A SIMPLE SYNTHESIS METHOD FOR POROUS COPPER TO IMPROVE ITS EFFICACY IN DECONTAMINATING POLLUTED WATER

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ABSTRACT

Monolithic nanoporous copper can be synthesized by a simple chemical etching method from a precursor copper alloy. While nanoporous gold has been reported to exhibit a greater antimicrobial efficacy than a nonporous gold surface, to the authors' knowledge there has been no research work conducted to investigate the antimicrobial properties of nanoporous copper. In this paper, the authors report a simple method to synthesize porous copper from household brass, using materials that are readily accessible in developing regions, which are compared against copper, zinc and untreated brass samples. The antimicrobial efficacy and ion release of the metal samples ion deionized water and water containing phosphate ions are compared, and the metal samples characterized using SEM and EDX analysis. The results of this study suggests a porous surface of copper synthesized from the dealloying of brass exhibits greater antimicrobial efficacy than a nonporous copper surface, and that the antimicrobial efficacy of the porous copper is less inhibited by the presence of phosphate ions in solution than that of untreated brass.

INTRODUCTION

The Problem of Water Security in Developing Regions

Throughout the history of mankind, water has been intertwined with our existence. In fact, some have commented that there is no other resource is as significant in every culture on this world as water [1]. Yet, despite its importance to human health, clean water remains an inaccessible resource to many. In less developed countries, 30,000 people die every day due to unclean water supply and poor sanitation [1], and 35% of people living in less developed countries die from problems related to a lack of clean water supply [1]. In the coming years, the problem of water security is likely to worsen; it is estimated in 2008 that in by 2028, the average per capita supply of clean water will decrease by more than 30% and that by 2025, more than half of the countries in the world will be facing a water supply deficit [1].

Despite the urgent need to improve water supplies in the developing world, the goal of providing universal access to clean water has yet to be reached [2]. Research by the World Health Organisation suggests that making household water decontamination methods accessible to communities in the developing world is important in improving water security [2]. However, comparing the water decontamination strategies available to such communities, chemical decontamination methods often requires the use of chemicals that are not accessible in developing regions; and UV irradiation of water under sunlight is only effective for clear water that is not turbid, and often requires long treatment duration [1]. Hence, there has been continued research work in engineering effective, affordable and viable solutions for water decontamination that can be used in developing regions.

The Use of Oligodynamic Metals in Decontaminating Water

In the search for an ideal method for water decontamination, there has been growing interest in the use of oligodynamic metals [1]. Oligodynamic metals such as copper and zinc have been known to exhibit antimicrobial characteristics against bacteria. Studies have shown that both copper and brass (a copper-zinc alloy) exhibit antimicrobial efficacy against pathogenic bacteria such as Escherichia coli in dynamic immersion assays. Fatoba et al. have shown that brass and copper slips are able to reduce the CFU of E. coli from 180 to 0 in 120min

[3], while Varkey has shown that copper plates were able to reduce the CFU of E. coli to 0 in 60min [4]. Copper and zinc ions exhibit the oligodynamic effect as they are able to disable key proteins in the bacterial cell by bonding to them [5]. In addition, copper ions can also result in the formation of reactive oxygen species which could cause oxidative damage to molecules in the bacterial cell [6]. As a result of their efficacy as antimicrobial agents, copper and copper alloys have been used for centuries to decontaminate water.

In developing countries, water has traditionally been stored in copper alloy pots prior drinking [5] as an affordable method to decontaminate water of pathogenic bacteria. Such a method of water decontamination not only employs materials accessible in developing regions, but also prevents the problem of re-contamination.

Nanoporous Metals as a Solution for Water Decontamination

The decontamination of water has been described as a problem on a nano-scale, hence there has been much research on the use of nano-structures in the decontamination of water [1]. However, structures synthesized using complex pathways and reagents inaccessible by most in the developing world would render such proposed solutions ineffective in practice.

