Efficient Bacteria Killing by Cu$_2$WS$_4$ Nanocrystals with Enzyme-like Properties and Bacteria-Binding Ability

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Supporting Information

ABSTRACT: Antibacterial agents with high antibacterial efficiency and bacteria-binding capability are highly desirable. Herein, we describe the successful preparation of Cu$_2$WS$_4$ nanocrystals (CWS NCs) with excellent antibacterial activity. CWS NCs with small size (∼20 nm) achieve more than 5 log (>99.999%) inactivation efficiency of both Staphylococcus aureus (Gram-positive) and Escherichia coli (Gram-negative) at low concentration (<2 μg mL$^{-1}$) with or without ambient light, which is much better than most of the reported antibacterial nanomaterials (including Ag, TiO$_2$, etc.) and even better than the widely used antibiotics (vancomycin and daptomycin). Antibacterial mechanism study showed that CWS NCs have both enzyme-like (oxidase and peroxidase) properties and selective bacteria-binding ability, which greatly facilitate the production of reactive oxygen species to kill bacteria. Animal experiments further indicated that CWS NCs can effectively treat wounds infected with methicillin-resistant Staphylococcus aureus (MRSA). This work demonstrates that CWS NCs have the potential as effective antibacterial nanozymes for the treatment of bacterial infection.

KEYWORDS: antibacterial activity, Cu$_2$WS$_4$ nanocrystals, bacteria binding, nanozyme, reactive oxygen species

Bacterial infection-related diseases are serious threats to human health and affect millions of people all over the world.1−3 Currently, antibiotics are the most conventional and effective treatment for bacterial infections.4−6 However, the extensive use of antibiotics has caused worldwide drug-resistance issues during the last decades, which brings great challenges to traditional treatment of bacterial infections.7,8 Consequently, alternative therapeutic ways to combat bacterial infections are urgently needed.

Recently, antibacterial nanomaterials have drawn increasing interest due to their distinct antibacterial mechanisms compared with antibiotics.5,9 Although their mechanisms have not been entirely understood at the molecular level, previous study has revealed that nanomaterials can kill bacteria generally by physical ways and chemical ways.9 For example, graphene and its derivatives can inactivate bacteria by physically disrupting the cell membrane structure of bacteria.10,11 Although this mode of action for killing bacteria seems relatively safe, the application is limited due to low antibacterial activity. In contrast, many nanomaterials can kill bacteria through chemical ways, including the release of toxic metal ions or generation of reactive oxygen species (ROS).1,12

As the mostly used commercial antibacterial nanomaterials, Ag nanoparticles (Ag NPs) have excellent wide-spectrum antibacterial activity and have been extensively adopted in biomedicine.13 The Ag$^+$ released from Ag NPs is usually considered as the main origin for their toxicity, which can damage the vital biomolecules of bacteria, such as DNA and enzymes.13,14 However, excess metal ions may be potentially harmful to humans.13,14 Furthermore, semiconductor nanomaterials (TiO$_2$, ZnO, etc.) have bactericidal effects by photo-
catalytic generation of ROS. Nevertheless, their antibacterial efficiency is significantly limited in dark conditions. Hence, although antibacterial nanomaterials have shown great potential as supplementary antibiotics, their further medical use is still limited due to potential toxicity or low antibacterial activity. To develop antibacterial nanomaterials which possess the characteristics of antibiotics, such as high antibacterial activity, low toxicity, and even selectivity, is still a great challenge.

Nanozymes are nanomaterials which can catalyze the reactions of biologically relevant substrates under mild conditions with similar reaction kinetics to natural enzymes and have shown great potential as effective antibacterial agents. Because of their capability to catalyze the formation of ROS, nanozymes with peroxidase- and oxidase-like properties have been explored as powerful tools to kill bacteria. For instance, V2O5 nanowires and CeO2−x nanorods with haloperoxidase-like activity were used to treat bacterial biofilms by the generation of hypohalous acids to disrupt microbial quorum sensing. Graphene quantum dots and MoS2 nanoflowers have peroxidase-like activity and can be utilized to efficiently generate hydroxyl radicals from hydrogen peroxide for wound disinfection. Pd nanoparticles with both oxidase- and peroxidase-like properties show broad-spectrum antibacterial activity. Although the production of ROS by nanozymes represents a promising strategy to design and prepare antibacterial agents, nanozymes also suffer from low antibacterial efficacy and potential side-effect issues that originate from the high reactivity and limited diffusion distance of ROS in biological systems. The conjugation of bacteria-targeting biomolecules, such as antibodies, to nanozymes can enhance their bacteria-binding ability, but the complex preparation and high cost will greatly offset its feasibility. Thus, it is an attractive strategy to develop antibacterial nanozymes with intrinsic bacteria-binding ability. However, to the best of our knowledge, nanozymes which simultaneously possess high antibacterial activity, selective bacteria-binding ability, and excellent biocompatibility have rarely been reported.

