Application of thiabendazole/bentonites hybrids as efficient antibacterial agent against Escherichia coli and Staphylococcus aureus

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Abstract
Clay minerals are commonly used in agriculture, light industry, cosmetics, pharmaceuticals and other fields because it has adsorption and cation exchange properties. In this work, cetyltrimethylammonium bromide (CTAB) as a modifier and thiabendazole (TBZ) as intercalation agent to prepare composite, and their potential use as antibacterial agent was evaluated. Thiabendazole is a kind of antiparasitic drug which began to be used abroad in the early 60’s. But soon after, it was discovered that it had a strong anti-fungal effect on many molds that affect vegetables, fruits, nuts and other crops. At present, it has been widely used in prevention and control abroad. Unmodified bentonite, modified bentonite and intercalated bentonite were characterized by x ray diffraction (XRD), Fourier transform infrared (FTIR), Energy dispersive x-ray spectroscopy (EDS) and Scanning electronic microscopy (SEM), and the antimicrobial properties of the compounds was investigated by Minimum inhibitory concentrations (MIC), Minimum bactericidal concentration (MBC) and Kirby-Bauer disc agar diffusion method. Among the three compounds, the ratio of modified bentonite to thiabenazole was 5:1 showed the highest antibacterial activity against Escherichia coli and Staphylococcus aureus. In the final, there are some figures about the SEM of E. coli and S. aureus that can indicate antibacterial of the antibacterial agent.

1. Introduction
Clay minerals are an important class of natural materials which are natural and low-cost widely available phyllosilicates [1–3]. Naturally occurring bentonite are usually superimposed between layers of hydrated ions by ultrathin crystalline aluminosilicates (around 1.0 nm). Therein, bentonite is an aluminosilicate in which octahedral sheet of Al³⁺ grid is sandwiched with two tetrahedral sheets of Si⁴⁺ [4–6]. The interlayer of bentonite is held together by electrostatic and hydrogen bonds, and other metal ions with different valence states can easily replace the central metal ions of the octahedron. So bentonite has excellent cation exchange property [7, 8]. Intercalation modification is a process in which exchangeable ions are replaced by intercalation agents and fixed between layers to increase the intercalation distance.

Bentonite is a common drug excipient and active substance. The intercalation of organic species into layered inorganic solids provides a useful and convenient method for the preparation of organic-inorganic hybrids that contain both inorganic host and organic guest properties in a single material [9]. In the preparation of composite materials, this property of bentonite is often used to improve the compatibility of inorganic and organic compounds in the composite materials. Using bentonite as the framework and carrier, functional substances are introduced into the bentonite to increase the interval between its layers. Through physical or chemical processes,
organic monomers or polymers are evenly distributed in the bentonite lamellae structure, and the nanocomposites with stable properties are prepared.

In recent years, bentonite clay embedded with drug molecules has attracted great interest due to its novel physical and chemical properties. Roxana-Mihaela Apetrei [10] filled dioctadecyl dimethyl ammonium chloride (DDAC) and methylene blue (MB) into bentonite, which through intercalation is able to occupy the interlayer site of the clay. In another study, novel molybdenum disulfide-bentonite were prepared used to inhibit bacterial growth [11]. Furthermore, organo-bentonite was synthesized by intercalation modification of sodium bentonite with cetyl trimethyl ammonium bromide (CTAB) and non-ionic nonylphenol polyoxyethylene ether (NPE) in order to achieve controlled—release [12]. This study also use CTAB as intercalating agent to occupy the interlayer site in order for antibacterial agent to enter the bentonite interlayer. CTAB is white or light yellow crystal to powder, has a stimulating odor, soluble in isopropyl alcohol, soluble in water, a large number of foams when oscillating, and anionic, non-ionic. It has excellent properties of penetration, softening, emulsification, antistatic, biodegradation and sterilization.

