

Zn Prolongs the Stability of Antibacterial Silver-Copper Nanoalloys

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Abstract

Addition of Zn to AgCu nanoalloys as a sacrificial anode, prolongs the stability of copper against oxidation which hampers the antibacterial characteristic of the AgCu nanoalloys. Copper oxidation was followed by X-Ray Photoelectron Spectroscopy without and after addition of Zn. The antibacterial behavior was investigated against *Escherichia coli* DH5 α strain to demonstrate the prolonged antibacterial activity of AgCu-Zn nanoclusters compared to AgCu.

Keywords

AgCu Nanoalloys; Antibacterial Nanoparticles; Sacrificial Anode; X-ray Photoelectron Spectroscopy; AgCu-Zn Nanoclusters

Introduction

Extensive studies have focused on the chemical, physical and biological properties of metallic nanoclusters and their applications. These smart nanoclusters have the advantage of easily tunable properties in terms of their electronic, optical, biological and chemical features. An assortment of methods including different techniques such as wet-chemical synthesis using various reducing agents, photo-reduction, electrochemical, microwave enhanced or biological routes are used to synthesize metallic nanoclusters [Feldheim, D. L. and Foss, C. A., 2002 - Gerbec, J. A., 2005 -Khanna, P. K., 2005 -Mukherjee, P., 2001 -Burda, C., 2005]. Moreover, surface modification of nanoclusters in solution is a commonly used technique in terms of ease in controlling reaction parameters such as temperature, pH, reducing agent or solvent without the need for expensive or complex equipment [Grouchko, M., 2009 -Henglein, A., 1993 - Reetz, M. T. and Helbig, W., 1994].

As an important issue for current research studies, single metal nanoparticles of silver, copper and zinc have been used in antibacterial applications attributable to their high surface area and activity, ability to penetrate through bacteria cell membrane causing deactivation or interaction with cytoplasmic components

and nucleic acids to inhibit respiratory enzymes. Antibacterial activity of these has been routinely tested against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus* species by different methods [Martinez-Castanon, G. A., 2008 -Panacek, A., 2006 -Qi, L., 2004 - Zhang, B., 2010 -Zhang, L., 2007 -Lok, C. N., 2007 - Stelzig, S. H., 2011 -Pang, M., Hu, J. and Zeng, H. C., 2010 -Lee, D., Cohen, R. E. and Rubner, M. F., 2005].

Unlike single metal nanoparticles like Ag, Au or Cu; synthesis, characterization and antibacterial investigation of bimetallic AgCu nanoalloys were established first time in literature in our previous work and we showed the superior antibacterial activity of bimetallic AgCu nanoalloys against *Escherichia coli* [Taner, M., 2011]. Ag and Cu metals in zero-oxidation state were synthesized through wet-chemical synthesis, by co-reduction of Ag and Cu ions using very strong reducing agent in the presence of protective agent and surfactant in solution. Because of high reactivity, relatively low reduction potential and low stability of copper under ambient conditions, disruption of AgCu nanoalloys with copper oxide formation during the synthesis and storage periods is the main issue. Furthermore, as copper oxide contaminates AgCu nanoalloys, the superior antibacterial activity of nanoclusters decreases sharply [Taner, M., 2011].

Synthesis processes are often performed in the presence of different kinds of stabilizers, oxygen free solvents, surfactants or very strong reducing agents in non-aqueous media or under inert atmosphere of nitrogen gas in order to protect copper against oxidation [Gao, F., 2009 -Grouchko, M., 2009 -Wang, H., 2004 -Wei, Y., 2010 -Huang, H. H., 1997]. However, during the storage period, copper oxide contamination becomes a matter of concern because interaction of protecting agents with water molecules increases while decreasing the protective property [Huang, H. H., 1997] and highly reactive oxygen species exist in

the solution.