Herein, we report the preparation of Cu2WS4 nanocrystals (CWS NCs) with small size (~20 nm) by a microwave irradiation method (Scheme 1a). In vitro antibacterial experiments showed that CWS NCs at low concentration (2 μg mL−1) achieved >99.999% inactivation of both Gram-negative and Gram-positive bacteria (Staphylococcus aureus, Escherichia coli, and methicillin-resistant S. aureus (MRSA)). CWS NCs exhibit much higher antibacterial activity of planktonic bacteria than typical antibacterial nanomaterials (Ag NPs and TiO2 NPs) and commonly used antibiotics (vancomycin and daptomycin) under the same experimental conditions. A mechanistic study revealed that CWS NCs not only have oxidase- and peroxidase-like activities for ROS production with or without light irradiation but also have good affinity toward the surface of bacteria (Scheme 1b). Moreover, CWS NCs can effectively treat MRSA-infected wounds in vivo and show therapeutic efficacy comparable to that of vancomycin. The as-prepared CWS NCs have good biocompatibility and negligible toxicity based on cytotoxicity, hemolysis, and animal experiments, which further demonstrates their potential use as highly efficient antibacterial agents.

RESULTS AND DISCUSSION

Synthesis and Characterization of Cu2WS4 Nano-crystals. As an attractive method for the preparation of inorganic nanocrystals, microwave-assisted synthesis has several advantages, such as homogeneous heating, fast reaction rate, high selectivity, and controllability. In this study, CWS NCs were successfully prepared through a microwave synthesis by using aqueous mixtures of (NH4)2WS4, CuBr, 3-
mercaptopropionic acid, and ammonia as precursors. Transmission electron microscopy (TEM) images show that the as-synthesized CWS NCs have cuboid-like morphology with average size of about 20 nm (Figure 1a and Figure S1), which is also confirmed by atomic force microscopy results (Figure S2). As shown by the high-resolution TEM (HRTEM) image in Figure 1b, the crystal lattice distance of about 0.50 nm corresponds to the (002) plane of body-centered tetragonal CWS (I-CWS).37 Furthermore, the dark-field TEM image and energy-dispersive spectroscopy (EDS) elemental mapping images (Figure 1c) show homogeneous and identical elements distribution of Cu, W, and S in CWS NCs. X-ray photoelectron spectroscopy (XPS) was used to investigate the compositions and valence states of CWS NCs. The peaks with binding energies of 953.1 and 933.2 eV belong to Cu 2p1/2 and Cu 2p3/2, respectively (Figure 1d); the peaks centered at 35.6 and 33.5 eV correspond to W 4f5/2 and W 4f7/2, respectively (Figure 1e); the peaks near 163.1 and 162.0 eV correspond to S 2p1/2 and S 2p3/2, respectively (Figure 1f).38 These results reveal the valence states of +1, +6, and −2 for Cu, W, and S in the CWS NCs, respectively. The X-ray diffraction (XRD) pattern (Figure 1g) of CWS NCs shows two diffraction peaks at 17.4 and 18.3°, which belong to the (002) and (101) planes of I-CWS, respectively.37 The Raman spectrum (Figure 1h) illustrates three characteristic peaks for different vibrational modes of CWS NCs, which are located at 218 cm⁻¹ (A1), 281 cm⁻¹ (B1), and 448 cm⁻¹ (E).34,36 Compared with bulk CWS, the wavenumber of the E mode of CWS NCs at 448 cm⁻¹ decreases by about 3 cm⁻¹, suggesting the as-prepared CWS has a small size.39 The zeta-potential result (Figure S3) indicates that CWS NCs in H₂O and saline are negatively charged. The ultraviolet–visible–near-infrared (UV–vis–NIR) absorption spectrum shows that CWS NCs have two distinct peaks at 329 and 458 nm, similar to those of previously reported I-CWS (Figure 1i).40 Although hydrothermal synthesis has also been reported for the preparation of CWS materials, the reaction time is very long (>24 h), and the size of CWS is in the submicron range.38,40,41 In comparison, microwave synthesis can significantly reduce both the reaction time (4 h) and the size of CWS NCs (~20 nm).

Antibacterial Activity of Cu₂WS₄ Nanocrystals. As shown in Figure 2a and Figure S4, CWS NCs (1.7 μg mL⁻¹) exhibited a 5 log (>99.999%) inactivation efficiency of E. coli within 4 h incubation in the dark, and a 6 log (>99.9999%) inactivation efficiency of E. coli within 4 h under indoor light. Moreover, scanning electron microscopy (SEM) images show

Figure 1. Characterization of CWS NCs. (a) TEM image of CWS NCs. (b) HRTEM image of CWS NCs. (c) Dark-field TEM image of CWS NCs and corresponding EDS elemental mapping images of Cu, W, and S. XPS spectra of (d) Cu 2p, (e) W 4f, and (f) S 2p orbitals for CWS NCs. (g) XRD pattern of CWS NCs and the standard diffraction pattern of I-CWS (PDF 01-074-3742, gray bars). (h) Raman and (i) UV–vis–NIR absorption spectrum of CWS NCs.
that the morphology of *E. coli* cells in the control group maintain a rod shape without cell membranes damage, whereas most *E. coli* cells become deformed and uneven after treatment with CWS NCs (Figure 2b). Meanwhile, a similar inactivation efficiency for *S. aureus* can be achieved by CWS NCs at even lower concentration (0.7 μg mL\(^{-1}\)) after 2 h incubation (Figure 2c and Figure S5), which reveals that CWS NCs have an antibacterial activity toward *S. aureus* that is higher than that toward *E. coli*. The reason may originate from the lack of an outer cell membrane in Gram-positive bacteria.\(^{1,13,42}\) SEM images in Figure 2d show that the *S. aureus* cells without the treatment of CWS NCs have intact sphere-shaped morphology with smooth cell membranes, whereas those treated by CWS NCs become wrinkled and damaged.