Thiabendazole (TBZ) is one of the various pesticides and it has fungicide action and is used before packing and transportation [13]. The pure product is white tasteless powder and it inhibits mitosis by binding to tubulin, thus affecting the growth and development of fungi. Thiabendazole is an internal fungicide with protective and therapeutic effects, which has shown good inhibitory effect on many microorganisms, especially on molds. It is also a benzimidazole that is used worldwide against gastrointestinal parasites and absorbed in the digestive tract [14, 15]. In addition, TBZ also has certain antibacterial effect on fungus. Thiabendazole is a kind of high-efficiency, broad-spectrum, universal fungicide. It can prevent and control many kinds of plant fungal diseases, used to treat fruits and vegetables after harvest, can prevent and control some diseases in storage, has been widely used at home and abroad. Figure 1 indicates the photographs of the structural formula of thiabenazole.

Bourakadi et al [16] prepared a new type of biological composite membrane using CS/PVA and modified thiobenlimidazole-bentonite as the substrate. Compared with pure CS/PVA membrane, the composite membrane had better antibacterial activity against Staphylococcus aureus and Escherichia coli. Setareh Ghiassi et al [17] synthesized silver bentonite nanocomposites in the layered space of bentonite using plant extract as reducing agent. However, most of the methods do not modify bentonite and do not take advantage of the advantage that the distance between bentonite layers can be increased. So by using the cation exchange property of bentonite, TBZ can be inserted into bentonite to prepare antibacterial agent. TBZ intercalated into bentonite has been deeply studied from an experimental point of view [1, 13, 18, 19].

The objectives of this study are to investigate the effects of thiabendazole addition and dosage on the structure and properties of modified bentonite, and to investigate the antibacterial effects of the resulting compounds. And the purpose of this experiment is to develop a kind of bentonite composite material with antibacterial and hygroscopic functions, and apply it in the field of packaging.

2. Experimental

2.1. Material and chemicals
The bentonite sample was donated by Shaanxi University of Science & Technology. The sample presented a cationic exchange capacity (CEC) of 78.2 mmol/100 g. Thiabendazole (C_{10}H_{7}N_{3}S, M = 201.25 gmol{\textsuperscript{−1}}) was purchased from Aladdin Industrial Corporation, (98% analytical grade). Cetyltrimethylammonium bromide (CTAB, C_{16}H_{33}BrN, M = 364.45 gmol{\textsuperscript{−1}}) was acquired from Da Mao Chemical Reagent Factory. Dimethyl sulfoxide (CH_{3}SO, M = 78.14 gmol{\textsuperscript{−1}}) was purchased from Da Mao Chemical Reagent Factory.

2.2. CTAB modified bentonite samples (BC)
12.0 g bentonite was dispersed in 500 ml deionized water and energetically stirred for 30 min. After being swell completely, 2.4 g CTAB was added into the solution for 1h. The systems was maintained under orbital at 333 K. The modified bentonite was isolated by vacuum filtering, washed by deionized water until AgNO_{3} could not
detect bromine ions. The sample prepared were dried at 353 K for 24 h and then grinding for characterization. The CTAB modified bentonite was named as BC (where B denoted bentonite, C denoted CTAB).

2.3. Synthesis of bentonite/drug hybrids

1 g TBZ was dispersed in the 250 ml HCl solution (0.05 mol l\(^{-1}\)) and then 5 g BC was added into the solution. The mixture stirred until BC well dispersed in solution. After ultrasonic, the obtained product dried at 353 K, and then ground and sieved. The final powder was named BC5T1 in short (where T denoted TBZ and the number 1 represented the mass of TBZ was 1 g and the number 5 represented the mass of BC was 5 g). With the same preparation processes, BC3T1 and BC4T2 were prepared. The preparation method of bentonite/drug hybrids is shown in figure 2.

2.4. Antibacterial activity

2.4.1. Minimum inhibitory concentrations (MIC)

The antibacterial activity of the synthesized compounds was evaluated against *Escherichia coli* and Gram-positive bacteria *Staphylococcus aureus*. The MICs of the compounds were determined by the broth microdilution method. Bacterial strains were cultivated with nutrient broth as growth media. The samples concentration were prepared from 25 μg ml\(^{-1}\) to 800 μg ml\(^{-1}\). The final DMSO concentration was less than 0.5%. The volume of 100 μl of bacterial culture and 100 μl of sample at various concentrations were dispensed in 96-well microtitration plates. The plates were incubated at 310 K, 24 h. Assays were carried out in triplicate. And then take a duplicate values.

2.4.2. Minimum bactericidal concentration (MBC)

Three concentrations higher than MIC (including MIC concentration) were selected for each sample, so there are nine sample concentrations.