Sacrificial anodes are often used to suppress the oxidation problem of metals involving metal-medium electrochemical potential, bringing the reactive metal to the immunity zone for oxide contamination [Reetz, M. T. and Helbig, W., 1994 -Rabiot, D., 1999]. For nanoparticles, rather than the oxidation by air or aqueous solutions, re-reduction of oxidized metal ions takes place straightforwardly, in the presence of sacrificial anodes [Dierstein, A., 2001 -Reetz, M. T. and Helbig, W., 1994]. Thence, with the idea of "sacrificial anode" as third metal, zinc has been added to the reduction medium of metal salts to prolong the stability of copper within the AgCu nanoalloys (NAs). Also the lower reduction potential of zinc (-0.76V) than copper (+0.34V) renders the inhibition of copper oxidation when Zn is added to AgCu nanoalloys in small amount via preferential oxidation of zinc. In addition, diminishing antibacterial activity of AgCu nanoalloys with respect to time as a result of oxide contamination of copper can be retarded with the help of AgCu-Zn ternary nanoalloy formation. This work presents the preparation and investigation of AgCu-Zn nanoalloys for prolonging stability and antibacterial property as compared to AgCu nanoalloys.

Materials and Methods

The water used in all experiments was prepared in a three-stage Millipore Milli-Q Synergy 185 purification system. Sodium citrate, zinc nitrate hexahydrate and sodium chloride were obtained from Sigma-Aldrich GmbH. Silver nitrate, copper acetate and cysteine were obtained from Fluka UK. Sodium hydroxide and hydrazine hydrate were obtained from Merck KGaA and BDH, respectively. Metal nanoparticles exhibit strong plasmon resonance extinction band in the visible spectrum in consequence of interaction between conduction electrons of metal nanoparticles and incident electromagnetic radiation [Feldheim, D. L. and Foss, C. A., 2002]. UV-visible spectroscopy that gives valuable information for samples, is the most commonly used optical technique because it is easy in application and use compared to other techniques. Double beam Thermo Scientific Evolution 160 UV-visible spectrometer was used for the optical characterization of synthesized AgCu and AgCu-Zn nanoalloys dispersed in water. Thermo Scientific K-alpha X-ray Photoelectron Spectrometer was used for further characterization of nanoalloys and nanoclusters since X-ray Photoelectron Spectroscopy

(XPS) is a very accurate technique in chemical identification, elemental analysis and determining chemical states of atoms for nanoparticle characterization [Hajati, S. and Tougaard, S., 2010 -Tunc, I., 2005 -Suzer, S., 2010 -Han, S. W., Kim, Y. and Kim, K., 1998].

One-step synthesis route for AgCu nanoalloys that was described in details in our previous work [Taner, M., 2011] was used for nanoalloys' preparation yielding no copper oxide formation as the end product. Shortly, AgCu nanoalloy synthesis route was performed after the stabilization of 0.01 M silver nitrate and copper acetate solutions by the use of 4ml mixture of 0.01 M cysteine and sodium hydroxide solutions. This stabilized reaction mixture was drop-wise and very slowly added to another reaction mixture containing 4×10^{-4} M reducing agent hydrazine hydrate (HH) and 0.01M complexing agent sodium citrate under ambient conditions and vigorous stirring. Subsequent to the 30 min reaction period dark, wine-brown color of the solution indicates the successful formation of AgCu nanoalloys without any oxide contamination. In AgCu-Zn nanoalloys' preparation for further stabilization, the same procedure described above was followed except for addition of 2.5×10^{-3} M zinc nitrate hexahydrate solution during the stabilization of silver and copper solutions. For characterization of AgCu and AgCu-Zn NAs via XPS, thin films were obtained by drop-casting and allowing for evaporation of synthesized nanoalloy solutions on microscope glass slides.

Antibacterial analysis on the AgCu and AgCu-Zn nanoalloys were achieved against *Escherichia coli* DH5 alpha strain in different methods. Using the standard dilution micro method, Minimum Inhibitory Concentration (MIC) was determined by following visual growth of bacteria (i.e. turbidity) as a result of overnight incubation in LB media supplemented with graded concentrations of nanoalloy solutions in range of 1 (corresponding to 30 µg/ml) to 1/512 (corresponding to ~60 ng/ml). The lowest concentrations at which the media remained clear at the end of the incubation time were chosen as MIC of the nanoalloy solutions. Minimum Bactericidal Concentration (MBC) represents the lowest concentration of NP or NA solutions that kills more than 99.9% of the bacteria after incubation period. MBC values were determined by plating 100 µl of LB broth, which were used for MIC analyses and had no visible bacterial growth, on agar plates containing no NAs. After 18 hours incubation at 37°C, MBC values

were selected as the lowest concentrations where no colony growth was observed on these plates.

