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Published in final edited form as:

Chem Res Toxicol. 2015 September 21; 28(9): 1815–1822. doi:10.1021/acs.chemrestox.5b00258.

Concentration Ranges of Antibacterial Cations for Showing the Highest Antibacterial Efficacy but the Least Cytotoxicity against Mammalian Cells: Implications for a New Antibacterial Mechanism

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Abstract

Antibacterial metal ions, such as Ag^{+}, Zn^{2+} and Cu^{2+}, have been extensively used in medical implants and devices due to their strong broad spectrum of antibacterial activity. However, it is still a controversial issue as to whether they can show the desired antibacterial activity while being toxic to mammalian cells. It is very important to balance their antibacterial effectiveness with minimal damage to mammalian cells. Toward this end, this study is to identify the suitable concentrations of these three ions at which they can effectively kill two types of clinically relevant bacteria (*Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*)) but show no obvious

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Notes

The authors declare no competing financial interest.

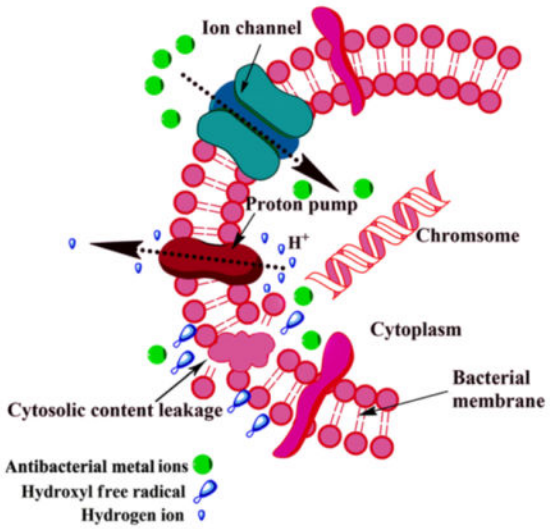
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00258.

Live and dead bacterial staining assay and cell morphology of L929 fibroblasts cocultured with different concentrations of antibacterial metal ions (PDF)

cytotoxicity on fibroblasts. Such concentration ranges are found to be 2.5×10^{-7} M–10^{-6} M, 10^{-5} M–10^{-4} M, and 10^{-5} M–10^{-4} M for Ag^{+}, Zn^{2+}, and Cu^{2+}, respectively. Investigation of their antibacterial mechanism shows that these three metal ions all show antibacterial property through a mechanism of damaging bacterial cell membranes by the generation of reactive oxygen species but surprisingly preserving the integrity of bacterial genomic DNA. The encouraging results indicate that antibacterial metal ions with controlled concentrations can bring considerable benefits to biomedical applications.

Graphical Abstract



INTRODUCTION

Antibacterial metal ions such as silver (Ag^{+}), zinc (Zn^{2+}), and copper (Cu^{2+}) are well known for their strong broad-spectrum antibacterial activity.[–] They have been widely used in *in vitro* and *in vivo* applications. For example, silver ions as well as silver nanoparticles can be exploited in medicine for prostheses, dental materials, catheters, wound dressing, textile fabrics, and water treatment. In addition, zinc ions also possess excellent antibacterial ability. Liu et al. found that Zn-incorporated TiO_2 coatings could inhibit the growth of both *S. aureus* and *E. coli*. Furthermore, zinc ions have been proven to have antibacterial activity in dental materials, while their salts have been incorporated into mouthwashes for the treatment of gingivitis.[,] Recently, there is an increasing interest in the use of copper as a sanitizing material. Moreover, copper salts have been incorporated into mouthwashes and toothpastes as antimicrobial agents for the treatment of gingivitis.

However, the relationship between antibacterial properties and the cytotoxicity of antibacterial metal ions has been found to vary, and the antibacterial mechanism of the metal ions is not well understood.[–] There has been no consistent data on the concentration range of antibacterial metal ions that can kill bacteria without impairing mammalian cell functions.[–] Thus, although antibacterial metal ions, such as silver, zinc, and copper ions, are well used

as antibacterial agents, the questions of clinical effectiveness and damage to mammalian cells and tissue were a subject of continuing controversy.[–] Therefore, a lot of attention has been paid to search for antimicrobial agents at concentration ranges below their cytotoxic level but above their antimicrobial minimum inhibitory concentrations (MICs).

In this work, the antibacterial activity of metal ions with different concentrations against *S. aureus* and *E. coli* was studied by the plate colony-counting method and Live/Dead bacterial staining assay. The cytotoxicity of antibacterial metal ions with different concentrations were assessed by an *in vitro* cell proliferation assay and cell morphology observation. We also found antibacterial metal ions in certain concentrations possessed good antimicrobial activity without cytotoxicity. To investigate the antibacterial mechanism, we have measured the production of reactive oxygen species (ROS) in the presence of antibacterial metal ions and used agarose gel electrophoresis for determining deoxyribose nucleic acid (DNA) degradation and a transmission electron microscope (TEM) for examining bacterial membrane damage. The results showed that the production of ROS and the consequent membranes damage might be the major mechanisms of the bactericidal activity of antibacterial metal ions. Surprisingly, we found that the genomic DNA of the bacteria was not damaged by the antibacterial metal ions, although the bacterial membrane was damaged to lead to bacterial death. These results provide the great potential applications of antibacterial metal ions in certain concentration ranges as candidate antibacterial agents for biomedical applications.