Mix 1 ml of the sample and 10 μl of bacterial solution in each test tube. After mixing, 100 μl mixture were added to the agar plates and coated evenly with sterilized coating stick. The plates were incubated at 310 K, 24 h. Assays were carried out in triplicate.

2.4.3. Kirby-Bauer disc agar diffusion method

The experimental samples are consistent with those in MBC experiment. 100 μl bacterial solution were added into agar plates and evenly coated. The same size pieces of qualitative filter paper (d = 8 mm) are placed in different positions on the agar plate. 50 μl sample solution of different concentrations were drawn with pipette. And then drop onto the surface of circular piece of papers placed symmetrically. Drops of 50 μl of 0.9% NaCl solutions were added to the middle circle as a blank control. The plates were incubated at 310 K, 24 h and observed the size of bacteriostatic circle.

3. Results and discussion

3.1. Characterization of bentonite/drug hybrids

The intercalation of thiabendazole into the interlayer space of bentonite has been confirmed by x-ray diffraction where the value of the basic distance within lamellar bentonite layers can be determined. XRD patterns of the unmodified bentonite, modified bentonite and BCnTn are presented in figure 3. According to the bragg’s equation, for the raw bentonite, the XRD patterns of the samples (Bent) exhibited the principal bentonite reflection at 2θ (d\(_{001}\) = 1.263 nm) and the layer spacing of bentonite increases from 1.263 nm to 1.784 nm after modification by CTAB. This increase in the interlayer space was obtained with surfactant CTAB, which has a relatively long alkyl chain. All d-spacing of modified bentonite were larger than the unmodified bentonite, which clearly confirmed that the CTAB or TBZ had been effectively intercalated into bentonite.
After TBZ intercalated modified bentonite experiment, a shift of the \( d_{001} \) value to higher angles was observed (1.449 nm). This decrease of around 0.3 nm was attributed to the diminution of water content due to the fungicide intercalation (or adsorption) and addition of dilute hydrochloric acid [17].

Fourier transform infrared (FTIR) was used to characterize different samples. Figure 4 shows that the absorption band of modified bentonite is only the superposition of CTAB and unmodified bentonite absorption band. Without the formation of new bands or the absence of bands indicates that there is no bond cooperation between the modifier and the original soil, but only physical adsorption. BC5T1 is the sample with thiabendazole intercalated bentonite. Compared with BC, BC5T1 can be seen that there is obviously the formation of new bands (3430, 1636 and 1387), indicating that there is a bonding relationship between thiabendazole and modified bentonite. And the EDS results (figure 5) shows that Element S appeared in the energy spectrum, but the bentonite does not contain element S, indicating that the TBZ intercalated into the bentonite.

The images of bentonite before and after modification are shown in figure 6. The particle shape of the unmodified bentonite is irregular. A similar behavior has been reported by Khadija El Bourakadi [16]. Nevertheless, the scanning electronic microscopy (SEM) result of modified bentonite shows that the physical appearance of bentonite particles changed remarkably. As stated before, the x-ray diffraction of the modified bentonite shows an improvement of the interlayer space. The platelets of bentonite modified by CTAB were stacked together in a disordered model to form agglomerates in some parts. This also directly indicates that the layer spacing of bentonite increases after modification, which corresponds to the previous increase of \( d_{001} \) value.
Figure 5. EDS results of the BC5T1.

Figure 6. SEM images of unmodified bentonite (a) and modified bentonite (b).
Figure 7. SEM images of BC5T1 (a), BC3T1 (b) and BC4T2 (c).

Table 1. Antimicrobial activities of synthesized compounds (BC5T1, BC3T1 and BC4T2).

| Compounds | E. coli (μg ml⁻¹) | S. aureus (μg ml⁻¹) |
|-----------|------------------|---------------------|
| BC5T1     | 100              | 100                 |
| BC3T1     | 200              | 200                 |
| BC4T2     | 200              | 200                 |
According to the XRD results above, after TBZ intercalated bentonite, the interval between the bentonite layers was smaller than that of the unintercalated ones. These results are also in good agreement with the SEM images (figure 7), which provide an important information about the morphology of the silicate.