Additionally, AgCu and AgCu-Zn nanoalloys were analyzed against *E.coli* DH5 α that were grown to optical density of 0.1 (3.5×10^8 CFU/ml) at 600nm constant wavelength. By using a Beckman DU 640 spectrophotometer, change in the optical density was examined before and after incubation periods at 37°C in order to monitor the bacterial growth. Bacteria were incubated in 10 ml liquid LB medium, under constant agitation at 225 rpm for minimizing any possible settlement or aggregation and also to ensure the constant aeration of the medium in order to facilitate the aerobic growth of bacteria. Control sets were prepared replacing AgCu or AgCu-Zn NAs with equal volumes of water. Colony forming abilities of bacteria in the presence of AgCu and AgCu-Zn NAs were determined by incubation of solid LB agar plates supplemented with different concentrations of NAs after 10^1 to 10^3 dilutions for 18 h at 37°C. *E. coli* DH5 α of 7×10^8 CFU ml $^{-1}$ with different dilutions in the range of 10^0 to 10^3 was used for colony growth. LB broth was used as carrier for all dilutions and all experiments were performed for the needed number of times in the direction of accuracy and reproducibility and all of the represented data corresponds to chosen results in duplicate manner.

Results and Discussions

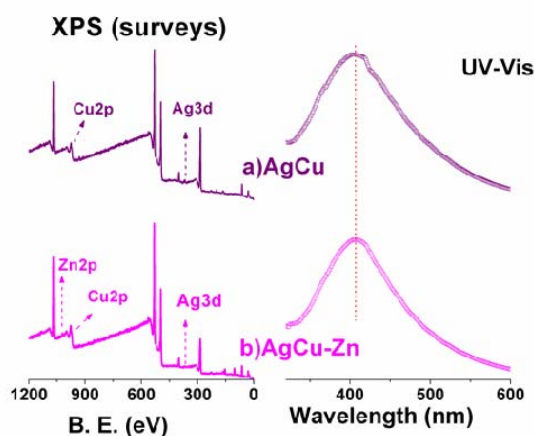


FIG. 1 XPS SURVEY AND UV-Vis ABSORPTIONSPECTRA OF AQUEOUS SOLUTIONS OF A)- AgCu BINARY AND B)-AgCuZn TERNARY NANOALLOYS

UV-visible and XPS spectra of AgCu and AgCu-Zn nanoalloys are represented in Figure 1. In the broadened peak of the AgCu NA no change was observed in its position after insertion of Zn into the binary nanoalloy. The peak at 420 nm in the absorption spectra now corresponds to the presence of

Ag, Cu and Zn atoms together in the same structure. Since small amount of Zn was used as compared to Ag and Cu (Ag:Cu:Zn = 25:25:1), Zn insertion does not cause any observable change in surface plasmon band position. As also mentioned in our previous publication, neither clear XRD pattern was observable for the binary nor the ternary alloys.

XPS spectra of AgCu and AgCu-Zn nanoalloys confirmed the presence of zero-valent silver, copper and zinc in synthesized nanostructures due to observed Ag3d, Cu2p and Zn2p doublets in the nanoalloys' survey spectrum. Because of the fact that relatively small amount of zinc was used for the synthesis process as compared to silver and copper, the intensity of the peaks in the survey spectrum is low, consistent with the synthesis conditions.

The rapid reduction of silver and copper ions by the strong reducing agent, zero-valent copper in AgCu nanoalloys was obtained without copper oxide formation. But during the storage, and under ambient conditions the Cu 0 atoms in the nanoalloy get oxidized to Cu $^+$ or Cu $^{2+}$ species due to high reactivity of copper as a result of its low reduction potential [Gao, F., 2009 - Grouchko, M., 2009 -Khanna, P. K., 2007 -Ohde, H., Hunt, F. and Wai, C. M., 2001]. The oxide contamination of copper particles can easily be followed by the color change of the synthesized nanoalloy solution. Fresh AgCu nanoalloy solution's color is dark wine-brown but when it is contaminated by oxide formation, color of the solution turns to very light yellow indicating that copper is oxidized from zero to +2 oxidation state [Huang, H. H., 1997 -Wang, H., 2004] (Figure 2). However, after the same storage period with AgCu NAs under the same conditions, AgCu-Zn nanoalloy solution's color stays darker because of the prevented oxide contamination.

