performed within a relatively short period of time (less than 6 hours). Comparing CuMPs, CuMPs/CuNPs, CuMPs/CuNPs/CuONPs and CuMPs/CuNPs/ Cu_2ONPs, the CuMPs/CuNPs/Cu_2ONPs are the most effective antibacterial agent in this research. Copper oxides exhibit immediate antibacterial effect since they can readily release copper ions. However, pure form coppers may provide delay antibacterial effects because they need to be oxidized first in order to release ions. Further studies about their effects on other species of microbes such as virus and fungi for the development of a broad-spectrum disinfectant, and other applications of these materials, such as electrodeposition of CuONPs and Cu_2ONPs on a surface of a conductor, are suggested.

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