Furthermore, a low concentration of CWS NCs (1.7 μg mL\(^{-1}\)) also shows efficient killing of MRSA with 4 log (~99.99%) inactivation efficiency in the dark and 5 log (99.999%) inactivation efficiency under indoor light after 2 h incubation (Figures S6 and S7). Moreover, the inactivation efficiency of CWS NCs for both *S. aureus* and *E. coli* under indoor light is about 1 log higher than that in the dark, which suggests that the photocatalytic generation of ROS is not a key factor for killing bacteria by CWS NCs, which is different from previously reported antibacterial semiconductor nanomaterials.\(^{19,20}\) Therefore, CWS NCs have excellent broad-spectrum antibacterial activity toward both Gram-negative and Gram-positive bacteria, and even the drug-resistant strain, whether under indoor light or in the dark.

The antibacterial activity of CWS NCs was further evaluated by comparison with common antibacterial nanomaterials (Ag NPs and TiO\(_2\) NPs) and antibiotics (vancomycin and daptomycin). As shown in Figure 2ef, CWS NCs (2 μg mL\(^{-1}\)) show 6 log and 2 log higher inactivation efficiency compared to that of TiO\(_2\) NPs and Ag NPs, respectively, toward both *E. coli* and *S. aureus* in the dark. Moreover, CWS NCs have similar inactivation efficiency under indoor light toward both *E. coli* and *S. aureus* at the same concentration compared with TiO\(_2\) and Ag NPs (Figures S8 and S9). More interestingly, the inactivation efficiency of CWS NCs is about 6 log higher than those of the commonly used antibiotics (vancomycin and daptomycin) toward both *E. coli* and *S. aureus* at 2 μg mL\(^{-1}\). Compared with other antibacterial nanomaterials reported in the literature (Table S1), CWS NCs achieve one of the highest antibacterial efficiencies for *E. coli*, which demonstrates their excellent antibacterial activity.

Although submicron Cu\(_2\)WS\(_4\) flake-like aggregates with antibacterial activity have been recently reported,\(^{41}\) these large-size CWS aggregates (2 μg mL\(^{-1}\)) showed very low inactivation efficiency (about 30%) to *E. coli* in our hands (Figure S10), which is about 5 log lower than that of the small-size CWS NCs prepared in this study. Until now, the exact reason for the different antibacterial efficiency between the CWS nanomaterials prepared by different methods is not completely understood, which may derive from the difference of size, morphology, or surface property.\(^{1,13,23,40}\)

**Antibacterial Mechanism of Cu\(_2\)WS\(_4\) Nanocrystals.**

Previous investigations have reported that the metal ions released from metal-containing nanomaterials play important roles in their antibacterial activity.\(^{9,45}\) Therefore, we first studied whether the copper ions released from CWS NCs play a key role for killing bacteria. A CWS NC aqueous dispersion (3.4 μg mL\(^{-1}\)) was first incubated at 37 °C for 4 h, and then CWS NCs were removed by centrifugation to obtain the supernatant. As shown in Figure S11, the supernatant from the CWS NC aqueous dispersion shows no obvious antibacterial activity to *E. coli*. Moreover, the UV-vis absorption spectra of...
the CWS NC aqueous dispersion before and after incubation show no obvious changes (Figure S12a), suggesting the decomposition of CWS NCs is negligible under the experimental conditions. Meanwhile, the concentration of copper ions of the supernatant from the CWS NC aqueous dispersion was quantitatively characterized by inductively coupled plasma optical emission spectroscopy (ICP-OES). Results indicate that the concentration of copper ions released from CWS NCs is only 1.75 ppb, and the same concentration of copper ions from CuCl₂ aqueous solution could not cause bacterial death (Figure S12b). According to these results, the antibacterial activity of CWS NCs may not originate from the copper ions leaked from the CWS NCs.

ROS are highly reactive oxygen-containing species, including superoxide radical anion (O₂⁻•), hydrogen peroxide (H₂O₂), hydroxyl radicals (•OH), and so on. As ROS can kill bacteria via oxidative damage of proteins, DNA, and other key biomolecules, the generation of ROS is considered to be an important antibacterial mechanism for nanomaterials, such as Ag, TiO₂, and ZnO. To study whether ROS play a key role in the antibacterial activity of CWS NCs, ROS-scavenging experiments were conducted. As shown in Figure S13, the antibacterial efficiency of CWS NCs in the presence of an O₂⁻• scavenger (2,2,6,6-tetramethyl-1-piperidinyloxy, TEMPO) does not differ from the control, suggesting that O₂⁻• does not account for the antibacterial effect of CWS NCs. As shown in Figure 3a, the bacterial inactivation efficiency significantly decreases from 5 log (>99.999%, Figure 2a) to ~1 log (in the dark) and ~2 log (under indoor light) in the presence of a H₂O₂ scavenger (catalase). Furthermore, Figure 3b shows that the bacterial inactivation efficiency is ~4 log (in the dark) and ~5 log (under indoor light) in the presence of an •OH scavenger (isopropyl alcohol), which is ~1 log lower than the inactivation efficiency of CWS NCs (5 log in the dark and 6 log under indoor light, Figure 2a). These results confirm that H₂O₂ and •OH are two key ROS for the antibacterial action of CWS NCs.

**Enzyme-like Properties of Cu₂WS₄ Nanocrystals.** Although the above results have shown that the high antibacterial activity of CWS NCs mainly originates from the existence of ROS (H₂O₂ and •OH), the mechanism of ROS generation by CWS NCs is not fully known. Recent studies have reported that some nanomaterials can kill bacteria by producing ROS through enzyme-like catalytic reactions. Therefore, it was of interest to investigate whether CWS NCs have enzyme-like properties.