Reduced and non-reduced species are followed by characteristic XPS binding energies, and peak shapes [Yoon, B. and Wai, C. M., 2005 -Xue, X., Wang, F. and Liu, X., 2008]. Cu2p region of XPS spectrum has strong indications for the chemical state of copper (Cu 0 or Cu $^+$ and Cu $^{2+}$) with characteristic shake-up satellite structure for the oxides [Jernigan, G. G. and Somorjai, G. A., 1994]. Accordingly, the effect of addition of zinc to the AgCu NAs on copper oxide formation during the storage period can easily be monitored by the Cu2p region. Figure 3 represents the Cu2p region of AgCu and AgCu-Zn NAs' XPS spectra, recorded, right after preparation, after 7, and 14 weeks. Fresh nanoalloys' Cu2p region contains only copper in zero

oxidation state for both AgCu and AgCu-Zn NAs. After 7 weeks, copper oxide formation in AgCu NAs can be distinguished easily by the oxide satellites formation and broadening of the main peaks due to change in its oxidation state, whereas AgCu-Zn NAs do not contain oxide satellites or broadened peaks for the same period. However, after 14 weeks of storage, it was observed that both of AgCu NAs and AgCu-Zn NAs (to a much lesser extent) have oxide contamination.

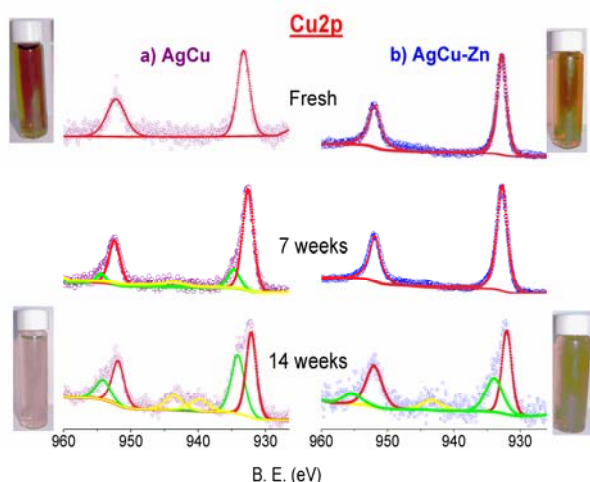


FIG. 2 Cu2p REGION OF XPS SPECTRA OF A)- AgCu BINARY AND B)-AgCuZn TERNARY NANOALLOYS RECORDED IN FRESH, AFTER AND 14 WEEKS

These results are consistent with the presence of a “sacrificial anode” [Reetz, M. T. and Helbig, W., 1994 - Shibli, SMA, Jabeera, B and Manu, R, 2007 -Dierstein, A., 2001 -Jabeera, B, Anirudhan, TS and Shibli, SMA, 2005] and are also the strong evidence for prolonged stability of copper against oxidation when Zn is added to the nanostructure as re-reduction of oxide contaminated copper ions into zero-valent copper takes place due to the rapid oxidation of Zn, furnishing anodic protection.

Novel strong antibacterial action of AgCu NAs was attested before, by confirming not only the bacteriostatic effect but also the bactericidal activity against *E. coli* [Taner, M., 2011], which is required for irreversible inhibition of bacterial growth in the presence of synthesized NAs. Likewise, the antibacterial nanoalloys composed of Ag, Cu and Zn possess similar dual antibacterial action. Figure 3 reveals the growth curves of bacteria grown in the presence of freshly prepared or 14-weeks-old AgCu (30 µg/ml) and AgCu-Zn (30 µg/ml) ternary nanoalloys, compared to water replaced control groups. In this non-destructive and fast technique,

absorbance at 600 nm optical density directly relates to the bacterial cell numbers, and thus, quantitating the number of bacteria in the solution at any given time, under desired conditions, such as in the presence of nanoalloys.