Table 2. The sterilization of synthesized compounds (BC5T1, BC3T1 and BC4T2).

| Compounds | E. coli (μg ml⁻¹) | S. aureus (μg ml⁻¹) |
|-----------|-------------------|---------------------|
| BC5T1     | 200               | 200                 |
| BC3T1     | 400               | 400                 |
| BC4T2     | 400               | 400                 |

Figure 8. Photographs of antibacterial properties of the compound against two kinds of bacteria.
nanocomposites. Compared with modified bentonite, the intercalated bentonite becomes more closely, and looser in structure than the original soil.

### 3.2. Antibacterial activity

MIC and MBC results of TBZ intercalated bentonite against the two bacteria are shown in tables 1 and 2 respectively. The highest antibacterial activity was found in the synthesized compounds BC5T1, which exhibited an inhibitory effect against *E. coli* and *S. aureus* with a MIC values 100 μg ml⁻¹. The product BC3T1 and BC4T2 showed moderate inhibitory effect against *S. aureus* and *E. coli* with a MIC of 200 μg ml⁻¹. As shown in table 2, the MBC value of BC5T1 was 200 μg ml⁻¹ for *E. coli* and *S. aureus*. And BC3T1 BC4T2 have a larger MBC than the other samples.

According to these results, the major factor for the antibacterial activity of the synthesized compounds was due to ratio of modified bentonite to TBZ. In general, the antibacterial effect was best when the ratio of bentonite to TBZ was 5:1. It is speculated that the dissolution rate of TBZ is inversely related to the concentration of TBZ. The lower the concentration of TBZ is, the more fully the dissolution will be.

The antimicrobial activity of the samples was further measured by the Kirby-Bauer disc agar diffusion method (figure 8). Nine samples (the concentration of 1, 2 and 3 samples is decreasing) were consistent with the samples in the MBC experiment and the middle circle papers was controlled trials. No antibacterial activity was observed as bentonite did not exhibit any inhibitory effect on the *S. aureus* and *E. coli* growth. However, clear inhibition zones were observed for other intercalated nanocomposites around each specimen. When the concentration drops from 1 to 3, it can be seen that, at the same sample proportion, the size of bacteriostatic circle decreases with the reduction of compounds. In addition, the compound has a relatively large inhibitory ability against *S. aureus* and a relatively small inhibitory effect on *E. coli*.

The antibacterial mechanism of interlayer nanocomposite includes the first step of adsorbing drugs on the surface of bacterial cells through electrostatic adsorption. Drug molecules bind to the plasma membrane, destroying the integrity of the membrane. As a result of this leakage of cytoplasmic components, microorganisms gradually die and a distinct inhibition zone emerges.

Figure 9 shows the scanning electron microscopy of bacteria. Figure A shows the SEM image of *E. coli* without the addition of bentonite antibacterial agent. It can be clearly seen from the figure that *E. coli* is rod-shaped, with blunt round ends and complete morphology. Figure b shows the SEM image of *E. coli* after the addition of bentonite antibacterial agent. It can be seen from the figure that the *E. coli* has many cracks and fractures, contraction and many forms are incomplete, indicating that the added bentonite antibacterial agent has a destructive effect on *E. coli* and has certain antibacterial effect. Similarly, the SEM image of *S. aureus*
without adding bentonite antibacterial agent is shown in figure C. It can be seen from the figure that S. aureus is full and smooth. Figure D shows the SEM image of Staphylococcus aureus after the addition of bentonite antibacterial agent. It can be seen from the figure that S. aureus has contraction phenomenon and some are eluted, which indicates that the bentonite antibacterial agent also has a certain antibacterial effect on S. aureus. In conclusion, the bentonite antibacterial agent has certain antibacterial effect on both Gram-negative bacteria E. coli and Gram-positive bacteria S. aureus, indicating that the prepared bentonite antibacterial agent has a certain broad-spectrum antibacterial effect.