To explore the oxidase-like property of CWS NCs, the oxidation of glutathione (GSH) by CWS NCs was studied. The oxidation capacity of GSH (0.8 mM) was 85.54 ± 0.19% after incubation with CWS NCs (3.4 μg mL⁻¹) for 4 h, and O₂ is essential for the oxidation of GSH (Figure 3c and Figure S14a). Moreover, XPS spectra (Figure S14b) confirm that there is no oxidation of the copper in CWS NCs during the oxidation of GSH. These results suggest that CWS NCs can catalyze the oxidation of GSH. As an antioxidant in cellular redox reactions, l-ascorbic acid (AA) was also used as an indicator to study the oxidase-like property of CWS NCs. Figure 3d shows that the absorbance of AA gradually decreased and completely disappeared within 15 min, indicating the oxidase-like property of CWS NCs.

Moreover, 3,3′,5,5′-tetramethylbenzidine (TMB) was used as a substrate to study whether CWS NCs have peroxidase-like activity. The peroxidase-like catalytic property of CWS NCs was evidenced by the UV absorbance decrease of TMB solutions at 650 nm under different conditions and corresponding photographs (inset, incubated for 4 h): I, TMB + H₂O₂; II, TMB + CWS + H₂O₂ (D); III, TMB + CWS + H₂O₂ (L). (f) Photoluminescence spectra of terephthalate (TA) incubated under different experimental conditions. D, dark; L, indoor light. Error bars are based on the three samples.
and H₂O₂ in the presence of O₂. Subsequently, the bacteria are catalyzed to produce the oxidative product A with bacteria, a scanning TEM (STEM) equipped with a bacteria surface. To further investigate the interaction of CWS NCs with bacteria, TEM images (Figure S16) show that CWS NCs only adhere on the surfaces of both E. coli and S. aureus cells, which suggests that CWS NCs. TEM and elemental mapping characterization were also used to further investigate the mode of antibacterial action of CWS NCs. TEM images (Figure S16) show that CWS NCs only adhered on the surfaces of both E. coli and S. aureus cells rather than entered into the bacteria cells, which implies the decomposition of H₂O₂ to form •OH by the catalysis of CWS. Furthermore, the •OH generation by CWS NCs was detected by using terephthalate (TA) as a fluorescent indicator. The fluorescence intensity in the presence of both CWS and H₂O₂ is significantly stronger than that of TA alone or TA + H₂O₂ both under indoor light and in the dark, confirming the generation of •OH and the peroxidase-like property of CWS NCs (Figure S1f). Moreover, the presence of •OH was further confirmed in the mixture of CWS and H₂O₂ by electron paramagnetic resonance (EPR) spectrometry (Figure S15). Based on the intrinsic enzyme-like properties (oxidase and peroxidase) and the main contribution of ROS (H₂O₂ and •OH) to the antibacterial activity of CWS NCs, we hypothesize that ROS can be generated by CWS NCs as follows:

\[
\begin{align*}
A H_2 + O_2 & \quad \text{CWS} \quad A + H_2O_2 \\
H_2O_2 & \quad \text{CWS} \quad •OH
\end{align*}
\]

As shown in eq 1, when oxidase-like CWS NCs interact with bacteria, physiologically relevant antioxidants (AH₂) in bacteria are catalyzed to produce the oxidative product A and H₂O₂ in the presence of O₂, Subsequently, the generated H₂O₂ is further catalyzed by peroxidase-like CWS NCs to form •OH (eq 2), which is also a strong antibacterial agent. Hence, the oxidase-like and peroxidase-like properties of CWS NCs make them excellent antibacterial agents toward various bacteria.

**Bacteria-Binding Ability of Cu₂W₅S₄ Nanocrystals.** TEM and elemental mapping characterization were also used to further investigate the mode of antibacterial action of CWS NCs. TEM images (Figure S16) show that CWS NCs only adhered on the surfaces of both E. coli and S. aureus cells rather than entered into the bacteria cells, which suggests that CWS NCs may interact with some specific components on the bacteria surface. To further investigate the interaction of CWS NCs with bacteria, a scanning TEM (STEM) equipped with a high-angle annular dark-field (HAADF) detector was used to investigate the distribution of CWS NCs in bacterial cells. Figure 4a shows that E. coli cells without CWS NC treatment have an intact cell structure with a smooth cell membrane. Elemental mapping images (Figure 4b–h) reveal that only carbon, oxygen, nitrogen, and sulfur elements were present in E. coli. As shown in Figure 4i, after CWS NC treatment, the surface of E. coli cells becomes rough with the presence of large amounts of CWS NCs. HAADF-STEM-EDS elemental mapping images (Figure 4j–p) show that strong copper and tungsten signals mainly originate from the surface of E. coli cells rather than the inner part, which suggests that CWS NCs have strong binding ability toward E. coli cells and exert their antibacterial activity from the surface of bacteria rather than from inside the bacteria.