MATERIALS AND METHODS

General Materials and Methods

Silver nitrate ($AgNO_3$, Bio Reagent), zinc chloride ($ZnCl_2$, Bio Reagent), and copper chloride ($CuCl_2.2H_2O$, Bio Reagent) were purchased from Sigma-Aldrich, and serial dilutions in purified water were made to avoid silver salt precipitation. The solution of antibacterial metal ions was prepared and filtered using a sterile, single-use filter of 0.22 μm (Millex-GS, Millipore, France). Other biological agents, such as tryptone and yeast extract, were supplied by Oxoid Ltd. *Staphylococcus aureus* (*S. aureus*, ATCC 25923) and *Escherichia coli* (*E. coli*, *ATCC 25922*) were obtained from Guangdong Provincial Key Laboratory of Microbial Culture Collection in China. Mouse fibroblast cells L929 (NO. CCL-1) were purchased from ATCC Cell Biology.

Antibacterial Activity Test of Ag^+, Zn^{2+}, and Cu^{2+}

The antibacterial properties of Ag^+, Zn^{2+}, and Cu^{2+} ions were evaluated using Gram-positive *S. aureus* and Gram-negative *E. coli*, which were cultivated in lysogeny broth (LB) medium (1% w/v tryptone, 0.3% w/v yeast extract, and 0.5% w/v NaCl) under shaking at 200 rpm at 37 °C for 12 h. The bacteria were adjusted to a concentration of 10^6 CFU/mL in the antibacterial assay. In the bactericidal rate test, the concentrations of silver, zinc, and copper ions were varied from 10^{-2} M to 10^{-8} M. Then, a 100 μL solution of metal ions was introduced to 900 μL of bacteria suspension. The resultant suspensions were incubated in a biochemical incubator at 37 °C for 24 h. The viable number of bacteria in the double distilled water was quantified by standard serial dilution. A culture suspension of 100 μL

from each tube was uniformly spread on the LB agar plates, and the number of viable bacterial colonies was counted after incubation at 37 °C for 24 h. To quantify the antibacterial ability, the bactericidal rate was calculated based on the following equation:

$$\text{antibacterial rate}(\%) = (N_{\text{control}} - N_{\text{solution}})/N_{\text{control}} \times 100$$

where N_{control} is the average number of bacteria in the control sample (CFU/sample), and N_{solution} is the average number of bacteria in the testing samples (CFU/sample).

Cell Culture and Cell Proliferation

The cell culture media were composed of HDMEM (4.5 g/L glucose, Gibco, USA) containing 10% fetal bovine serum (FBS, Sigma, USA). The medium was replaced every other day. The cells were cultured in 25 cm^2 flasks (Corning Incorporated, USA) at 37 °C in an atmosphere of 5% CO_2 at 95% humidity. To assess the cell proliferation, a 100 μL suspension of fibroblast cells L929 was seeded in a 96-well plate at a density of 2×10^4 cells/cm^2 and then cultured in HDMEM with 10% FBS. After 24 h, a 100 μL solution of metal ions was added into each well. After the cells were cultured for 1, 3, and 5 days, cell proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, at each prescribed time point, the solution was gently transferred to a new 96-well plate. The MTT solution was added to the antibacterial metal ions solution and incubated at 37 °C to form formazan. The formazan was then dissolved using dimethyl sulfoxide (DMSO, sigma, USA), and the optical density (OD) was measured at 490 nm using a microplate reader (Multiskan Go, Thermo Scientific, USA).

Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS level in the bacterial cells was measured by the Intracellular ROS Assay Kit (Green Fluorescence, Beyotime, China). Bacteria and antibacterial metal ions were cocultured in an incubator at 37 °C for 24 h. The blank group was prepared similarly without metal ion treatment. Later, 20 μM DCFH-DA was added into the mixed solution in an incubator at 37 °C for 30 min. H2-DCFDA is a cell-permeant indicator for ROS that is nonfluorescent and deacetylated by cellular esterases to form nonfluorescent 2′,7′-dichlorodihydrofluorescin (DCFH). The DCFH can be rapidly oxidized into highly fluorescent 2′,7′-dichlorofluorescein (DCF) by ROS. Stained cells were washed with PBS twice. Fluorescence was analyzed on an inverted fluorescence microscope (Olympus BX51, Japan) with a 485/538 nm filter set and 530 nm cut off.

