Antibacterial Activity and Mechanism of ZnO/ Cu²⁺-Chitosan/Montmorillonite

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Abstract: A new composite antibacterial material ZnO/Cu²⁺-Chitosan/Montmorillonite (ZCCM) was prepared with montmorillonite as carrier, Zn(Ac)₂·2H₂O, Cu(NO₃)₂·3H₂O and chitosan as raw materials. ZCCM was characterized by X-ray diffraction, nitrogen physical adsorption, scanning electron microscopy and energy dispersion spectrometry. The antibacterial activity of ZCCM against *Escherichia coli*, *Salmonella typhimurium*, and *Staphylococcus aureus* was evaluated by minimal inhibitory concentration, minimum bactericidal concentration and the influence of growth curves. ZCCM displays excellent antibacterial activity which is higher than ZnO-Montmorillonite, Cu²⁺-Montmorillonite and ZnO/Cu²⁺-Montmorillonite. In addition, the antibacterial mechanism of ZCCM was investigated by analyzing bacterial morphology, integrity of cell membrane, lipid peroxidation and the effect of histidine on antibacterial activity of materials. It is found that cell morphologies of bacteria are damaged and bacterial cells are shrunken. With the increase of cell membrane permeability, the intracellular dissolved matters leak continuously. What's more, the reactive oxygen species are generated and biomacromolecules are oxidized.

Key words: montmorillonite; chitosan; ZnO; Cu²⁺; antibacterial mechanism

1 Introduction

The emergence of multi-resistant organisms has led to ineffective treatment of currently available drugs, posing a great threat to public health and food technology sectors^[1]. Safety issues related to drug-resistant microorganisms have emphasized the need to modify traditional antimicrobial compounds or find other promising alternatives. Many inorganics have strong antibacterial properties, including Cu^[2], Zn^[3], and ZnO^[4], but the use conditions and occasions of them are very limited^[5,6]. In addition, lots of organic antibacterial agents have the characteristics of fast sterilization speed but easy to decompose^[7].

While, composite antibacterial materials^[8-10] always exhibit superior comprehensive performance than a single component and broaden the application

range of antibacterial materials. Studies had reported that when organic antibacterial agents were loaded on inorganic silicate clay, the hydroxyl and charge characteristics on surface of silicate clay could help control the release of organic antibacterial agents and improve antibacterial stability of organic antibacterial agents, the morphology and pore structure characteristics of salt clay could load organic antibacterial agents into it and effectively reduced the biotoxic side effects of organic antibacterial agents^[11]. Besides, the fabrication of montmorillonite (MMT) decorated with lysozyme-modified silver nanoparticles was reported^[12]. Coupling the bactericidal activity of the lysozyme with AgNPs, along with the high porous structure and large specific surface area of MMT, prevented aggregation of AgNPs and promoted nanomaterial-bacteria interactions, resulting in a greatly enhanced bactericidal capability.

MMT is used as an inorganic carrier for antibacterial materials because of its good adsorption ability, drug-carrying capability, high cation-exchange capacity, high surface area and chemical inertness^[13-15]. Chitosan (CS) is a natural biopolymeric cation that can be embedded in MMT by cation exchange and hydrogen bonding processes, and resulting bio-

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nanocomposite exhibits interesting structural and functional properties^[16]. It has been reported that modification of MMT with quaternized CS could enhance the antimicrobial activity of MMT in a weak acidic or weak basic medium^[17]. The negatively charged interlayer region of the MMT is primarily filled with exchangeable positively charged ions^[18]. Thus, ZnO and Cu²⁺ can be accommodated in the interlayer space to provide a long lasting action time for the material^[19, 20]. ZnO is a widely-used antibacterial agent that inhibits both Gram-negative and Gram-positive bacteria^[21]. Cu²⁺ possesses the advantages of broader spectrum of antimicrobial activity compared with copper nanoparticles and better oxidation resistance properties than silver nanoparticles^[22].

According to the above analysis, ZnO/Cu²⁺-Chitosan/Montmorillonite (ZCCM) was prepared by loading CS, ZnO and Cu²⁺ onto MMT. The antibacterial activity of ZCCM against *Salmonella typhimurium* (*S. typhimurium*), *Escherichia coli* (*E. coli*), and *Staphylococcus aureus* (*S. aureus*) was evaluated with minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Besides, the antibacterial mechanism of ZCCM was determined by measuring the bacterial morphology, integrity of cell membrane, lipid peroxidation and the effect of histidine (His) on antibacterial activity, which provides scientific basis for the development of such antibacterial materials.