Hence, it is important to reveal the possible interaction between CWS NCs and bacteria. Figures S3 and S17 show that the zeta-potentials of CWS NCs and E. coli cells in saline are both negative, which excludes electrostatic attractions from playing a role in the binding to the bacteria. As we know, both Gram-positive and Gram-negative bacteria have cell walls of peptidoglycan consisting of amino acid residues, which may coordinate to the metal ions in the CWS NCs. To investigate the bacteria-binding ability of CWS NCs to different chemical groups, molecules with different functional groups, including oleyl alcohol (−OH), oleylamine (−NH₂), and oleic acid (−COOH), were used as hydrophobic ligands to transfer CWS NCs from an aqueous dispersion into a hydrophobic solvent (methylene dichloride). As shown in Figure S18, CWS NCs show strong affinity for oleylamine (C₁₇H₃₅CH₂NH₂) compared with oleyl alcohol (C₁₇H₃₅CH₂OH) and oleic acid (C₁₇H₃₅COOH), indicating that the bacteria-binding ability of CWS NCs may be attributed to the interaction between the Cu atom of CWS NCs and the amino groups of the bacterial cell wall. Due to the inherent high reactivity, ROS usually have a short half-life and limited diffusion distance, which will limit their efficacy for killing bacteria. The bacteria-binding ability can facilitate the attachment of nanozymes to the surface of bacteria and...
decrease the diffusion distance of the released ROS, which will greatly facilitate the bacterial killing by ROS. Thus, the bacteria-binding ability is the key feature for CWS NCs in determining their high antibacterial activity.

Toxicity Evaluation of Cu2WS4 Nanocrystals. It is essential to complete a toxicity assessment of nanomaterials prior to their applications in biomedicine. The in vitro cytotoxicity of CWS NCs was tested by using lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. HeLa cells are commonly used as the model cell lines for toxicity assessment in vitro, and they were also adopted in this study.52,53 After incubation with CWS NCs for 24 h, the viability of HeLa cells was approximately 100%, even at the concentration of CWS NCs up to 64 μg mL\(^{-1}\) (Figure 5a). SEM and elemental mapping images (Figure S19) show that few CWS NCs attach to the surface of HeLa cells, though HeLa cells were exposed to 100 μg mL\(^{-1}\) of CWS NCs. The low binding ability of CWS NCs to HeLa cells may originate from the weak interaction between CWS NCs and the cell surface containing lipids and membrane proteins.54 This may account for the low cytotoxicity to HeLa cells.

The hemolytic property of CWS NCs was studied by using rat erythrocytes. As illustrated in Figure 5b, CWS NCs displayed a negligible hemolysis ratio (~1.4%) even at high concentration of CWS NCs (up to 64 μg mL\(^{-1}\)), indicating that CWS NCs have good blood compatibility. Furthermore, the toxicity of CWS NCs was further studied in vivo. After intravenous injection of CWS NC saline dispersions (34 μg mL\(^{-1}\), 100 μL) into female mice (Balb/c), the photomicrographs of the major organs stained with hematoxylin and eosin (H&E) after treatment with saline or CWS NCs for 14 days show no assessable damage or abnormality, which indicate that CWS NCs have no obvious toxicity.

As we know, Cu extensively exists in the earth’s crust and is a required trace element for many aerobic organisms.55,56 The concentration of copper ions released during the antibacterial experiments is very low (<2 μg L\(^{-1}\)), which is far below the acceptable concentration of Cu in drinking water.57 For the in vivo toxicity assessment, the dose of CWS NCs used is 0.17 mg kg\(^{-1}\), much lower than the concentration of Cu in the human body.55 Moreover, tungsten is not an essential element for the human body.58,59 Ionic species of tungsten have low bioaccumulation and can be excreted from the body through feces and urine, which make them have relatively low toxicity to humans.58,59 Sulfur is considered as an essential element for the human body. As known, sulfur-containing compounds at high concentration may be toxic, whereas they can be detoxified by enzymes in the human body.60 Although previous studies and the primary toxicology evaluation in this work have suggested that the toxicity of CWS NCs is limited at the dose used, systematic study of their accumulation, distribution,
metabolism, and potential toxicity should also be carefully performed before their clinical translation.

**In Vivo Treatment of MRSA-Infected Wounds by Cu2WS4 Nanocrystals.** To assess the in vivo antibacterial efficacy of CWS NCs, an animal model of a MRSA-infected wound was constructed. As shown in Figure 6a,b, the wound areas of the mice treated with CWS NCs and vancomycin are much smaller compared to those with saline, which indicates that CWS NCs can effectively promote the MRSA-infected wound healing process to an extent similar to that of vancomycin. Moreover, the numbers of bacteria surviving in the wound tissues after different treatments were quantified by a standard plate count method. Compared with the saline group (Figure 6c), bacteria in the wound tissues after CWS NCs treatment are 5 orders of magnitude fewer at the seventh day post-treatment, indicating their excellent in vivo antibacterial efficacy, which is comparable with the vancomycin group. The wound healing was further evaluated by histological analyses (Figure 6d). H&E staining results show that the wound in the control group (saline) still has a large scab with an incomplete epidermal layer formation at the seventh day post-treatment, whereas the treated groups show small scabs and intact epidermal layers, suggesting good wound healing. Furthermore, Masson’s trichrome staining results also indicate that both the CWS NCs and vancomycin groups show better collagen fiber (blue) deposition than the control group (saline). These results clearly demonstrate that CWS NCs exhibit excellent wound disinfection efficacy in vivo that is even comparable to that of vancomycin at the same dose.

