Journal of Inorganic Biochemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

The synergistic antibacterial activity and mechanism of multicomponent metal ions-containing aqueous solutions against *Staphylococcus aureus*

Xiaolan Wang [a,b,1], Shaoxiang Liu [c,b,1], Mei Li [b], Peng Yu [a], Xiao Chu [b], Lihua Li [b], Guoxin Tan [d], Yingjun Wang [a], Xiaofeng Chen [a], Yu Zhang [b,*], Chengyun Ning [a,e,**]

[a] School of Materials Science and Engineering, South China University of Technology, Guangzhou 510641, China
[b] Guangdong Key Lab of Orthopedic Technology and Implant Materials, Key Laboratory of Trauma & Tissue Repair of Tropical Area of PLA, Guangzhou General Hospital of Guangzhou Military Command, Guangzhou 510010, China
[c] Second Military Medical University, Shanghai 200433, China
[d] Institute of Chemical Engineering and Light Industry, Guangdong University of Technology, Guangzhou 510006, China
[e] Guangdong Key Lab of Biomedical Engineering, South China University of Technology, Guangzhou 510006, China

A R T I C L E I N F O

Article history:
Received 27 March 2016
Received in revised form 19 July 2016
Accepted 26 July 2016
Available online xxxx

Keywords:
Antibacterial activities
Multicomponent metal ions
Synergistic antibacterial mechanism

A B S T R A C T

Silver (Ag^{+}), zinc (Zn^{2+}) and copper (Cu^{2+}) ions, are well known for their broad-spectrum antibacterial activities while generating low resistance. However, whether or multiple metal ions in aqueous solutions acted synergistically or antagonistically antimicrobial properties, remained unknown. Therefore, it was of great significance to investigate the antibacterial properties of multicomponent metal ions-containing aqueous solutions. In this study, the antibacterial activities of multicomponent metal ions-containing aqueous solutions were investigated for the first time. We found that the antibacterial activities of multicomponent metal ions-containing aqueous solutions were higher than those of single metal ion-containing aqueous solution. Furthermore, the synergistic antibacterial mechanism of these multicomponent metal ions-containing aqueous solutions was first investigated. The generation of reactive oxygen species (ROS) through electron transfer in the enzymes and Fenton reactions formed the main synergistic antibacterial mechanism of the multicomponent metal ions-containing aqueous solutions. Therefore, the encouraging results demonstrate the great potential applications of multicomponent metal ions for the design of new biomaterials or prosthesis containing Ag-Cu-Zn alloy which can release Ag^{+}, Zn^{2+} and Cu^{2+} and minimize the risk of hospital acquired infection.

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1. Introduction

Recently, infection has been regarded as the most severe and devastating complication associated with the use of biomaterials [1,2]. The World Health Organization (WHO) recently published a report showing that resistance to common bacteria had reached alarming levels in many regions of the world, underlining the risk of a global post-antibiotic era in which common infections and minor injuries can kill healthy people. Over the past few decades, Ag^{+} [3], Zn^{2+} [4] and Cu^{2+} [5] have gained attention due to their strong broad-spectrum antibacterial activities and the considerably low resistance generated. In contrast to metallic Ag, metallic Zn and Cu are found abundantly in the earth as well as essential trace elements in most living organisms [6,7]. In addition, metallic Zn and Cu are also environmentally benign and relatively inexpensive. In other words, Zn^{2+} and Cu^{2+} maybe partially replace with Ag^{+} in biomaterials as antibacterial agents if Ag^{+}, Zn^{2+} and Cu^{2+} act synergistically antimicrobial properties. Therefore, it was necessary to investigate the antimicrobial properties of multicomponent metal ions-containing aqueous solutions. Moreover, there has been no previous research on the antibacterial properties of such multicomponent metal ions-containing aqueous solutions.