However, research has shown that a simple monolithic nanoporous copper structure can be synthesized from copper alloys by a simple chemical etching pathways [8-10]. Promisingly, recent reports suggest that such metal and metal containing frameworks exhibit an improved antimicrobial effect [11-12]. In nanoporous gold, it has been reported that the metal atoms on the surface of the porous structure existing in a more unstable state of low coordination and thus catalyzing the decomposition of the bacterial cell surface [11]. While there has been no research thus far on the antimicrobial efficacy of porous copper, Gao et. al report that a Cu/C nanorod material exhibits antimicrobial efficacy against certain strains of pathogenic bacteria [12]. Results from Gao et. al appear to suggest that an increased surface area is the reason for the high antimicrobial efficacy of the copper-carbon nanoscale framework [12].

This paper proposes simple synthesis method for preparing porous copper from household brass using a dilute solution of sodium hydroxide, which involves starting materials readily accessible in less developed regions. The ion release and antimicrobial efficacy of brass treated for various durations will be compared against copper, brass and zinc samples. Ion release and antimicrobial efficacy studies will be repeated for an immersion medium containing phosphate ions, to simulate water contaminated by agricultural run-off. The samples will also be subject to SEM and EDX analysis for characterization of surface properties.

MATERIALS AND METHODS

Metal Samples:

Four types of metals were investigated for their killing efficacy, zinc, copper, brass and dealloyed brass. Stainless steel was used as a negative control. Copper (99.9% purity), brass (composed of 63% copper, 37% zinc), zinc (99.9% purity) and stainless steel (AISI 304) foil sheets of 0.25 mm thickness were obtained from Goodfellow, and sectioned into samples of 1cm x 1cm.

Dealloying of Brass to Achieve Porosity

To achieve porosity, brass samples of 1cm by 1cm were immersed in 0.5M NaOH solution. Qi et. al has shown the ability of sodium hydroxide solution in removing aluminum, which like zinc can react with hydroxides in solution, from a Cu-

Al alloy [13]. The duration of the dealloying treatment was varied to investigate its effect. All samples were immersed in ethanol and sterilized in a sonicator for 15 minutes prior to use. Samples will be referred to as "dealloyed brass" in this report.

Characterisation of the Release of Copper and Zinc Ions by Metal Samples:

Disodium hydrogen phosphate Na2HPO4 was dissolved in deionised water to prepare the phosphate solution, with the addition of H3PO4 to adjust the pH to 7.12. The resultant phosphate solution had a concentration of phosphate (V) ions of 63.74 ppm as measured by the Orion Colourimeter A4000 (Thermo Fisher Scientific). Sterile LB broth was diluted 100 times by the phosphate solution, and 2.5 ml was pipetted into tubes containing either dealloyed brass, brass, zinc or copper plates, and subsequently incubated at 37°C with shaking (220 rpm). After 20 hours of immersion, a 2 ml sample of solution was extracted and diluted 5 times with deionised water. Copper and zinc ion concentration in each sample was then characterised by Atomic Absorption Spectroscopy AAnalyst 400 (Perkin Elmer). This procedure was then repeated for all the metal samples, replacing phosphate solution with deionised water as the immersion medium. All samples were conducted in triplicates.

Characterisation of the Antibacterial Efficacy of Immersed Metal Samples:

E. coli was cultivated to log phase (McFarland Tube Number 0.4 to 0.7), and was diluted 100 times with autoclaved phosphate solution. Brass, copper, dealloyed brass and stainless steel samples were immersed in the phosphate solution, with the latter acting as a negative control. The samples were incubated with shaking as described above. At 60 min, 120 min and 180 min, 100 µl was extracted and serially diluted with sterile PBS to a factor of 10-5. Three droplets of 20 µl from each dilution factor including the stock were pipetted onto sterile LB agar as per the Miles and Misra method. The plates were incubated overnight at 37°C and enumerated the next day after 16 hours. This procedure was then repeated for all the metal samples, replacing phosphate solution with deionised water as the immersion medium. All samples were conducted in triplicates.