2 Experimental

2.1 Materials

MMT (cation exchange capacity, CEC = 124 mmol/100g), CS (degree of deace stylation $\geq 95\%$), Zinc acetate dehydrate ($\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$) and copper nitrate hydrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$) were purchased from Xuzhou Tianhong Chemical Reagent Co., Ltd. The strain of *S. aureus* (ATCC6538), *E. coli* (CMCC44102), and *S. typhimurium* (ATCC14028) were purchased from Shanghai Luwei Microbial SCI.&TECH. Co., Ltd.

2.2 Synthesis of ZCCM

CS in 1% acetic acid was prepared (pH = 4.0). Then MMT was introduced into the solution (CS to MMT mass ratios of 1/12). The mixture was continuously stirred at 60 °C for 12 h. After washing with distilled, the CS/MMT was dried and ground to powder. CS/MMT powder was dispersed in $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$ (0.75 times CEC of the MMT) aqueous

solution under the condition of 60 °C water bath for 24 h; NaOH (2.5 times of $\text{Zn}(\text{Ac})_2 \cdot 2\text{H}_2\text{O}$) was added to the solution. Then the mixture was washed, dried and calcined at 125 °C. ZnO-CS/MMT was dispersed in $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (0.75 times CEC of the MMT) aqueous solution under the condition of 60 °C water bath for 24 h. After washing and drying, ZCCM was ground, and then passed through a 300-mesh sieve.

The ZnO contents in ZCCM, ZnO/Cu²⁺-MMT, and ZnO/MMT were 6.6wt%, 6.6wt%, and 6.7wt%, respectively. The Cu²⁺ concentrations in ZCCM, ZnO/Cu²⁺-MMT, and Cu²⁺-MMT were 5.4wt%, 5.4wt%, and 5.3wt%, respectively. The CS concentration in ZCCM was 6.0wt%.

2.3 Characterization

Various characterizations techniques were used to test the morphologies and structures of antibacterial materials, including X-ray diffraction (XRD, Bruker D8 advance), scanning electron microscopy (SEM, Hitachi SU8020), coupled with energy dispersion spectrometry (EDS, HORIBA EX-350), and physical adsorber (BET, Micromeritics ASAP 2460).

2.4 Antibacterial activity test

The inhibitory efficacy of antibacterial materials against *S. typhimurium*, *E. coli*, and *S. aureus* was evaluated by MIC and MBC. Antibacterial materials were added to a bacterial suspension having a concentration of about 10⁵ CFU/mL, and then cultured in a 37 °C incubator for 24 h with shaking. The lowest concentration of antibacterial materials that resulted in no visible turbidity was considered as the MIC^[23]. Futher 100 μL of the samples were transferred onto the NA medium and incubated at 37 °C for 24 h. The MBC was defined as the minimum concentration of antibacterial materials that caused more than 99.9% of bacterial population killed^[24]. Growth curves of *S. typhimurium* were determined based on the absorbing value of OD₆₀₀.

2.5 The bacterial morphology

To determine the morphological changes of bacteria, *S. typhimurium* cells were treated with ZCCM. The samples were incubated at 37 °C for 12 h, respectively. And samples were taken every two hours during this period. After incubation, cells were harvested by centrifugation (5000 rpm, 10 min) and washed twice with phosphate buffer solution, then fixed with 2.5% (v/v) glutaraldehyde in phosphate buffer solution overnight at 4 °C. After that, cells were further dehydrated using a graded series of ethanol (5%, 25%, 50%, 75%, 95%, and 100%) followed dried.

Finally, the samples were fixed on SEM support, then sputter-coated with gold under vacuum, followed by microscopic examinations using a scanning electron microscope (ZEISS EVO18).

2.6 Integrity of cell membrane

The integrity of the cell membrane was determined by measuring the release of cell constituents including proteins and nucleic acids according to the method described by Zhao *et al.*^[25] with some modifications. *S. typhimurium* cells from liquid medium were collected by centrifuged for 10 min at 3500 rpm, washed three times with phosphate buffer solution, and resuspended in the same buffer. Series of 10 mL of cell suspension were incubated for 6 h at 37 °C under agitation in the presence of antibacterial materials at 2 mg/mL. Then, each sample was collected and centrifuged at 3500 rpm for 10 min. Control group containing the MMT-treated bacterial supernatant was tested similarly. Deionized water was used as blank control, and the change in concentration of nucleic acids in supernatant was estimated by detecting absorbance at 260 nm. The change in concentration of proteins in supernatant was determined by Bradford assay. The mixed reagents of 1 mL of diluted supernatants and 5 mL of Coomassie Brilliant Blue G-250 reagents were thoroughly mixed and allowed to stand for 3 minutes, and the absorbance were measured at 595 nm.