4. Conclusion

Thiabendazole was loaded onto the CTAB modified bentonite in different proportions (B:T = 5:1, B:T = 3:1, B: T = 4:2). The effect of ratio of thiabendazole on the structure of bentonite as well as the structural, morphological and antibacterial properties of BCnTn composites was investigated. The results obtained in this approach showed that the interval of modified bentonite and intercalated bentonite increased when compared with the unmodified bentonite. After the modification of bentonite, the layers became loose. MIC and MBC values also changed with the change of thiabendazole content. Moreover, by observing the images of the two bacteria before and after contact with antimicrobial agents, we can also see the antibacterial properties of antimicrobial agents. Accordingly, the whole results presented here suggested that in the future perspectives, the modified bentonite compound has an excellent potential as antibacterial agent. In addition, it was found that the bentonite compound had good stability and remained unchanged in dry environment at least one year. The samples can be used in the field of antibacterial coating and antibacterial packaging. Not only can effectively prevent and control bacteria, but also can play a important role in extending the shelf life of product.

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Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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References

[1] Cavalcanti G R S et al 2019 Thiabendazole/bentonites hybrids as controlled release systems Colloids Surf B Biointerfaces 176 249–55
[2] Sanchez I M, Alvarez V A and Ollier K P 2019 Acid-treated bentonite as filler in the development of novel composite PVA hydrogels Journal of Applied Polymer Science 136 47663
[3] Alexander J A et al 2016 Physicochemical characteristics of surface modified Djiah-Monkin bentonite Particulate Science and Technology 36 287–97
[4] ZHOU C H 2011 An overview on strategies towards clay-based designer catalysts for green and sustainable catalysis Applied Clay Science 53 87–96
[5] Kostenko L S et al 2019 Bentonites with grafted aminogroups: synthesis, protolytic properties and assessing Cu(II), Cd(II) and Pb(II) adsorption capacity Applied Clay Science 172 49–56
[6] Kadhom M and Deng R 2019 Thin film nanocomposite membranes filled with bentonite nanoparticles for brackish water desalination: a novel water uptake concept Microporous and Mesoporous Materials 279 82–91
[7] Kaufhold S et al 2019 Mg and silica release in short-term dissolution tests in bentonites Applied Clay Science 172 106–14
[8] Zhuang G et al 2015 A new ball milling method to produce organo-montmorillonite from anionic and nonionic surfactants Applied Clay Science 104 18–26
[9] Joshi G V et al 2009 Montmorillonite as a drug delivery system: intercalation and in vitro release of timolol maleate International Journal of Pharmaceutics 374 53–7
[10] Apetrei R M and Camurlu P 2020 The effect of montmorillonite functionalization on the performance of glucose biosensors based on composite montmorillonite/PAN nanofibers Electrochimica Acta 353 136484
[11] Wang W et al 2020 Adsorption toward Co(II) and inhibitory effect on bacterial growth occurring on molybdenum disulfide-montmorillonite hydrogel surface Chemosphere 248 126025
[12] Yan H, Chen X et al 2020 Synthesis and assessment of CTAB and NPE modified organo-montmorillonite for the fabrication of organo-montmorillonite/alginate based hydrophobic pharmaceutical controlled-release formulation Colloids Surf B Biointerfaces 191 110983
[13] Lombardi B 2003 Optimization of parameters and adsorption mechanism of thiabendazole fungicide by a montmorillonite of North Patagonia, Argentina Applied Clay Science 24 43–50
[14] Barron-bravo O G et al 2020 Susceptibility of entomopathogenic nematodes to ivermectin and thiabendazole Chemosphere 253 126658
[15] Zhang C et al 2015 Synthesis and biological evaluation of thiabendazole derivatives as anti-angiogenesis and vascular disrupting agents Bioorg Med Chem 23 3774–80
[16] Bourakadi K E et al 2019 Chitosan/polyvinyl alcohol/thiabendazole–montmorillonite bio-nanocomposite films: mechanical, morphological and antimicrobial properties Composites Part B: Engineering 172 103–10
[17] Ghiassi S et al 2018 Plant-mediated bio-synthesis of silver–montmorillonite nanocomposite and antibacterial effects on gram-positive and -negative bacteria Journal of Nanostructure in Chemistry 8 353–7
[18] Gamba M et al 2017 Insight into thiabendazole interaction with montmorillonite and organically modified montmorillonites Applied Clay Science 137 59–68
[19] Mothilal K K et al 2004 Synthesis, x-ray crystal structure, antimicrobial activity and photodynamic effects of some thiabendazole complexes J Inorg Biochem 98 322–32