Extracting Bacterial DNA and Agar Gel Electrophoresis

Five milliliter bacterial broths treated by metal ions were centrifuged at 10000 rpm for 1 min. Then, 400 μL of lysosome was added to *S. aureus* at 20 °C and 600 rpm for 1 h. Genomic DNA was extracted using TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). The concentration and purity of the bacterial DNA were detected by Nano-Drop 2000 (Thermo Fisher Scientific, USA). Agarose gel was prepared with 1× TAE buffer, and ethidium bromide (5 μL per 100 mL of the gel) was used to monitor the size of the analyzed

fragment. Electrophoresis (Bio-Rad, Hercules, CA, USA) was run at 60 V for 30 min. The bands were visualized by a UV transilluminator (Bio-Rad, GDS-7500, USA).

TEM Sample Preparation

To directly observe the morphological changes of the internal structure of bacterial cells after treatment by silver, zinc, and copper ions, TEM (Hitachi H-7650, Japan) was employed. The collected bacteria treated by antibacterial metal ions were fixed with 2% glutaraldehyde and 1% osmium tetraoxide at room temperature. Then, the dehydration process was conducted with 30%, 50%, 70%, 80%, 95%, and 100% of alcohol. The fixed bacteria were embedded with Epon 812 at 37 °C overnight. The blocks of bacteria in the Epon were cut with an ultratome to form ultrathin sections, which were then observed by TEM.

Statistical Analysis

The assays were performed in triplicate, and data were expressed as the means ± standard deviations. The statistical analysis was done by using a Graph Pad Prism statistical software package 5. The statistical significance of the difference was measured using one-way analysis of variance. $p < 0.05$ was regarded as statistical difference and $p < 0.01$ was considered as significant difference.

RESULTS AND DISSCUSION

Minimal Inhibition Concentrations (MICs) and Cytotoxicity of Ag^+, Zn^{2+}, and Cu^{2+}

The antibacterial rate against *S. aureus* and *E. coli* and the cell proliferation results on the fibroblast cells L929 in the presence of Ag^+, Zn^{2+}, and Cu^{2+} were evaluated, and the results are shown in Figure 1. In the bactericidal rate test, the MICs of silver ions against *S. aureus* and *E. coli* were both 10^{-7} M, as shown in Figure 1A on the left. The L929 cell proliferation rate in the presence of silver ions was increased with decreasing Ag^+ concentrations (Figure 1A, right). The results indicated that when the concentration of silver ions was 10^{-6}–10^{-7} M, they had certain antimicrobial activity without cytotoxicity. According to the same analytical method, the MICs of zinc ions were determined to be 10^{-7} M, and the cell proliferation rate was over 80% at a concentration below 10^{-4} M of Zn^{2+} concentration, as shown in Figure 1B. Therefore, the optimal concentration range of zinc ions was 10^{-4}–10^{-6} M. Similarly, the best concentrations range of Cu^{2+} ions were 10^{-4}–10^{-5} M (Figure 1C). Hence, by regulating the metal ions concentrations, we can choose proper metal ions, which can be against various bacteria *in vitro* (Figure S1) but do not damage mammalian cells (Figure S2).

Minimum Bactericidal Concentrations (MBCs) of Ag^+, Zn^{2+}, and Cu^{2+}

The MBCs were tested using the viable cell counting method. The results for the MBCs of silver, zinc, and copper ions are depicted in Figure 2. The results demonstrated that 0.25 μM silver ions can kill 99% of *S. aureus* and *E. coli* in 24 h of contact, i.e., the MBC of the silver ions was 0.25 μM. No viable bacteria were observed with 10 μM zinc ions, i.e., the MBC of the zinc ions against both *S. aureus* and *E. coli* was 10 μM (Figure 2B). According to the

same calculation method, the MBC of copper ions against *S. aureus* and *E. coli* was 25 μM and 10 μM, respectively (Figure 2C).

Agarwal et al. have reported that the concentration of silver nitrate solution from 0.05 mM to 5 mM killed over 90% of the bacteria. Wen et al. found that the zinc concentrations from 0.1 μM to 10 μM measured in the liquid media possessed an optimal microbial growth *in vitro*. It has been reported that on the titanium implants, which released galvanically deposited copper at concentrations ranging from 0.3 mM to 1.75 mM, growth of planktonic *S. aureus* was blocked. These results are all in agreement with the MICs and MBCs of antibacterial metal ions discovered by us. Our results indicate that controlling the concentrations of antibacterial metal ions is very important. Hence, when metal ions are released from nanoparticles, controlling the release kinetics is of great importance for achieving the best antibacterial effect but minimizing the toxicity to mammalian cells.[–]

In fact, the best optimal concentrations of antibacterial metal ions need to have more than 90% of antibacterial rate and over 80% of cell proliferation rate on L929. The results (Figures 1 and 2) showed that the silver ions in the range of 2.5×10^{-7} M–10^{-6} M, zinc ions in the range of 10^{-5} M–10^{-4} M, and copper ions in the range of 10^{-5} M–10^{-4} M resulted in over 90% of antibacterial rate and no cytotoxicity on L929.