Characterisation with Scanning Electron Microscopy (SEM) and Energy-Dispersive X-Ray (EDX) Spectroscopy:

The metal samples that were immersed in deionised water and phosphate solution for 180 minutes were imaged using SEM and the surface elemental composition was characterised by EDX (JEOL JSM-6010LA Analytical Scanning Electron Microscope) to understand the passivation behaviour of the samples during immersion in the media. The formation of passivation layers on the metal samples inhibits the release of antimicrobial metal ions from the metal surface, thus affecting the antimicrobial performance of the metals.

RESULTS

EDX and SEM Characterisation

Figures 1 and 2 show the surface of brass and dealloyed brass samples after immersion as viewed under an SEM microscope. Zinc, brass and copper samples, following immersion in deionised water and phosphate solution, were observed to have a nonporous surface under the SEM. However, as seen from Figures 1 and 2, dealloyed brass treated for 20 hours was observed to exhibit a porous structure with cavities. Dealloyed brass treated for 60 minutes did not exhibit porosity.

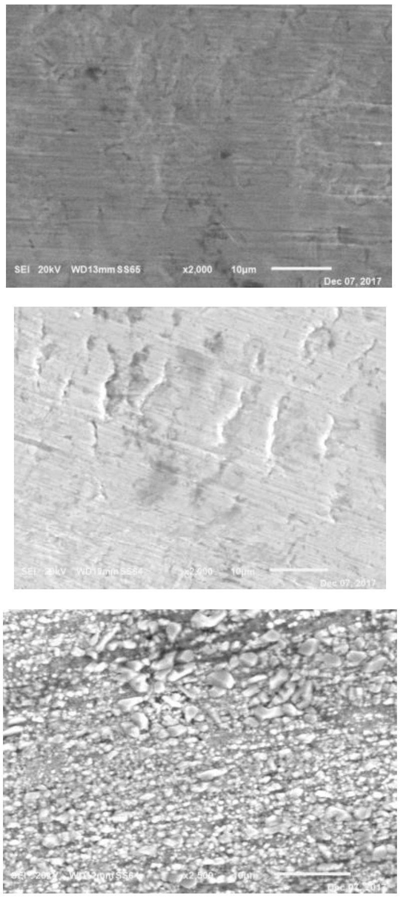

Figure 1: SEM images of (from left) brass, brass dealloyed for 60 minutes and brass dealloyed for 20 hours, imaged after immersion in deionised water for 180 minutes

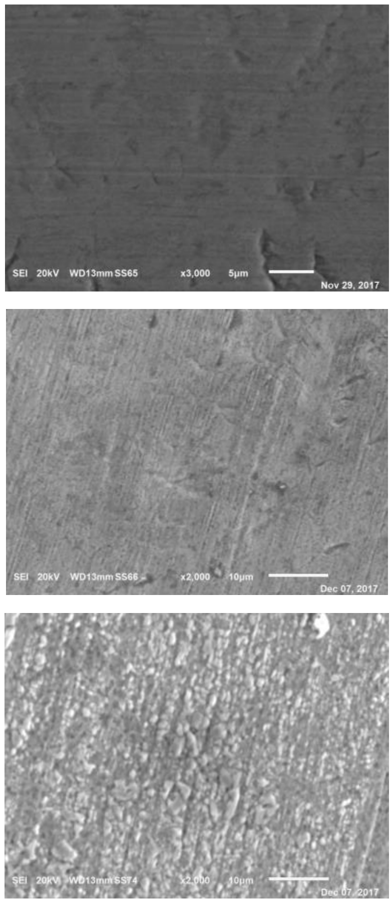

Figure 2: SEM images of (from left) brass, brass dealloyed for 60 minutes and brass dealloyed for 20 hours, imaged after immersion in phosphate solution for 180 minutes

From EDX analysis, zinc samples immersed in deionised water had 45.27% of oxygen detected on the surface. Copper samples immersed in deionised water had 4.86% oxygen detected, while copper samples immersed in the phosphate solution had 4.77% oxygen and 0.52% phosphate detected. Brass samples immersed in deionised water had 7.71% oxygen detected, and brass samples immersed in the phosphate solution had 6.58% of oxygen and 0.07% of phosphate detected. Brass dealloyed for 60 minutes immersed in deionised water had only 2.91% of zinc remaining on the surface, with 2.94% oxygen detected, while dealloyed brass immersed in the phosphate solution had no detectable zinc remaining on the surface, and 77.11% oxygen.