**CONCLUSIONS**

In this work, cuboid-like CWS NCs were successfully synthesized by microwave irradiation in aqueous solution. The small-size CWS NCs (~20 nm) achieved more than 5 log (>99.999%) inactivation efficiency of both E. coli (Gram-negative) and S. aureus (Gram-positive) at very low concentration (~2 μg mL⁻¹) whether in the dark or under ambient light, which is far superior to the typical antibacterial nanomaterials (Ag and TiO₂ nanoparticles) and antibiotics (vancomycin and daptomycin). A mechanism study revealed that CWS NCs have both oxidase-like and peroxidase-like properties and can generate ROS (H₂O₂ and ⋅OH) to kill the bacteria. More importantly, CWS NCs can efficiently attach to the surface of bacteria through the interaction of copper atoms from CWS NCs and amino groups from peptidoglycan in the bacteria cell wall. Hence, the ROS produced by CWS NCs can kill the bacteria in situ, which significantly improves the antibacterial efficiency and reduces the possible collateral damage to normal mammalian cells. In vitro and in vivo experiments indicated that CWS NCs exhibit both excellent antibacterial activity and good biocompatibility. Animal studies showed that CWS NCs have better therapeutic efficacy for the methicillin-resistant S. aureus (MRSA) infected wounds in mice compared to that with vancomycin. This work provides an example of antibacterial nanozyme (CWS NCs) with both enzyme-like properties and bacteria-binding ability. The results show that the bacteria-binding capability is a key factor for enhancing the antibacterial activity of CWS NCs and reducing their toxicity. Moreover, animal experiment results further demonstrate that CWS NCs have excellent antibacterial activity and biocompatibility, suggesting their potential medical use as antibacterial agents.

**METHODS**

Preparation of Cu2WS₄ Nanocrystals. CWS NCs were synthesized in a microwave system (Explorer-48, CEM, USA) working at 2450 MHz. CWS precursor solution was first prepared. Typically, 1 mmol (NH₄)₂WS₄ was dissolved in 100 mL of H₂O. Then 1 mmol CuBr was dispersed in 9 mL of 3-mercaptopropionic acid aqueous solution (0.1 M), and 1 mL NH₃·H₂O was added into the (NH₄)₂WS₄ aqueous solution under stirring; the color of the solution immediately changed to red. This CWS precursor solution (15 mL) was transferred into a vitreous vessel and irradiated with microwave at 140 °C. The reaction was stopped at 4 h. The final products were collected by centrifuging at 8000 rpm for 15 min. The as-prepared CWS NCs were washed five times using ultrapure water and finally dispersed in ultrapure water and stored at 4 °C. The concentration of CWS NCs in aqueous dispersions was determined by using ICP-OES (Optima 5300DV, PerkinElmer, USA).

**Bacterial Culture.** E. coli (ATCC25922), S. aureus (ATCC29213), and MRSA (ATCC43300) were used as antibacterial indicators. A monoclonal of bacteria was cultured in 5 mL of Luria–Bertani (LB) medium (NaCl 10 g L⁻¹, yeast extract 5 g L⁻¹, and tryptone 10 g L⁻¹) under shaking with 220 rpm at 37 °C over 12 h and further diluted to 2 × 10⁶ CFU mL⁻¹ with saline (0.85% NaCl). The concentration of bacteria was estimated by the optical density

**Figure 6. Treatment of MRSA infected wounds by CWS NCs.** (a) Photographs of the wounds on mice after different treatments. (b) Changes of wound area during treatment. (c) Number of the surviving bacteria in the infected wound tissues. (d) H&E staining and Masson’s trichrome staining of infected tissue slices after different treatments at day 7. Scale bar is 500 μm. Van, vancomycin. D, day. Error bars are based on three samples.
value at the wavelength of 600 nm (OD$_{600}$ of 0.1 corresponds to $\sim$10$^8$ CFU mL$^{-1}$).\textsuperscript{51–63}

**In Vitro Antibacterial Activity of Cu$_2$WS$_4$ Nanocrystals.** The bacteria suspension (100 $\mu$L, 2 $\times$ 10$^6$ CFU mL$^{-1}$) was mixed with CWS NCs (100 $\mu$L, 1.4 or 3.4 $\mu$g mL$^{-1}$) in 1.5 mL centrifuge tubes. For the control, sterilized saline was used instead of CWS NCs. Then the bacteria were incubated with CWS NCs at 37 °C for different time. The solutions were diluted 1, 10, 100, or 1000 times with saline. One hundred microliters of the bacteria dilution was spread onto the LB agar plates. After incubation at 37 °C for 18 h, the number of colony forming units (CFU) was counted and recorded. For comparisons, the antibacterial activities of CWS NCs, Ag NPs (20 nm), TiO$_2$ NPs (21 nm), vancomycin, and daptoycin with concentration of 4 $\mu$g mL$^{-1}$ were examined over 2 h at 37 °C by using the aforementioned method. These tubes were covered with aluminum foil to avoid the illumination of ambient light for the antibacterial activity study in the dark.

**Scavenger Quenching Assays.** The role of ROS in bacteria inactivating was evaluated by three kinds of scavenger quenching assays. TEMPO (0.1, 1, and 10 mM), 20 U mL$^{-1}$ of catalase, and 0.1 mM of isopropyl alcohol were used as the scavengers for quenching O$_2^•⁻$, HO$•$, and H$_2$O$_2$, respectively. The E. coli suspensions (100 $\mu$L, 2 $\times$ 10$^6$ CFU mL$^{-1}$) were incubated with CWS NCs (100 $\mu$L, 3.4 $\mu$g mL$^{-1}$), then the scavengers were added into the mixtures. Bacteria concentration was determined by measuring every hour for samples both in the dark and under indoor light. The samples were diluted 1, 10, and 1000 times with saline. Finally, 100 $\mu$L of bacteria dilution was streaked on the LB agar plate and incubated at 37 °C for 18 h before counting the number of CFUs. All assays were performed as triplicates.