Antibacterial Mechanism of Ag^{+}, Zn^{2+}, and Cu^{2+}

To better understand the antibacterial properties of metal ions and to ascertain whether the cell membranes were damaged by metal ions, the antibacterial mechanism of silver, zinc, and copper ions was investigated by using an ROS assay kit, TIANamp Bacteria DNA kit, and TEM characterization.

Lim et al. proposed that the mechanism of microorganism inhibition involved the entry of heavy metal ions (Ag^{+}, Zn^{2+}, Cu^{2+}, etc.) to the metabolic system of an organism with consequent formation of toxic secondary metabolites. Furthermore, some changes in bacterial genetic information such as in the gene 16S rRNA could occur due to the presence of heavy metals. 16S rRNA, conferred by 16S rDNA, is one of the components of small subunit of prokaryotic ribosomes. Recently, it was reported that the antibacterial activities of antibacterial metal oxides were related to the formation of ROS.[,] Ag^{+}, Zn^{2+}, and Cu^{2+} as transition metal ions, could induce a Fenton reaction, resulting in the production of ROS. Leong et al. found that the ROS generated by the metal nanoparticles could induce the damage to the bacterial cell membrane and human cells. Their work further indicates that ROS formation is one possible antibacterial mechanism of the metal ions.[,]

A high ROS level can increase oxidative stress in cells, which can damage both the cell membranes and the proteins.[,] Hence, we measured intracellular ROS formation in the presence of antibacterial metal ions by using 2′,7′-dichlorodihydrofluorescein diacetate (H2-DCFDA) as a fluorescence staining probe (Figure 3). We examined the intracellular ROS production after 1 h of incubation. Fluorescence images of ROS in *S. aureus* and *E. coli* treated with antibacterial metal ions (Figure 3) showed that the green fluorescence intensity of *S. aureus* increased when they were treated by the antibacterial metal ions. The green fluorescence intensity of *E. coli* has a similar trend (Figure 3). The prevailing hypothesis is

that antibacterial metal ions produce ROS, leading to oxidative damage to cell membranes and proteins and subsequently resulting in cell death. There may be other possible mechanisms of damaging the membranes. For example, metal ions could interact with the respiratory and transport proteins, causing their deactivation and subsequent damage of cell membranes. Bacterial cells exposed to metal ions could also undergo morphological and physiological changes such as cytoplasm membrane detachment from cell membranes. In addition, the metal ions themselves could destroy the electrolytic balance around the membranes, leading to the damage of cell membranes.

Genomic DNA from *S. aureus* and *E. coli* with antibacterial metal ions was extracted using a TIANamp bacteria genomic DNA extraction kit. The agar gel electrophoresis profiles for direct analysis of genomic DNA are shown in Figure 4. The analysis of agar gel electrophoresis demonstrated that antibacterial metal ions could not induce the decomposability of genome DNA. However, it should be noted that our agar gel electrophoresis analysis cannot exclude the possible base damage by the antibacterial ions.

Previous studies showed that metal nanoparticles, such as silver nanoparticles,[,] zinc oxide nanoparticles, and copper oxide nanoparticles could lead to the degradation of bacterial DNA, which further resulted in the death of bacteria. The comparative results showed that antibacterial metal ions did not damage bacterial DNA as the nanoparticles but maintained the integrity of genome DNA.

The morphological change in *S. aureus* and *E. coli* cells caused by antibacterial metal ions was examined by TEM. Figure 5 shows the TEM images of *S. aureus* and *E. coli* treated and untreated with antibacterial metal ions for 12 h. It is observed that cells were typically grape-shaped in the control group and that the cell membranes were intact. However, *S. aureus* treated with antibacterial metal ions exhibited morphological changes, and the structure of the cell membranes was damaged. Figure 5 also shows that *E. coli* cells of the control group were indeed rod-shaped and that their cell membranes were intact. However, in the presence of antibacterial metal ions, there were many fragments on the cell surface, and the structure of the cell membranes was significantly damaged. This research showed that antibacterial metal ions changed not only the morphology of the cell surface but also resulted in the formation of cell fragments through the damage of cell membranes. The permeability of cell membranes or leakage of cell contents could also be caused by ROS. However, there are some differences in the morphological destruction caused by antibacterial metal ions between Gram positive *S. aureus* and Gram negative *E. coli*. *S. aureus* showed slightly less bactericidal activities than *E. coli*. This difference was possibly attributable to the difference of the peptidoglycan layer of the bacterial cells between Gram positive *S. aureus* and Gram negative *E. coli*.

Figure 6 schematically illustrates the antibacterial process of the antibacterial cations. The antibacterial cations can enter the cells through the ion channels across the bacterial cell membranes, which in turn induce the formation of ROS such as hydroxyl free radicals, as confirmed by the fluorescent ROS staining assay (Figure 3). The ROS increases the oxidative stress in the cells and the permeability of the cell membranes, resulting in the penetration and disruption of the bacterial cell membranes and the subsequent breaking of

the cells into fragments that can be observed by TEM (Figure 5). However, surprisingly the ROS generated by the antibacterial cations does not destroy the bacterial genomic DNA (Figure 4). The detailed mechanism of the antibacterial effect of antibacterial metal ions still awaits further exploration. Nevertheless, our investigation provides a direction in the design of new and more effective antibacterial agents, which might help combat emerging pathological bacteria. In essence, our present study demonstrates a new antibacterial mechanism of antibacterial metal ions against *S. aureus* and *E. coli* mainly by the formation of ROS but not by destroying the genomic DNA.