The dual antibacterial action of both freshly prepared AgCu and AgCu-Zn ternary NAs is apparent in Figure 3a, depicting both bacteriostatic property and irreversible inhibition of the bacterial growth, which are directly related to the concentration of nanoalloys as well as to the initial number of bacteria. Relatively high bacterial concentration, 3.5×10^8 CFU/ml which is very rarely found in real-life systems was used in antibacterial investigations as well, to demonstrate the high effectiveness of the synthesized nanoalloys and their superior activity in potential practical application. When bacterial growth profiles in fresh and 14-weeks-old nanoalloys were compared (Figure 3a and 3b), a sharp decline in antibacterial activity was clearly observable in the case of AgCu nanoalloys resulting from oxide contamination during the 14 weeks storage period. Likewise, Ruparelia and co-workers [Ruparelia, J. P., 2008] showed superior antibacterial activity of nano-sized copper particles over oxide contaminated ones against different kinds of bacteria. Hence, higher concentrations of nano-copper oxide particles are needed to obtain similar antibacterial efficiency with copper-only nanoparticles [Ren, Guogang, 2009]. Notwithstanding the decrease in antibacterial activity of AgCu-Zn ternary nanoalloys, they were still more effective in inhibition of bacterial growth, consistent with the lower oxide contamination of AgCu-Zn ternary nanoalloys than of AgCu nanoalloys.

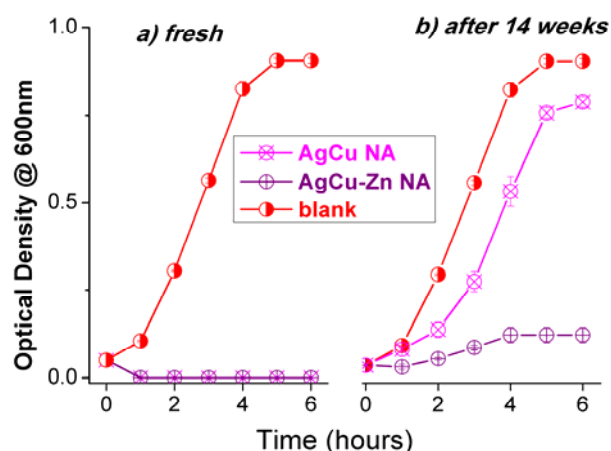


FIG. 3 REPRESENTATIVE BATCH GROWTH PROFILES IN THE PRESENCE OF AgCu AND AgCuZn NANOALLOYS WITH CONCENTRATION AT 30 µg/ml A)- FRESH B)-AFTER 14 WEEK. THE ERROR BARS REPRESENTS THE STANDARD DEVIATION OF REPLICATE EXPERIMENTS

The relative antibacterial characteristics of freshly prepared, 7-weeks-old and 14-weeks-old AgCu and AgCu-Zn nanoalloys were assessed by determining the MIC values of NAs in liquid LB, where lower MIC values correspond to the better antibacterial activity [Andrews, Jennifer M, 2001]. Absence of visual growth of bacteria in LB indicates the effective antibacterial property for nanoalloys. In order to distinguish complete bactericidal effect, Minimum Bactericidal Concentration (MBC) of nanoalloys was determined as described [Andrews, Jennifer M, 2001 -Taylor, PC, 1983]. If the NAs do not kill 100% of the bacteria, but only inhibit the growth of them, recovery of bacteria colonies should be observed on the agar plates. The results are presented in Table 1.

TABLE 1 COMPARISON OF THE ANTIBACTERIAL ACTIVITY (MIC AND MBC VALUES) OF FRESH, 7WEEKS-OLD AND 14WEEKS-OLD SILVER-COPPER NANOALLOYS AND SILVER-COPPER-ZINC TERNARY NANOALLOYS TOWARDS *E. COLI* DH5-ALPHA

Samples	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)
Fresh AgCu	0.5	0.5
7 weeks-old AgCu	1.0	1.0
14 weeks-old AgCu	2.0	2.0
Fresh AgCu-Zn	1.0	1.0
7 weeks-old AgCu-Zn	0.5	0.5
14 weeks-old AgCu-Zn	1.0	4.0

