

# Antibacterial Effect of Chitosan-Modified Fe<sub>3</sub>O<sub>4</sub> Nanozymes on *Acinetobacter baumannii*

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The aim of this study was to determine whether the antibacterial activity of chitosan-modified Fe<sub>3</sub>O<sub>4</sub> (CS@Fe<sub>3</sub>O<sub>4</sub>) nanomaterials against *Acinetobacter baumannii* (*A. baumannii*) is mediated through changes in biofilm formation and reactive oxygen species (ROS) production. For this purpose, the broth dilution method was used to examine the effect of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on bacterial growth. The effects of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on biofilm formation were measured using a semi-quantitative crystal violet staining assay. In addition, a bacterial ROS detection kit was used to detect the production of ROS in bacteria. The results showed that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles had a significant inhibitory effect on the colony growth and biofilm formation of drug-resistant *A. baumannii* ( $p < 0.05$ ). The ROS stress assay revealed significantly higher ROS levels in *A. baumannii* subjected to CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticle treatment than the control group ( $p < 0.05$ ). Thus, we demonstrated for the first time that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles had an inhibitory effect on *A. baumannii* in vitro, and that the antibacterial effect of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on drug-resistant *A. baumannii* was more significant than on drug-sensitive bacteria. Our findings suggest that the antibacterial mechanism of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles is mediated through inhibition of biofilm formation in drug-resistant bacteria, as well as stimulation of *A. baumannii* to produce ROS. In summary, our data indicate that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles could be used to treat infections caused by drug-resistant *A. baumannii*.

**Keywords:** Nanozyme, antibacterial activity, *Acinetobacter baumannii*

## Introduction

Nanozymes, nanomaterials with enzyme-like catalytic activity, are a new generation of artificial enzyme that can catalyze the substrates of biological enzymes under near physiological conditions. Their catalytic behavior, reaction kinetics and catalytic mechanisms are similar to those of natural enzymes. In 2019, Jiao *et al.* [1] at the Chinese Academy of Sciences first reported that Fe<sub>3</sub>O<sub>4</sub> magnetic nanoparticles exhibited mimetic enzyme activities similar to those of natural peroxidases. Since then, more than 300 types of nanomaterials have been found to have enzymatic activity. Compared with natural enzymes and traditional mimic enzymes, nanozymes have high catalytic efficiency, multiple functions, good stability, low cost and easy large-scale preparation [2]. In the biomedical field, nanozymes can be used in clinical detection, tumor diagnosis and treatment, cell protection, anti-aging, and other important areas related to human health [3].

The Fe<sub>3</sub>O<sub>4</sub> nanozyme is a typical metal oxide nanozyme. Fe<sub>3</sub>O<sub>4</sub> nanoenzymes have the same catalytic characteristics as horseradish peroxidase (HRP), and can catalyze H<sub>2</sub>O<sub>2</sub> to destroy biofilm matrices [5]. However, due to their toxic effect on cells, subsequent studies have developed various modification methods to reduce their cytotoxicity. Chitosan nanoparticles can be applied to deliver antimicrobial drugs, which further enhances the efficiency and stability of the antimicrobial agent [6]. The proposed antibacterial mechanism of chitosan under acidic conditions involves the binding of the negatively charged bacteria to the protonated amino group on the chitosan molecular chain, leading to disruption of the cell, and subsequent inhibition of bacterial growth and reproduction [4]. Tian *et al.* [7] demonstrated that the chitosan-modified Fe<sub>3</sub>O<sub>4</sub> (CS@Fe<sub>3</sub>O<sub>4</sub>) nanozyme had lower cytotoxicity than the sodium oleate-modified Fe<sub>3</sub>O<sub>4</sub> nanozyme, thereby leading to the clinical application of CS@Fe<sub>3</sub>O<sub>4</sub> nanozymes.