CONCLUSIONS

This study identified the concentration range of antibacterial metal ions that allow the metal ions to possess excellent antibacterial activities against *S. aureus* and *E. coli* while not showing cytotoxicity on fibroblasts. This work indicates that antibacterial metal ions can penetrate the cell membranes and disrupt the cell membranes but preserve the integrity of bacterial genome DNA, subsequently killing the bacterial cells. The stronger antibacterial activities of metal ions result from ROS generation. As a future direction, one of the alternative methods of studying the antibacterial properties and cytotoxicity of metal ions released from medical implants will be to coculture the bacteria with the 3D cell spheroids in the presence of metal ions to mimic the case of medical implants.[–] Our work will enable the exploration of proper antibacterial agents for biomedical devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

We gratefully acknowledge the financial support of National Basic Research Program of China (Grant No. 2012CB619100) and the National Natural Science Foundation of China (Grant Nos. 51372087, 81271957, 51232002 and 51072057). Y. Zhu and C.B.M. thank National Institutes of Health (EB015190), National Science Foundation (CMMI-1234957), Department of Defense Congressionally Directed Medical Research Program, Oklahoma Center for Adult Stem Cell Research (434003), and Oklahoma Center for the Advancement of Science and Technology (HR14-160) for financial support.

ABBREVIATIONS

S. aureus	*Staphylococcus aureus*
E. coli	*Escherichia coli*
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
MICs	minimum inhibitory concentrations
MBCs	minimum bactericidal concentrations
ROS	reactive oxygen species

TEM	transmission electron microscope
ATCC	American Type Culture Collection
LB	lysogeny broth
CFU	colony forming units
HDMEM	high glucose Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	dimethyl sulfoxide
DCFH	2′,7′-dichlorodihydrofluorescin
DCF	2′,7′-dichlorofluorescein
PBS	phosphate buffered saline
TAE	tris-acetic acid
3D	three-dimensional

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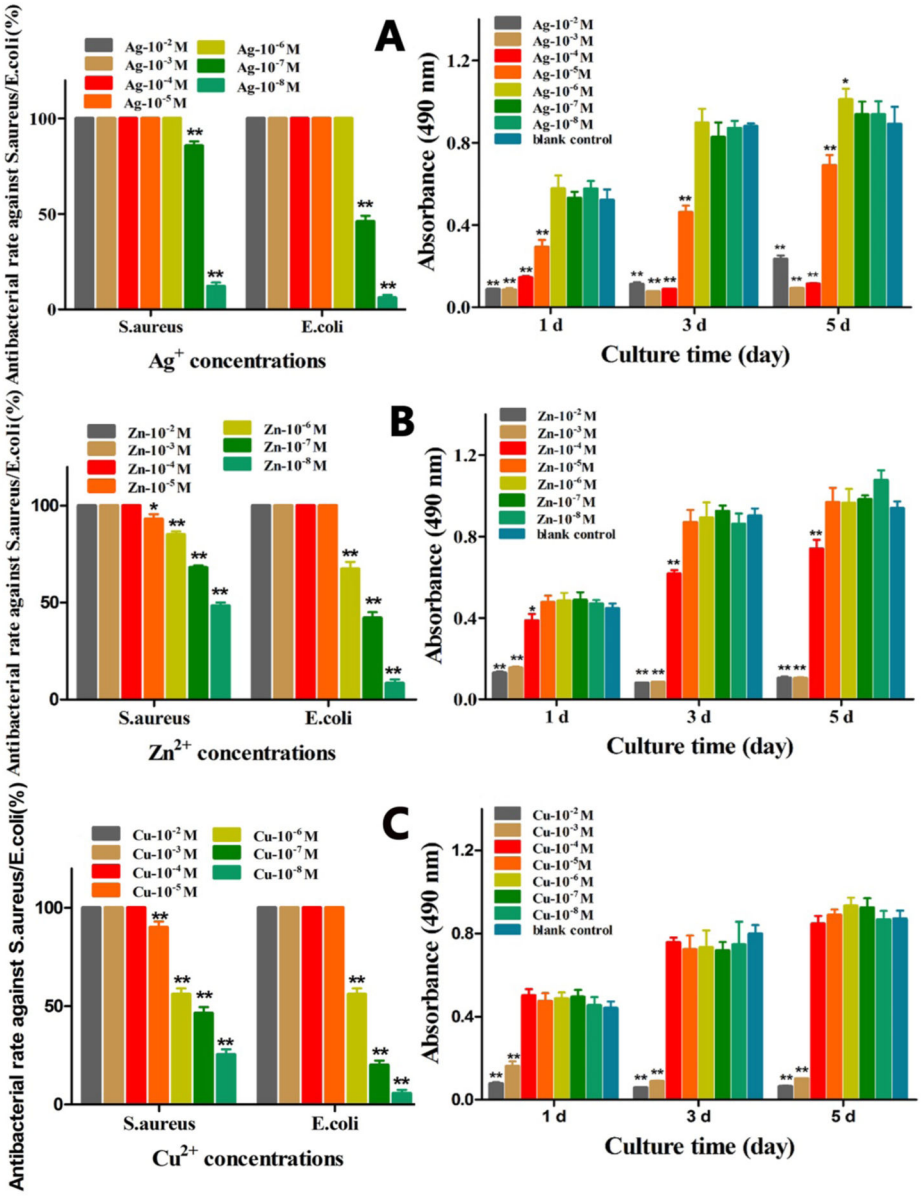