Release of Copper and Zinc Ions by Metal Samples

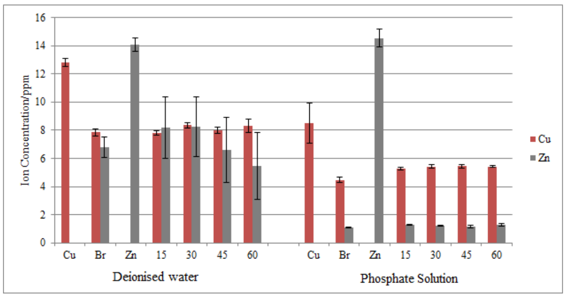

Figure 3: Concentration of copper and zinc ions released by copper (Cu), brass (Br), zinc (Zn), and brass dealloyed for 15, 30, 45 and 60 minutes in NaOH into deionised water, and phosphate solution after 20 hours of dynamic immersion

From Figure 3, in both media of immersion, copper samples released the highest concentration of copper ions, with copper ion release of 12.8ppm in deionised water and 8.5ppm in phosphate solution. In deionised water, dealloyed brass was observed to released comparable concentrations of copper ions as compared to brass, while in the phosphate solution, dealloyed brass released around 20% more copper ions than brass. For all samples, copper ion release was lower in the phosphate solution immersion medium than in deionised water. Copper samples released 50.6% more copper ions in deionised water than in phosphate solution, while brass samples released 75.1% more copper ions in deionised water than in phosphate solution.

In both deionised water and phosphate solution, zinc samples released the highest concentration of zinc ions, with 14.1ppm in deionised water and 14.5ppm in the phosphate solution. Treated brass samples exhibited similar zinc ion release to brass samples in both media of immersion. For brass and treated brass samples, release of zinc ions was lower in the phosphate solution than in deionised water. However, zinc ion release by zinc samples was comparable in the phosphate solution and deionised water.

Antimicrobial Efficacy of Immersed Metal Samples

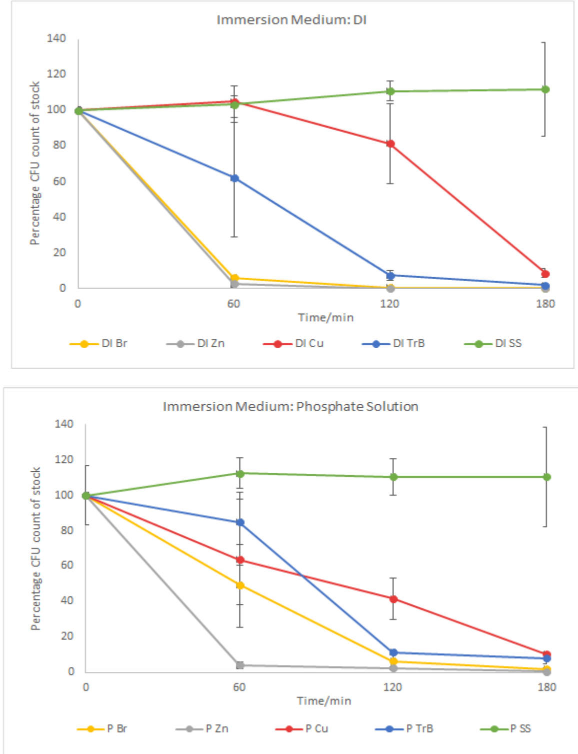

Figure 4: Antimicrobial efficacy of copper (Cu), brass (Br), zinc (Zn), and brass dealloyed for 60 minutes in NaOH (TrB) against E. coli in a deionised (DI) water immersion medium, and a phosphate (P) solution immersion medium