**Ellman’s Assay.** The thiol concentrations of glutathione (GSH) were quantified using Ellman’s assay.\textsuperscript{64} Typically, 600 $\mu$L of GSH (0.8 mM) bicarbonate buffer solution (50 mM, pH 8.7) was mixed with 600 $\mu$L of CWS NC aqueous solution (3.4 $\mu$g mL$^{-1}$) in a 2 mL centrifuge tube. Aluminum foil was used to cover these tubes to prevent illumination of light. After being incubated for 1–4 h at room temperature, 314 $\mu$L of Tris-HCl (50 mM, pH 8.5) solution and 6 $\mu$L of DTNB (5 mM) were added into 180 $\mu$L of the mixtures. CWS NCs were removed by centrifugation at 10 000 rpm for 4 min. Then, 150 $\mu$L of supernatant (removed CWS NCs) was transferred into a 96-well plate. The absorbance of each well was measured at 412 nm by a microplate spectrophotometer. Meanwhile, ultrapure water was mixed with the GSH solution as the negative control. Moreover, the loss of GSH was calculated according to the following equation:

$$\text{loss of GSH} (\%) = \frac{(A_n - A_i)/A_i} \times 100\%$$

where $A_n$ represents the average absorbance of the negative control at 412 nm and $A_i$ represents the absorbance of the sample at 412 nm. All assays were performed as triplicates.

**Oxidase-like Activity of Cu$_2$WS$_4$ Nanocrystals.** l-Ascorbic acid (AA) was used as the oxidase substrate to investigate the oxidase-like property of CWS NCs. CWS NC aqueous solutions (3.4 $\mu$g mL$^{-1}$) were added into 10 mM AA aqueous solution. Then, a UV–vis–NIR spectrometer was used to record the changes of UV–vis absorption spectra at different times.

**Peroxidase-like Property of Cu$_2$WS$_4$ Nanocrystals.** The peroxidase mimic property of CWS NCs was evaluated using TMB as a substrate after incubation for 0.5–4.5 h at room temperature. Three groups of assays, including TMB + H$_2$O$_2$, TMB + CWS NCs + H$_2$O$_2$ (dark), and TMB + CWS NCs + H$_2$O$_2$ (indoor light), were investigated at pH = 4. Briefly, 100 $\mu$L of CWS NC aqueous solution (3.4 $\mu$g mL$^{-1}$) with NaAc/HAc buffer (0.2 M/0.2 M), 100 $\mu$L of TMB solution in DMSO (2 mM), and 100 $\mu$L of H$_2$O$_2$ (10 mM) were mixed within 4.5 h. The absorbance of different mixtures for TMB at the wavelength of 650 nm was measured by using a microplate spectrophotometer. All assays were performed as triplicates.

**Detection of Hydroxyl Radical (•OH).** The generation of •OH was investigated by using terephthalate (TA) to form 2-hydroxyterephthalate as a fluorescence indicator.\textsuperscript{27,28} Four experimental groups including TA (5 mM), H$_2$O$_2$ (10 mM) + TA, CWS NCs (34 $\mu$g mL$^{-1}$) + TA + H$_2$O$_2$ (D), and CWS NCs (34 $\mu$g mL$^{-1}$) + TA + H$_2$O$_2$ (L) were evaluated. All samples were incubated at room temperature for 4 h. Afterward, the mixtures were withdrawn and their fluorescence was detected using 312 nm as excitation wavelength. The fluorescence of emission at wavelength of 425 nm is related with •OH production. D: dark; L: indoor light.

Moreover, the generation of •OH signal was further detected by EPR. CWS NC aqueous dispersion (0.2 mL, 34 $\mu$g mL$^{-1}$) and H$_2$O$_2$ solution (0.2 mL, 10 mM) were mixed and stored at room temperature for 4 h. In comparison, the 10 mM H$_2$O$_2$ solution was used as a control. Twenty microliters of S,S-dimethyl-1-pyrrrole-N-oxide (DMPO) was added into the mixture and tested by EPR immediately.

**Bacteria-Binding Ability of Cu$_2$WS$_4$ Nanocrystals.** The bacteria suspensions (500 $\mu$L, $\sim$10$^8$ CFU mL$^{-1}$) were mixed with CWS NCs (500 $\mu$L, 100 $\mu$g mL$^{-1}$) in a 15 mL centrifuge tube at 37 °C for 4 h. Saline was used as the control. After different treatments, the bacteria cells were harvested by centrifugation at 10 000 rpm within 3 min.

TEM samples were prepared as follows: the bacteria cells were washed three times with saline before they were fixed overnight with 2.5% glutaraldehyde aqueous solution for 2 h and dehydrated with graded ethanol solution (30, 50, 70, 90, and 100%). The bacteria samples were further embedded in epoxy resin and polymerized at 70 °C overnight. Thin sections (70–90 nm) were cut by using a Leica EM UC7 ultramicrotome. Grids were stained with uranyl acetate and lead citrate stains.