Figure 1.

Antibacterial rate against *S. aureus*/*E. coli* (left) and cytotoxicity on L929 fibroblasts (right) in the presence of metal ions with different concentrations. (A) silver ions; (B) zinc ions; and (C) copper ions. The bacteria concentration was 10^6 CFU/mL. The density of L929 cells is 2×10^4 cells/cm^2. Data are presented as the mean ± standard deviation. Significant difference of antibacterial rate was observed with respect to the experiment groups with metal ion concentration of 10^{-2} M. Significant difference of MTT was observed between experiment groups and the blank control ($n = 4$, *$p < 0.05$, **$p < 0.01$).

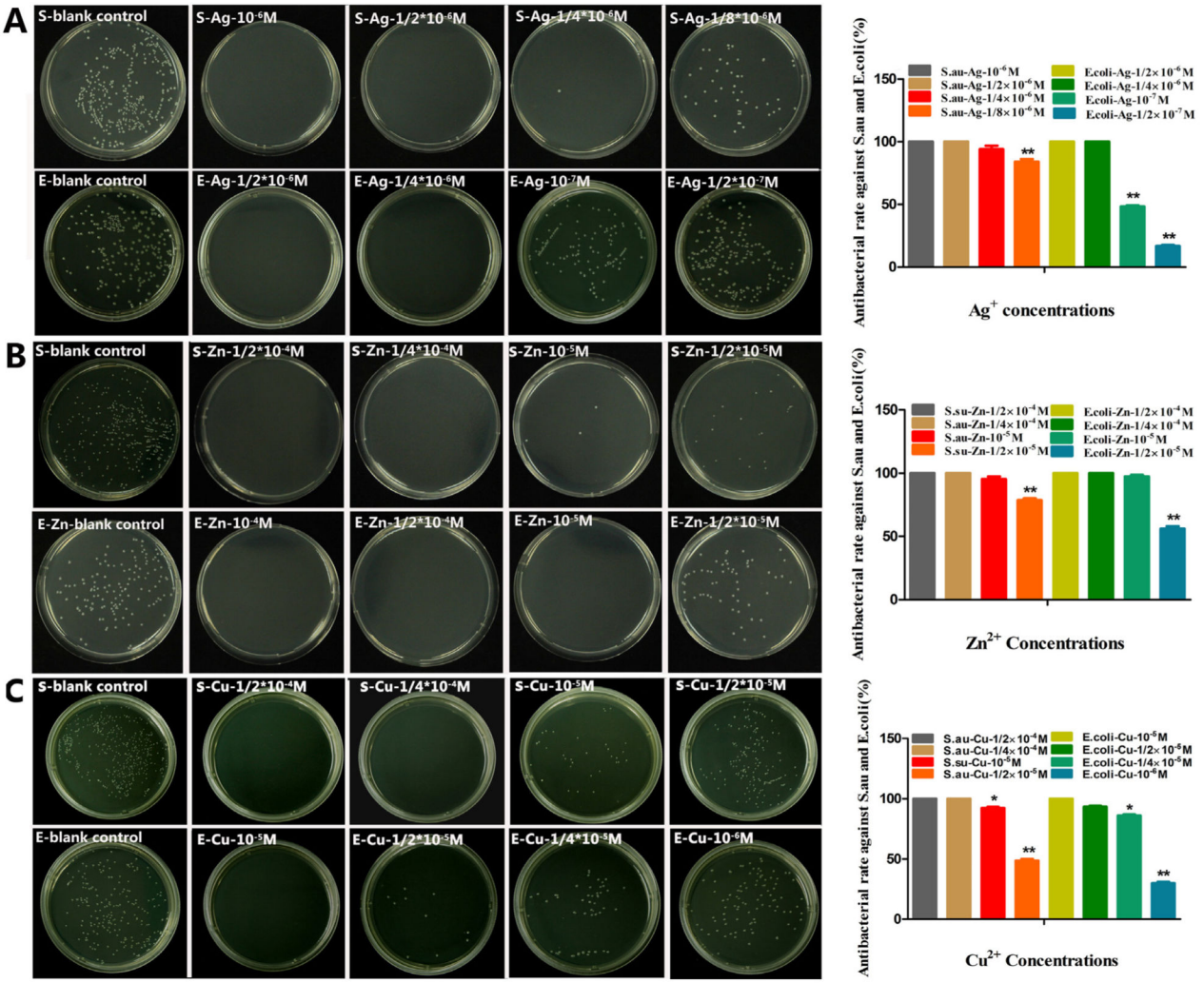

Figure 2.