	Percentage decrease in CFU count for *E. coli*/%			
	Brass dealloyed in NaOH for 60 minutes		Brass dealloyed in NaOH for 20 hours	
	DI Water	P Solution	DI Water	P Solution
60 minutes	37.6	15.0	99.9	99.8
120 minutes	92.4	88.6	100	100
180 minutes	98.1	91.9	100	100

Table 1: Comparison of the antimicrobial efficacy of brass dealloyed for 60 minutes and brass dealloyed for 20 hours, in deionised water and phosphate solution immersion media

Comparing the Antimicrobial Efficacy of Metal Samples

As seen from Figure 4, in deionised water immersion medium, brass and zinc exhibited similar antimicrobial efficacy in terms of reduction in bacterial CFU count, with both brass and zinc resulting in a decrease in bacterial count of more than 90%. In deionised water, brass and zinc exhibited greater antimicrobial efficacy compared to brass dealloyed for 60 minutes, with brass dealloyed for 60 minutes exhibiting a greater antimicrobial efficacy as compared to copper. In the first 60 minutes of immersion in deionised water, copper samples did not show any observable bacterial killing. After 180 minutes of immersion in deionised water, copper, zinc, brass and dealloyed brass samples were able to reduce bacterial count by more than 90%.

From Figure 4, in the phosphate solution medium, zinc exhibited greater antimicrobial efficacy compared to the other samples, reducing E. coli CFU count by more than 95% at 60 minutes. In phosphate solution, brass exhibited greater antimicrobial efficacy than copper and brass dealloyed for 60 minutes, reducing bacterial count by more than 50% at 60 minutes. At 60 minutes, copper exhibited greater antimicrobial efficacy (-36.3%) than dealloyed brass (-15.0%) in phosphate solution, while at 120 minutes, dealloyed brass exhibited greater antimicrobial efficacy (-88.6%) than copper (-58.2%). After 180 minutes of immersion, copper, brass, zinc and dealloyed brass samples were able to achieve 90% reduction in CFU count.

From Table 1, brass dealloyed for 20 hours exhibited greater antimicrobial efficacy than brass dealloyed for 60 minutes, in both deionised water and phosphate solution media. In both deionised water and phosphate solution, brass dealloyed for 20 hours was able to reduce E. coli CFU count by more than 99% in 60 minutes.

The Effect on Phosphate Ions on Antimicrobial Efficacy

Zinc samples exhibited similar antimicrobial efficacies in deionised water and phosphate solution. In 60 minutes of immersion, zinc samples reduced bacterial count by 97.3% in deionised water and 95.9% in the phosphate solution. Brass samples exhibited greater antimicrobial efficacy in deionised water than in the phosphate solution, killing 93.8% of bacteria in deionised water after 60 minutes, but only 50.6% of bacteria in phosphate solution at the same time point. Similarly, brass dealloyed for 60 minutes was more effective at bacterial killing in deionised water than in phosphate solution. After 60 minutes of immersion, brass dealloyed for 60 minutes reduced CFU count by 37.5% in deionised water, but only 15.0% in phosphate solution. However, copper exhibited greater antimicrobial efficacy in phosphate solution than in deionised water. At 60 minutes, copper samples reduced bacterial count by 36.3%, but there was no obvious reduction in deionised water.

DISCUSSION

In this section, the antimicrobial efficacy of the metal samples first will be compared, and the effect of phosphate ions on antimicrobial efficacy will be discussed. Following which, the efficacy of the dealloying treatment method applied to brass will be evaluated.

Comparison of the Antimicrobial Efficacy of Metals

In deionised water, copper did not exhibit observable killing in the first 60 minutes of immersion. This is consistent with results from Santo et. al, suggesting that a threshold immersion time is required for copper ions in solution to reach the minimum inhibitory concentration (MIC) required for bacterial killing [14]. This initial plateau is not observed for studies which have low initial bacterial count, such as Fatoba et. al. (initial CFU/ml: 120) [3] and Varkey (initial CFU/ml: 250) [5], due to a lower MIC of copper ions required for decrease in bacterial count (rate of bacterial killing > rate of bacterial growth).