Additionally, as consistent with the MIC values and bacterial growth curves, Table 2 represents the colony forming abilities of different densities of bacteria in different concentrations of fresh and old (14 weeks) AgCu and AgCu-Zn NAs, as the number of colonies on each agar plate. The results indicate that, the observed antibacterial activities of the NAs are weaker on solid media than in liquid media (comparing Table 1 and Table 2). This, though, might be expected since the growth properties of *E.coli* might differ on solid media and in liquid media. It can be anticipated that in the liquid media, access of nanoparticles to the bacteria will be greater than that on the agar surface. Moreover, the MBC represents the concentration at which 99.9% of the bacteria are killed, which leaves a number of bacteria in the media alive, and these cannot be detected via inspection with eye or spectrophotometry, and may form colonies when plated over solid media without NAs. Since the experiments regarding colony forming abilities of bacteria start with direct plating of a high number of bacteria (i.e. 1.7×10^4 to 1.7×10^5) over the plates, the remaining living bacteria might form countable number of colonies. In fact, 417 colonies, which are counted in presence of 3 $\mu\text{g/ml}$ of AgCuZn, account for 1% of the initial number of bacteria. Combined with lesser accessibility of NAs to the bacteria, the

results seem reasonable. When MIC values of freshly prepared AgCu and AgCu-Zn NAs are compared, it was seen that fresh AgCu NAs with MIC at 0.5 $\mu\text{g/ml}$ were more effective than fresh AgCu-Zn with MIC at 1 $\mu\text{g/ml}$ ternary NAs. On the other hand, when 7 and 14-weeks-old samples' MIC values were compared, it is clear that effectiveness of AgCu NAs was declined due to oxide contamination. But while AgCu-Zn NAs show less antibacterial activity at the beginning of storage period, they were more active and have lower MIC value of 0.5 $\mu\text{g/ml}$ than their AgCu counterparts in terms of antibacterial activity at the end of 7 weeks. This phenomenon can be explained by the prolonged stability of ternary nanoalloys with the incorporation of Zn and the formation of strong and tough antibacterial zinc-oxide species [McConnell, W.P., 2000 -Zhang, L., 2007]. At the end of 14 weeks, although AgCu-Zn ternary NAs have a decline in their antibacterial activity, these particles are still more effective than AgCu NAs, consistent with the XPS results, where more copper oxide formation was detected for AgCu binary alloys.

TABLE 2 EFFECT OF DIFFERENT CONCENTRATIONS OF NANOPARTICLE AND NANOALLOYS ON DILUTION SERIES OF *E. COLI* DH5-ALPHA

NAs	DF	Number of colonies for given dilution factors (DF)			
		1	10^1	10^2	10^3
Fresh AgCu (30 $\mu\text{g/ml}$)		0	0	0	0
AgCu (3 $\mu\text{g/ml}$)		0	0	1	0
AgCu (0.3 $\mu\text{g/ml}$)		972	201	32	10
AgCu-Zn (30 $\mu\text{g/ml}$)		0	0	0	0
AgCu-Zn (3 $\mu\text{g/ml}$)		147	24	0	4
AgCu-Zn (0.3 $\mu\text{g/ml}$)		1332	84	38	4
14 Weeks Old AgCu (30 $\mu\text{g/ml}$)		0	1	0	0
AgCu (3 $\mu\text{g/ml}$)		417	17	3	2
AgCu (0.3 $\mu\text{g/ml}$)		1984	194	64	23
AgCu-Zn (30 $\mu\text{g/ml}$)		1	0	0	0
AgCu-Zn (3 $\mu\text{g/ml}$)		347	5	3	2
AgCu-Zn (0.3 $\mu\text{g/ml}$)		1224	225	36	15
No NAs		2240	279	46	34

Comparison of the bactericidal effectiveness of the nanoalloys (MBC) revealed that the concentrations at which the bacteria growth was inhibited, were also adequate to irreversibly inhibit the growth of 100% of the bacteria in the growth medium, while the 14 weeks old AgCu-Zn ternary nanoalloys inhibited observable bacterial growth effectively at lower concentrations compared to AgCu, the bactericidal effect of the

former diminishes, and remained behind the latter.

Conclusions

Silver-copper nanoalloys were synthesized by co-reduction of their metal salts in aqueous media without any copper oxide contamination and showed superior antibacterial characteristics against *E.coli* DH5 α strain. But during the storage period antibacterial activity of AgCu nanoalloys declines as a result of copper oxide formation. In order to prolong the stability of copper in nanoalloys and antibacterial effectiveness, zinc was added to the nanoalloys as sacrificial anode. AgCu-Zn ternary nanoalloys lengthen the stability of copper against oxidation and prolong the antibacterial effectiveness as compared to AgCu counterparts.

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