2.7 lipid peroxidation

To determine the effect of antibacterial material on lipid peroxidation of bacteria, 2.0 mg/mL antibacterial materials were mixed with equal value *S. typhimurium* suspension and incubated at 37 °C for 8 h, then added equal value 10% ice-cold TCA and further incubated for 20 min. The mixtures were centrifuged at 4000 rpm for 10 min, then 2.0 mL of supernatant was added to 2.0 mL of 0.6% TBA and boiled for 30 min. After samples were cooled to room temperature, it were centrifuged at 4000 rpm for 4 min after cooled to room temperature, and the absorbance of supernatant at 532 nm was determined by a UV/visible spectrophotometer (UV 3300, Mapada).

2.8 The effect of His

As an active oxygen scavenger, His can inhibit the bactericidal effect of antibacterial materials^[26]. *S. typhimurium* cells were activated and cultured, then inoculated in three groups of NB medium. Then, antibacterial materials having a concentration of MBC, antibacterial materials and His, and MMT were added respectively. The mixtures were cultured at 37 °C and determined based on the absorbing value of OD₆₀₀.

3 Results and discussion

3.1 Characterization analysis

X-ray diffraction is an effective method to study the microstructure of materials. And the XRD patterns of MMT, CS/MMT, and ZCCM are compared in Fig.1. The XRD patterns of ZCCM show peaks related to ZnO at 2θ of 31.01°, 35.07°, and 36.03°, which are indexed to the (100), (002), and (101) planes, respectively, indicating that ZnO is synthesized on MMT. MMT shows a characteristic reflection peak at 5.79°, indicating the layer-to-layer spacing of 1.52 nm (d_{001} spacing). After intercalation of CS macromolecules into MMT interlayer apace, the layer-to-layer spacing of MMT was enlarged^[27]. While, the characteristic reflection peak of CS/MMT is found at 6.87°, with corresponding d_{001} spacing of 1.31 nm. The characteristic reflection peak of ZCCM is found at 5.85°, with corresponding d_{001} spacing of 1.51 nm (0.2 nm larger than CS/MMT), and the main diffraction peaks assign to the MMT phases modify both in terms of shape and intensity. These results suggest that CS is adsorbed on the inner and outer surfaces of MMT, and the immobilized of Cu²⁺ and ZnO leads a slight local distortion of the layered structures.

Fig.1 XRD patterns of MMT, CS/MMT, and ZCCM

The morphologies of MMT and ZCCM are examined by SEM (Fig.2). Fig.2(a) is MMT particles which has a relatively smooth surface. While, the surface of ZCCM has a small amount of particles and becomes rough (Fig.2(b)). It can be seen that the introduction of CS, ZnO, and Cu²⁺ changes the shape of MMT particles, which can also be analyzed from XRD patterns (Fig.1). Moreover, to reveal the distribution of CS, ZnO, and Cu²⁺ in the ZCCM, elemental mapping is performed. Table 1 indicates that there is no Zn, Cu, and N in the composition of MMT. Therefore, Zn, Cu, and N (N came from CS) are selected for element mapping to detect the distribution of CS, ZnO, and Cu²⁺. As presented in Figs.2(c)-2(f), the SEM and

corresponding mapping distribution of ZCCM showed the existence of Zn, Cu, and N, which reflects that ZnO, Cu²⁺, and CS are distributed in ZCCM.

Fig.2 SEM images of as-prepared samples: (a) MMT, (b) ZCCM, (c) SEM images ZCCM, (d) the corresponding elemental mappings of Zn, (e) Cu, and (f) N

Table 1 Each element content of MMT

Element	Weight/%	Atomic/%
O	59.49	71.71
Mg	2.42	1.91
Al	7.74	5.53
Si	30.36	20.85
Totals	100.00	100.00

The pore structure of antibacterial materials is an important factor affecting their antibacterial effect. As shown in Table 2, ZCCM has the largest surface area (107.84 m²/g) amongst all samples, which makes it have less opportunities to agglomerate and more opportunities to contact with bacteria. When ZnO is introduced into MMT, the surface area (67.75 m²/g) and pore volume (0.12 cm³/g) of ZnO-MMT decrease to 32.83 m²/g and 0.09 cm³/g, respectively. However,

after Cu²⁺ is incorporated into MMT, the surface area and pore volumes of Cu²⁺-MMT increase to 102.83 m²/g and 0.15 cm³/g, respectively. This might be due to the fact that Cu²⁺ enters MMT pores and increases its specific surface area; while, ZnO entered interlayers of MMT and covers its surface, blocking part of the interlayer and surface pores, thereby reducing the specific surface area; and CS leads to an increase in the porous structure of MMT^[28].