Results from this study suggest that zinc is a better antimicrobial agent than copper in dynamic immersion in both deionised water and phosphate solution. This result can be considered surprising, given the electrochemical behaviour of copper and zinc. Unlike copper, which can exist in either the +1 or +2 oxidation state, zinc is not electrochemically active, hence it is unable to catalyse the production of reactive oxygen species in the bacterial cell, a pathway proposed for the antimicrobial effect of copper [15]. Santo et. al hypothesised that the electrochemical activity of copper is the reason behind the greater antimicrobial efficacy of copper compared to zinc that was observed in his study of dry contact killing [14]. However, the results from this study appears to suggest the opposite trend in a dynamic immersion assay. In past research by the authors, this was hypothesised to be due to greater zinc ion release compared to copper ion release. However, results from AAS analysis shows that for brass samples, there is comparable release of copper and zinc ions. This suggests that against E. coli, zinc ions are more bactericidal than copper ions in solution.

The antimicrobial efficacy of brass and zinc was similar in deionised water. This could be because at 60 minutes, the first sampling timepoint, the release of zinc ions by both brass and zinc samples had reached a sufficiently high concentration to result in almost complete bacterial killing, thus resulting in a killing efficacy of greater than 90%.

Effect of Phosphate Ions on Antimicrobial Efficacy

In developing countries, water sources are often contaminated by agricultural runoff; a study by Taboada-Castro found a concentration of 28.59 mg/L of phosphate (V) ions when a water catchment was deliberately polluted, by an entry of slurry, to simulate agricultural runoff for the research [16]. Concurring with results from a prior study [17], inhibitory effects of phosphate ions were observed for the antimicrobial efficacy of brass and dealloyed brass samples. AAS analysis also shows that for both brass and dealloyed brass samples, there was decreased release of copper and zinc ions in the phosphate solution compared to in deionised water.

The inhibitory effect of phosphate ions against metal ion leaching by copper and copper alloys are well known [18-20]. However, sources differ on the passivation formed in the presence of phosphate ions. Valcarce et. al and Drogowska et. al suggest that the presence of phosphate ions would result in increased solubility of the Cu_2O passivation layer on brass and copper, favouring the formation of a more protective passivation layer of CuO which inhibits metal ion release [8, 21]. Yohai et. al further suggest that in brass, dezincification first occurs, leaving a copper rich surface which is oxidised to form a CuO passive layer. Following which, a layer of $Zn_3(PO_4)_2$ is deposited on the surface from the reaction between zinc ions and phosphate ions [18]. Another proposed inhibition mechanism is be the formation of a layer of $Cu_3(PO_4)_2$ on the copper alloy surfaces, which is more insoluble than a passivation layer of $Cu(OH)_2$ [20].

However, EDX analysis suggests that under the conditions of this study, there was no incorporation of phosphate into the passivation layer of the metal samples immersed in the phosphate solution. Hence, phosphate inhibition of brass samples are unlikely to be due to the formation of a phosphate passivation layer. Dealloyed brass exhibited a greater surface percentage of oxygen when immersed in phosphate solution than in deionised water, suggesting the formation of a thick O-rich passivation layer, concurring with the hypothesis of CuO formation proposed by Yohai et. al. A possible reason why the $Zn_3(PO_4)_2$ observed by Yohai et. al was not observed on brass in this study could be the differing medium of immersion used; Yohai et. al used an immersion medium that contained sulfates, chlorides and nitrates in addition to the phosphate corrosion inhibitor [18]. The presence of these additional ions could have affected the passivation behaviour of brass in solution.