**In Vitro Cytotoxicity.** The LDH assays were used to evaluate the cytotoxicity of CWS NCs. Briefly, 2 $\times$ 10$^5$ of HeLa cells (100 $\mu$L) suspended in DMEM medium were seeded in a 96-well plate for 24 h at 37 °C and then incubated with different concentrations of CWS NCs (1, 2, 4, 8, 16, 32, and 64 $\mu$L of 1 $\mu$L) suspended in DMEM medium (150 $\mu$L). HeLa cells cultured without CWS NCs and with a medium containing 1% Triton X-100 were used as low control and high control, respectively. After being incubated at 37 °C for 24 h, 100 $\mu$L supernatant was transferred carefully into other 96-well plates. After the LDH reaction mixtures were added to each well, the plates were incubated in the dark for 15 min at room temperature. Microplate spectrophotometer was used to measure the absorbance of all samples at 495 nm. The cell viability can be calculated by the following formula:

$$\text{cell viability} = 100\% - (OD_{495 \ (test \ sample)})$$

$$- OD_{495 \ (low \ control)}/OD_{495 \ (high \ control)}$$

$$- OD_{495 \ (low \ control)} \times 100\%$$

The cytotoxicity of CWS NCs was further studied by using MTT assays. After HeLa cells and CWS NCs were incubated at 37 °C for 24 h, the medium was removed and 50 $\mu$L of MTT solution was added into each well of a 96-well plate for 6 h. The resulting crystals formed during the MTT assay were dissolved in 150 $\mu$L of DMSO. The native cells and medium were used as controls. The absorbance of all samples was measured by a microplate spectrophotometer at 490 nm (OD$_{490}$). The cell viability can be calculated by the following formula:

$$\text{cell viability} = OD_{490 \ (sample)}/OD_{490 \ (control)} \times 100\%$$

**Hemolysis Assay of Cu$_2$WS$_4$ Nanocrystals.** All animal procedures were performed in accordance with the Guidelines for
Care and Use of Laboratory Animals of Nanjing University and experiments were approved by the Animal Ethics Committee of Nanjing University.

Hemolysis assay was performed by using fresh animal blood from a 10 week old female SD rat (Qinglong Mountain Company, China). First, the red blood cells (RBCs) were collected via centrifugation at 1500 rpm for 15 min and washed three times with saline. Then 0.3 mL of a RBC saline suspension (5%) was resuspended in 6 mL of saline. RBC suspensions (0.1 mL) were added into 1 mL of CWS NC saline dispersions with different concentrations (4, 8, 16, 32, and 64 μg mL⁻¹). The mixtures were incubated at 37 °C for 3 h. Ultrapure water and saline were used as the positive control and negative control, respectively. Finally, the absorbance of the supernatant was measured at 540 nm after centrifugation at 12 000 rpm for 15 min. The ratio of hemolysis was calculated by the formula:

\[
\text{hemolysis ratio (\%) = } \frac{(A_S - A_N)/(A_P - A_N)}{100}
\]

where \(A_S\) represents the absorbance of RBCs exposed to CWS NCs, \(A_N\) represents the absorbance of RBCs exposed to saline, and \(A_P\) represents the absorbance of RBCs exposed to ultrapure water. All assays were performed as triplicates.

In Vivo Treatment of MRSA-Infected Wounds. The bacteria-infected wound models were established on the back of female mice (Balb/c, 6–8 weeks, 18–22 g, Qinglong Mountain Company, China). The mice were anesthetized by injecting 100 μL of chloral hydrate (3.5%) in the abdominal cavity. The wounds of \(d = 4 \text{ mm}(\sim 12 \text{ mm}^2)\) were established by a surgical scissor after removing the dorsal hair of mice. After being infected by 100 μL of MRSA suspension (1 × 10⁷ CFU mL⁻¹) within 24 h, all wounds were covered by medical gauze. Then the nine mice were divided into three groups. The mice with bacteria-infected wounds were treated by CWS NC gel containing 0.3% agar (20 μL, 50 μg mL⁻¹) with 24 h interval for 7 days. Meanwhile, saline gel (0.3% agar) and vancomycin gel (0.3% agar, the same concentration with CWS NCs) were used as the controls. The mice were sacrificed at therapeutic day 4 and day 7. To count the number of CFUs in the wound, the homogenized wound tissues were then placed in saline by ultrasonication for 15 min at a working power of 104 W. The obtained bacteria suspensions were plated onto LB agar plates and were further cultured for 18 h at 37 °C.

Histological Analysis. The treated mice were sacrificed at therapeutic day 7. The wound tissues were harvested and fixed in 4% paraformaldehyde solution. Then the tissues were paraffinized, sectioned, and then analyzed by Masson’s trichrome staining and H&E staining. All samples were examined using a microscope (Olympus IX-71).

In Vivo Toxicity Evaluation. Six mice were divided into two groups. CWS NC saline dispersions (100 μL, 34 μg mL⁻¹) were intravenously injected into mice. Saline was used as the control. At day 14, the mice were sacrificed, and then the major organs (spleen, liver, heart, lung, and kidney) were harvested and fixed by using 4% paraformaldehyde solution. Finally, all organs tissues were sectioned and stained by H&E. The samples were examined using a microscope (Olympus IX-71).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.9b03868.

Details of chemical reagents, characterization, study of the binding ability of CWS NCs to cells, and experiment of interaction between CWS NCs and different chemical groups; Figures S1–S19 showing size distribution, AFM image and height distribution, zeta-potential of CWS NCs, with other typical antibacterial nanomaterials (PDF)

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