Variable numbers of colonies and antibacterial rate in the presence of metal ions at different concentrations and cocultured for 24 h against *S. aureus* and *E. coli.* The bacteria suspension concentration was 10^6 CFU/mL. (A) silver ions; (B) zinc ions; and (C) copper ions. Significant difference was observed with respect to the group with metal ions of 100% antibacterial rate ($n = 4$, $^*p < 0.05$, $^{**}p < 0.01$).

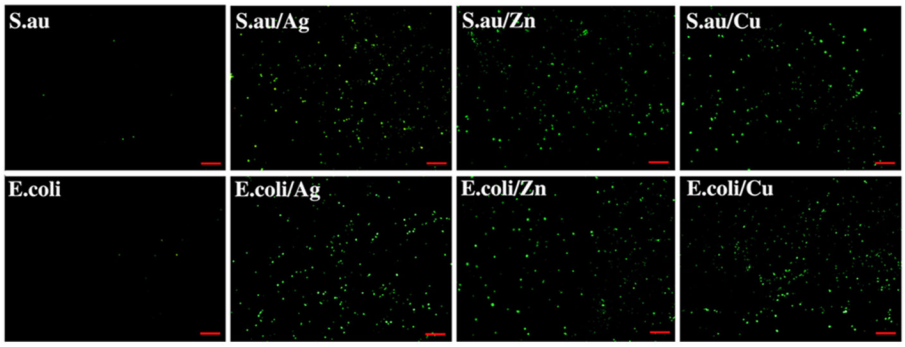

Figure 3.

DCF fluorescence in *S. aureus* and *E. coli* treated with antibacterial metal ions after coculture for 12 h. Scale bar: 50 μm. The concentrations of *S.au*/Ag, *S.au*/Zn, *S.au*/Cu, *E. coli*/Ag, *E. coli*/Zn, and *E. coli*/Cu were 0.25 μM, 10 μM, 10 μM, 0.25 μM, 10 μM, and 5 μM, respectively.

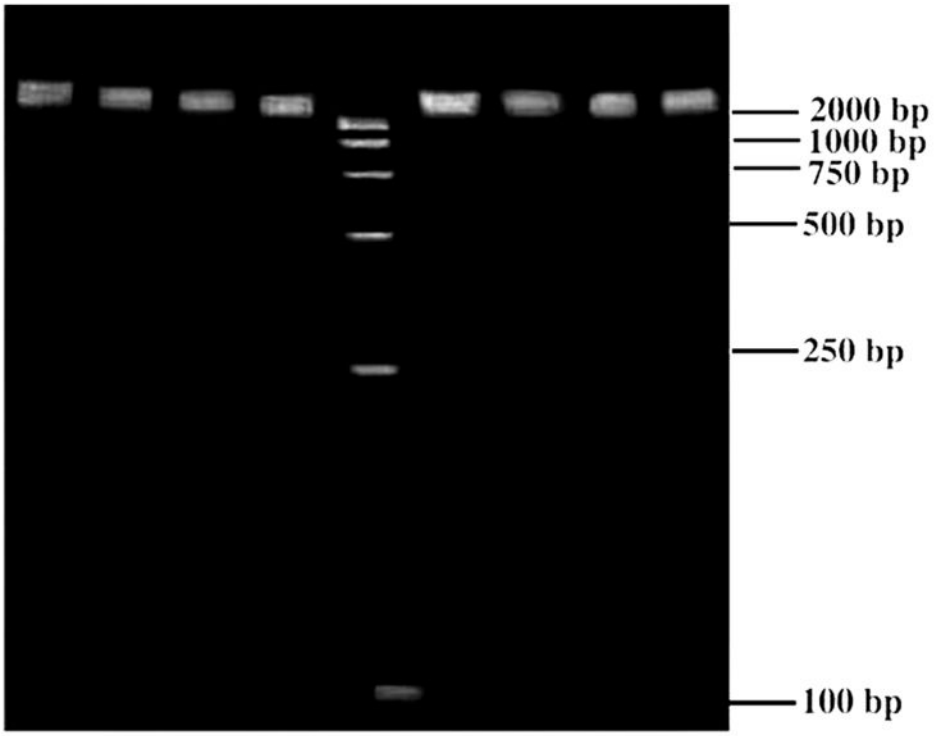

Figure 4.

Bacterial agar gel electrophoresis profiles of genomic DNA obtained from direct DNA extracts of the samples. The bands from left to right in turn correspond to *S.au*/Ag, *S.au*/Zn, *S.au*/Cu, marker, *E. coli*/Ag, *E. coli*/Zn, and *E. coli*/Cu.