Table 2 Surface characteristics and pore structure of materials

	MMT	ZnO-MMT	Cu ²⁺ -MMT	ZnO/Cu ²⁺ -MMT	ZCCM
Surface area/(m ² /g)	67.75	32.83	102.83	92.07	107.84
External Surface area/(m ² /g)	59.92	29.99	74.94	74.50	80.62
Pore volume/(cm ³ /g)	0.12	0.09	0.15	0.15	0.17
Pore diameter/nm	7.05	10.73	5.73	6.37	6.36

3.2 Antibacterial activity

The MIC and MBC of ZnO-MMT, Cu²⁺-MMT, ZnO/Cu²⁺-MMT, and ZCCM against three test bacteria are shown in Table 3. It is seen that the MBC of the ZCCM against *S. aureus*, *E. coli*, and *S. typhimurium* are 1.0, 2.0, and 2.0 mg/mL, respectively, lower than ZnO/Cu²⁺-MMT, Cu²⁺-MMT, and ZnO-MMT, which demonstrates that ZCCM shows higher antibacterial activity against the three test bacteria. Based on systematically characterization and analyses, it could be attributed to the synergistic effect resulted from CS, ZnO, and Cu²⁺. Besides, the composite material prepared by using Cu²⁺, Zn²⁺, cetylpyridinium, and MMT, was reported that it has synergistic antibacterial effect against *E. coli* and *S. typhimurium*^[29]. This indicates that single antibacterial agents play synergistic bactericidal effect in composite antibacterials.

The growth curves of *S. typhimurium* treated with ZCCM are shown in Fig.3. *S. typhimurium* reaches exponential phase rapidly with the absence of ZCCM. At the concentration of 0.5×MIC, ZCCM inhibits

Table 3 The MIC and MBC values of antibacterial material against *S. aureus*, *E. coli*, and *S. typhimurium*

	<i>S. aureus</i>		<i>E. coli</i>		<i>S. typhimurium</i>	
	MIC/(mg/mL)	MBC/(mg/mL)	MIC/(mg/mL)	MBC/(mg/mL)	MIC/(mg/mL)	MBC/(mg/mL)
MMT	-	-	-	-	-	-
ZnO-MMT	4.0	8.0	4.0	8.0	8.0	16.0
Cu ²⁺ -MMT	8.0	16.0	8.0	16.0	8.0	16.0
ZnO/Cu ²⁺ -MMT	1.0	2.0	2.0	4.0	2.0	4.0
ZCCM	0.5	1.0	1.0	2.0	1.0	2.0

55.0% of bacteria growth after 12 h of incubation. The ZCCM of 1.0×MIC reduces 94.4% of bacteria growth. At the concentration of 2.0×MIC, ZCCM inhibits bacterial growth completely and has bactericidal ability, which is consistent with the result of MBC. It could be seen that the higher the concentration of bacteria, the better the inhibition effect on bacteria.

Fig.3 The growth curves of *S. typhimurium*

3.3 Bacteria morphology

Fig.4 SEM images of *S. typhimurium* and treated with ZCCM for 0 h (a), 4 h (b), 8 h (c), and 12 h (d)

Morphological changes of bacterial cells were evaluated by SEM analysis to understand the antibacterial mechanism. The electron micrographs of *S. typhimurium* cells treated with or without ZCCM are shown in Fig.4. Untreated cells show regular and typical short rod-like morphology with a plump and

smooth surface (Fig.4(a)). However, bacterial cells are damaged when treated with ZCCM at the MBC for 4 h (Fig.4(b)), showing depressions on the surfaces. *S. typhimurium* cells treated with ZCCM for 8 h (Fig.4(c)) and 12 h (Fig.4(d)) show adhesions, irregular wrinkles and accumulation of cell debris on its surface. This confirms the adsorption of ZCCM on bacteria, indicating that ZCCM could improve the contact opportunities with bacteria and thus show antibacterial performance advantages. Taken together, these SEM images are strong evidence that bacterial structure are damaged by the antibacterial action of ZCCM, and the degree of damage of cells is exacerbated over time.