However, several studies have been conducted to investigate the synergistic effects of combining elemental Ag, Zn and Cu in biomaterials. Quirynen et al. [8] find that the presence of Ag^{+} and Zn^{2+} improve the antibacterial effectiveness of toothbrushes and that the combined effect is greater than the use of Ag^{+} or Zn^{2+} alone, suggesting that Ag^{+} and Zn^{2+} possess synergistic antibacterial activities. Kaali and his colleagues [9] find that the presence of Zn^{2+} or Cu^{2+} as individual ion in biomaterials does not result in significant antimicrobial activities but that a binary Zn^{2+}-Cu^{2+} system decrease the number of viable bacteria on the surface of biomaterials. These results show that metal ions in biomaterials are more effective in fighting off bacteria in conjunction with one another than as single entities. From a commercial standpoint, the use

* Corresponding author.
** Correspondence to: C. Ning, School of Materials Science and Engineering, South China University of Technology, Guangzhou 510641, China.
E-mail addresses: luck_2001@126.com (Y. Zhang), ning_lab@hotmail.com (C. Ning).
[1] The authors contributed equally to this work and should be considered co-first authors.

http://dx.doi.org/10.1016/j.jinorgbio.2016.07.019
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of a binary Ag^{+}-Zn^{2+} or Ag^{+}-Cu^{2+} was better than only using Ag^{+} in the field of biomaterials because Zn and Cu are more abundant in the earth and are cheaper than silver.

According to our previous research results [10], Ag^{+}, Zn^{2+} or Cu^{2+} has a specific concentration range in which it will kill bacteria while leaving mammalian cells unaffected. We find that metal ions kill bacteria by inducing the production of ROS (ROS are chemically reactive chemical species containing oxygen. Examples include peroxides, superoxide, hydroxyl radical, and singlet oxygen), which damage bacterial cell membranes. In this paper, we evaluated the synergistic antibacterial effects of the combination Ag^{+}-Zn^{2+}, Ag^{+}-Cu^{2+}, Zn^{2+}-Cu^{2+} and Ag^{+}-Zn^{2+}-Cu^{2+} aqueous solutions. However, the underlying synergistic antibacterial mechanisms of the multicomponent metal ions-containing aqueous solutions have not been studied before and require further investigations.

In this research, the antibacterial properties of multicomponent metal ions-containing aqueous solutions against *S. aureus* were studied using a plate colony-counting method and a Live/Dead bacterial staining assay following an orthogonal experimental design. The cytotoxicities of multicomponent metal ions-containing aqueous solutions were assessed by an *in vitro* cell proliferation assay [11]. To investigate the synergistic antibacterial mechanisms of the multicomponent metal ions-containing aqueous solutions, ROS in the simultaneous presence of different metal ions were measured, genomic deoxyribonucleic nucleic acid (DNA) degradation was detected through agarose gel electrophoresis and gene sequencing, the integrity of bacterial membrane was identified with transmission electron microscope (TEM).

2. Materials and methods

2.1. Materials preparation

Silver nitrate, zinc chloride and copper chloride were purchased from Sigma-Aldrich Chemical Co., (St. Louis, USA) and serial dilutions were made in purified water to avoid silver salt precipitation. Aqueous solutions of the metal ions were prepared and filtered using a nonpyrogenic, sterile, single-use filter of 0.22 μm (Millex®-GS, Millipore, France). Other biological reagents, such as tryptone and yeast extract, were supplied by Oxoid Ltd. (Basingstoke, England).

S. aureus ATCC 25923 was obtained from Guangdong Provincial Key Laboratory of Microbial Culture Collection in China, and mouse fibroblast cells L929 (NO. CCL-1™) were purchased from *ATCC* Cell Biology (Maryland, USA).

2.2. Antibacterial tests of multicomponent metal ions-containing aqueous solutions

The antibacterial properties of the multicomponent metal ions-containing aqueous solutions were evaluated using gram-positive *S. aureus*, which were cultivated in lysogenic broth (LB) medium (1% w/v tryptone, 0.3% w/v yeast extract, 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. Based on our previous research results [10], when the final concentrations of the Ag^{+}, Zn^{2+} or Cu^{2+} are 10^{-8}–10^{-6} M, 10^{-6}–10^{-4} M and 10^{-6}–10^{-4} M, respectively, they could effectively kill *S. aureus* and show no obvious cytotoxicity on fibroblasts. So we chose them to do the synergistic antibacterial tests. In the experiment, multicomponent metal ions-containing aqueous solutions (100 μL) were introduced to the bacterial suspensions. Then, the mixed aqueous solutions were co-incubated in a biochemical incubator at 37 °C for 24 h. The viable numbers of bacteria in double-distilled water were quantified by standard serial dilutions. A suspension of 100 μL from each tube was uniformly spread on an LB agar plate, and the number of viable bacterial colonies was counted after incubation at 37 °C for 24 h. The control group was only bacterial suspensions with a concentration of 10^{6} CFU/mL. To quantify the antibacterial abilities, antibacterial ratio was calculated using the following equation:

$$\text{Antibacterial ratio } (\%) = (\text{Nc} - \text{Nt})/\text{Nc} \times 100\% \quad (1)$$

where Nc is the average number of bacteria in the control group (CFU/mL) and Nt is the average number of bacteria in the testing groups (CFU/mL).