While brass and dealloyed brass experienced phosphate inhibition of antimicrobial efficacy, the presence of phosphate ions in immersion media improved the antimicrobial efficacy of copper samples. However, AAS results show that copper released a lower concentration of copper ions in the phosphate solution than in deionised water. The apparent phosphate-enhancement of copper antimicrobial efficacy could be due a synergistic antimicrobial effect between copper and phosphate ions. Trisodium phosphate is hypothesised to be able to disrupt the cytoplasmic membrane of gram-negative bacteria through the surfactant properties of phosphate ions [21,22]; such disruption of the membrane by phosphate ions could allow easier entry of copper ions into the gram-negative E. coli bacterial cell, thus increasing susceptibility to copper toxicity. Synergistic effects between phosphates and lysozyme and nisin have been documented [21]. Phosphate-enhancement of the antimicrobial properties of brass not being observed could be due to the inhibition of metal ion release outweighing the effect of phosphate-enhancement of antimicrobial effect.

Phosphate ions did not affect the antimicrobial efficacy of zinc samples. Results from AAS analysis also shows comparable zinc ion release by zinc samples in both phosphate solution and deionised water, with slightly higher zinc ion release of around 0.5ppm in the phosphate solution. De Pauli et. al report that phosphate ions encourage zinc dissolution from zinc surfaces, as phosphate ions react with water at the zinc-water interface to generate hydroxide ions, which react with the zinc surface to form soluble zinc-hydroxide complexes [23]. The passivation film formed on zinc surfaces in phosphate solution is hypothesised to be a highly polymerised zinc phosphate layer, which is permeable and allows for diffusion of ions through the passivation layer [24]. Hence, unlike for copper and brass, the presence of phosphates in solution does not reduce ion release from zinc samples, thus zinc samples do not exhibit a phosphate inhibition of antimicrobial efficacy.

Evaluation of Treatment Method

The antimicrobial efficacy of brass dealloyed for 60 minutes was lower than that of brass and zinc in both media of immersion. This could be due to the depletion of zinc from the surface of the sample following dealloying in NaOH; results suggest that zinc is a better antimicrobial agent than copper. However, for such a short duration of dealloying treatment, the surface does not exhibit porosity (Figures 1 and 2).

From Table 1, brass dealloyed for 20 hours exhibits a greater antimicrobial efficacy compared to brass dealloyed for 60 minutes, resulting in near complete killing of bacteria after 60 minutes of immersion in both media. Under SEM imaging, brass that had undergone dealloying treatment for 20 hours exhibits a nanoporous structure on the surface while brass that had been dealloyed for only 60 minutes does not (Figures 1 and 2). Hence a longer duration of dealloying treatment such that a porous copper surface structure is attained does increase the antimicrobial efficacy of the metal. In addition, in the phosphate solution medium, brass dealloyed for 20 hours exhibits a greater percentage reduction of bacterial CFU count than untreated brass, suggesting that the porous copper structure is more resistant to inhibition by phosphate ions than the nonporous surface of brass. As agricultural runoff is likely to introduce phosphates into water sources [16], the use of porous copper storage vessels could allow for a reduction in treatment time needed for the decontamination of water.

Hence, the results of this study suggest that surface porosity of oligodynamic metals does improve their antimicrobial efficacy in dynamic immersion, making them candidates for an effective and easily-synthesized water decontamination solution. Future work can investigate the synthesis of a copper and zinc containing porous structure for water decontamination, as the results of this study suggests that zinc is a better antimicrobial agent than copper in dynamic immersion killing.

CONCLUSION

While simple chemical etching methods for the synthesis of nanoporous copper has been investigated, to the author's knowledge there has been no investigation into the antimicrobial efficacy of such structures in decontaminating polluted water. The results of this study suggests that a porous surface of copper synthesized from the dealloying of brass exhibits greater antimicrobial efficacy than a nonporous copper surface, and that the antimicrobial efficacy of the porous copper is less inhibited by the presence of phosphate ions in solution than untreated brass. The results of this study highlight the potential of simple porous metal structures, synthesized using materials that are accessible in developing regions, in the decontamination of polluted water.

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