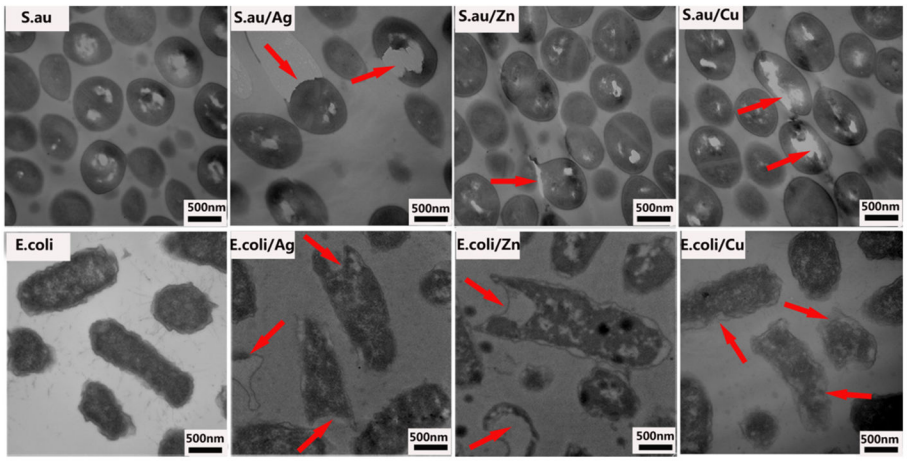

Figure 5.

TEM images showing the surface morphology of *S. aureus* and *E. coli* treated with different antibacterial metal ions. Scale bar = 500 nm. Red arrows highlight the damage of cell membranes.

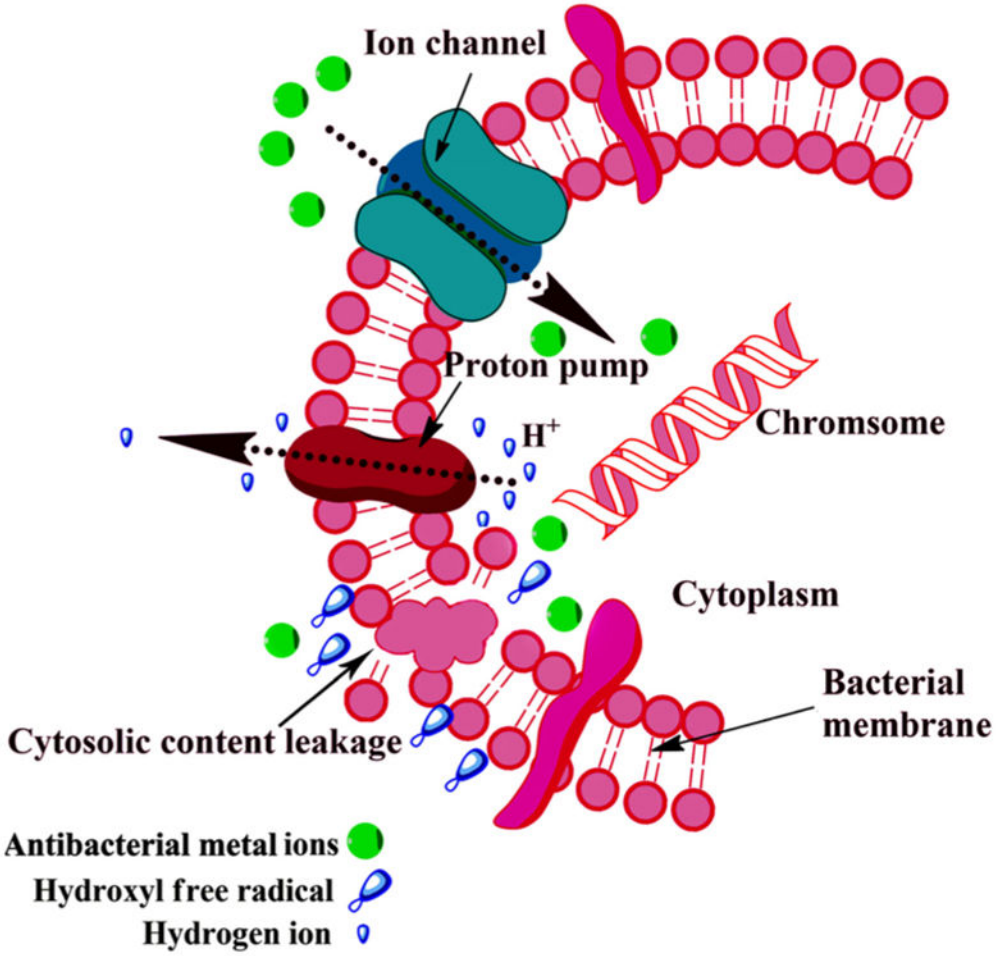

Figure 6.
Schematic illustration of the possible antibacterial mechanism of the antibacterial metal ions.