2.3. Cell culture and cell proliferation assay

The cells growth media was composed of Dulbecco's Modified Eagles Medium (DMEM) (high glucose, Gibco, USA) containing 10% fetal bovine serum (FBS, Sigma, USA). The medium was replaced every other day. The L929 cells were cultured in 25 cm^2 flasks (Corning Incorporated, USA) at 37 °C in an atmosphere of 5% CO_2 and 95% humidity.

L929 cells suspensions (100 μL) were seeded in each 96-well plate at a density of 2×10^{4} cells/cm^2. After culturing for 2 days, 200 μL aliquots of the multicomponent metal ions-containing aqueous solutions were added into each well. Cell proliferation assay was assessed using a cell counting kit-8 assay after 24 h. In brief, at a prescribed time point, an aqueous CCK-8 solution was added to the co-cultured aqueous solutions, and the mixture was incubated at 37 °C for 1 h. Then, the optical densities (OD) of the samples were measured at 450 nm using a microplate reader (Multiskan Go, Thermo Scientific, USA).

2.4. Measurement of ROS

ROS in the bacterial cells were measured using an Intracellular ROS Assay Kit (Green Fluorescence, Beyotime, China). H_2-DCFDA is a cell-permeant indicator for ROS that is nonfluorescent and is deacetylated by cellular esterases to non-fluorescent 2′, 7′-dichlorodihydrofluorescin (DCFH), which is rapidly oxidized to highly fluorescent 2′, 7′-dichlorodihydrofluorescein (DCF) by ROS. In the experiment, bacteria and multicomponent metal ions-containing aqueous solutions were co-cultured in an incubator at 37 °C for 24 h. A blank group was prepared similarly without treatment with metal ions. Later, 20 μM DCFH-DA was added into the mixed aqueous solutions, and the resulting solutions were incubated at 37 °C for 30 min. Stained cells were washed with phosphate-buffered saline (PBS) twice and the fluorescence was analyzed on an inverted fluorescence microscope (Olympus BX51, Japan) with a 485/538 nm filter set and a 530 nm cut off.

2.5. Bacterial DNA extracting and agar gel electrophoresis

Bacteria broth samples (5 mL) were treated with metal antibacterial ions and centrifuged at 10,000 rpm for 1 min. Genomic DNA was extracted using a TIANamp Bacteria DNA Kit (Tiangen, Beijing, China). The concentration and purity of the bacterial DNA were detected by a NanoDrop 2000 (Thermo Fisher Scientific, USA). Then, the gene information of the bacterial genomic DNA was obtained by agar gel electrophoresis. In brief, agarose gel (1% v/v, low melt, Mercury, San Diego, CA, USA) was prepared by boiling 1 × TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid) for 2 min in a microwave. Then, the gel was cooled to 60 °C and ethidium bromide was added (5 μL/100 mL of the gel). The gel was transferred into an electrophoretic bath containing 1 × TAE buffer. Samples prepared with 5% (v/v) bromophenol blue and 3% (v/v) glycerol loaded into a gel in 30 μL aliquots. A DNA ladder (Takara, Japan) within a size range from 250 bp to 15,000 bp was used to monitor the sizes of the analyzed fragments. Electrophoresis (Bio-Rad, USA) was conducted at 90 V for 45 min. The bands were visualized by a UV transilluminator (Bio-Rad, GDS-7500, USA).

Please cite this article as: X. Wang, et al., The synergistic antibacterial activity and mechanism of multicomponent metal ions-containing aqueous solutions against *Staphylococcus aureus*..., J. Inorg. Biochem. (2016), http://dx.doi.org/10.1016/j.jinorgbio.2016.07.019

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