3.4 Cell membrane permeability

Information on the release of cell constituents including proteins and nucleic acids reveals the integrity of cell membrane. When cell membrane is intact, proteins and nucleic acids mainly exist inside the cell. If cell membrane is damaged, the proteins and nucleic acids inside the cell would leaked out, increasing the concentration of proteins and nucleic acids outside the cell. It is evident that proteins (Fig.5(a)) and nucleic acids (Fig.5(b)) of *S. typhimurium* cells are released into cell suspension and the order of leakage concentration is: ZCCM > ZnO/Cu²⁺-MMT > Cu²⁺-MMT > ZnO-MMT. The tendency is in accordance with the results of antibacterial activity test. These clearly indicates that the bacterial cell membrane integrity has been compromised after exposed to antibacterial materials. Cell membrane is a semi-permeable membrane that is placed in close proximity to the cell wall and its main function is to selectively absorb and transport substances^[30]. The experimental results show that ZCCM destroys the cell membrane structure and apparently enhances the permeability of membrane for proteins and nucleic acids in cells. It confirms that increasing of membranous permeability plays an important role in antibacterial action of ZCCM.

Fig.5 (a) Leakage of reducing proteins and (b) nucleic acids from *S. typhimurium*

3.5 Lipid peroxidation injury

Fig.6 The concentration of MDA in *S. typhimurium*

The toxicity of reactive oxygen species (ROS) to bacteria was attributed to their high reactivity and oxidizing property^[31]. Such reactive species mainly included superoxide anion (O_2^-), hydroxide (OH^-), and hydrogen peroxide (H_2O_2). Unsaturated fatty acids in cell membrane are easily oxidized by ROS and participate in a series of chain reactions leading to destruction of biomolecules. Lipid peroxidation damage could be detected by measuring malondialdehyde (MDA), which is an oxidation product of polyunsaturated fatty acids^[32]. As shown in Fig. 6, the addition of ZnO-MMT, ZnO/Cu²⁺-MMT, and ZCCM (2.0 mg/mL) significantly increase the concentration of MDA in *S. typhimurium*, indicating that antibacterial materials cause oxidative injury and produce ROS during antibacterial process^[33]. It is seen that ZnO/Cu²⁺-MMT and ZCCM do not cause more MDA than ZnO-MMT at the same concentration, which

indicates that ZnO plays an important role in causing lipid peroxidation injury. Several studies indicate that excessive formation of ROS as the main mechanism is responsible for ZnO antibacterial activity^[34,35].

3.6 The effect of His

As shown in Fig. 7, after the addition of His, bacteria cells in the four groups of experiments are grown. After adding His to culture for 12 h, ZnO-MMT, Cu²⁺-MMT, ZnO/Cu²⁺-MMT, and ZCCM inhibit the growth of bacteria by 71.7%, 60.0%, 20.5%, and 48.3%, respectively. So, His as an active oxygen scavenger has inhibitory effect on the antibacterial activity of materials and follows the order of: ZnO/Cu²⁺-MMT > ZCCM > Cu²⁺-MMT > ZnO-MMT. These indicate that intracellular ROS is generated after antibacterial materials treatment, which is in accordance with the results of lipid peroxidation experiment. It is noted that the inhibitory effect of His on ZnO/Cu²⁺-MMT is greater than that of ZCCM. This may be due to that CS increase the antibacterial activity of ZCCM^[36]. A smaller amount of Cu²⁺ do not cause bacterial cells lipid peroxidation according to the result of experiment (Fig. 6). However, Cu²⁺ could catalyze the production of ROS, which might further lead to their toxicity^[37,38]. The similar result is deduced based on the effect of His on antibacterial activities of antibacterial materials. The results of this experiment verify that active oxygen sterilization mechanism plays an important role in the sterilization process of ZCCM.

Fig.7 Effects of His on antibacterial activities of (a) ZnO-MMT, (b) Cu²⁺-MMT, (c) ZnO/Cu²⁺-MMT, and (d) ZCCM

4 Conclusions

Compared with ZnO-MMT, Cu²⁺-MMT, and ZnO/Cu²⁺-MMT, ZCCM showed higher antibacterial activity against *S. aureus*, *E. coli*, and *S. typhimurium*. Based on present research, the antibacterial mechanism of ZCCM included that it directly destroyed the morphology of bacterial cell; the permeability of cell membrane increased; bacteria produced ROS and were damaged by lipid peroxidation. The results demonstrated that ZCCM had great antibacterial activity and potentially be applied as antibacterial material in food, packaging, cosmetics, medical, health care and other industries.

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