*Acinetobacter baumannii* (*A. baumannii*) is a gram-negative bacilli mainly associated with community-acquired pneumonia and hospital infection. *A. baumannii* can secrete many virulence factors, which are related to its pathogenicity and drug resistance [8]. In addition, the extracellular matrix secreted by *A. baumannii* can adhere to the body, and most *A. baumannii* can form biofilms, which further enhance its pathogenicity [9]. *A. baumannii* infection leads to prolonged disease and high mortality. In addition, most *A. baumannii* strains have now

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developed resistance to a variety of drugs, including quinolones, aminoglycosides, broad-spectrum cephalosporins and other common clinical antibiotics [10]. In 2014, China's Ministry of Health issued technical guidelines for the prevention and control of hospital infections caused by multidrug-resistant bacteria, clearly defining multidrug-resistant bacteria as bacteria that are resistant to three or more types of antibiotics in clinical use at the same time. In recent years, the increasing occurrence of multidrug-resistant *A. baumannii* has had a serious impact on human health. Indeed, *A. baumannii* has become one of the most intractable pathogens in global healthcare institutions [11].

Here, CS@Fe<sub>3</sub>O<sub>4</sub> nanozymes were used in in vitro antibacterial experiments to determine their effects on *A. baumannii* biofilms, as well as their impact on reactive oxygen species (ROS) levels in *A. baumannii*.

## Materials and Methods

### Bacteriostasis Assay Using the Broth Dilution Method

A 500 µg/ml working solution of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles (Ruixi Biotechnology, China) was prepared in double-distilled water. An *A. baumannii* colony was diluted in 4 ml Mueller-Hinton broth medium to prepare a bacterial suspension working solution ( $1 \times 10^7$  CFU/ml, OD 600 nm = 0.06) (Huankai Microbial Technology, China). The CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles were added to the bacterial suspension (final concentration of 125 µg/ml), and the final working solution was placed in 96-well plates and cultured for 24 h. In the control group, double-distilled water replaced the CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles.

### Semi-Quantitative Crystal Violet Staining Assay

Biofilm formation was measured after 24 and 48 h, as described previously [12, 13]. An *A. baumannii* suspension ( $1 \times 10^7$  CFU/ml, OD 600 nm = 0.06) was prepared. CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles were diluted to a concentration of 500 µg/ml. In the experimental group, the CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticle working solution (50 µl) and bacterial suspension (150 µl) were added to 96-well plates. In the control group, the CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticle working solution was replaced with double-distilled water. After incubation for 24 h, the culture medium was removed, and each well was washed three times with PBS. The biofilms were then fixed with methanol (200 µl) for 15 min, air-dried, and stained with 2% crystal violet solution (200 µl) for 5 min at room temperature. After the excess stain was removed, samples were washed three times with PBS and air-dried at room temperature. Adherent crystal violet was dissolved in 95% alcohol (200 µl) and the plates were shaken for 20 min. The optical density (OD) was measured at 570 nm using a microplate reader (Biotek, USA).

### Microscopic Analysis of Crystal Violet Staining

CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticle working solution (125 µl) and bacterial suspension (375 µl) were added to 24-well plates containing a clean sterile glass cover slip. In the control group, the CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticle working solution was replaced with double-distilled water. After incubation for 24 and 48 h, the glass cover slips were gently rinsed with PBS to remove non-adherent bacteria and then stained with 2% crystal violet for 30 min. Slides were washed with water to remove excess crystal violet and then visualized using an Olympus CKX41 microscope (Japan).

### ROS Stress Test

The ROS stress test was carried out as described previously [14] using a bacterial ROS fluorescence detection kit (Beibo Biotechnology, China). The experimental group was treated with a CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticle working solution (500 µg/ml) prepared using double-distilled water. The control group was treated with double-distilled water (200 µl). The fluorescence intensity (excitation light was 488 nm, emission light was 530 nm) was measured with a fluorescence enzyme reader (Biotek). After the absorbance had been measured, the bacterial solution was centrifuged at 12,000 ×g for 20 min, then placed on a glass slide and observed using an Olympus fluorescence microscope BX43.

### Statistical Methods

The paired *t*-test was used to analyze the biofilm inhibition and ROS stimulation data. The Wilcoxon nonparametric test was used to analyze the 24 h data. All statistical analyses were performed using SPSS version 20.0 (IBM), and all figures were generated using GraphPad Prism 5.01 (GraphPad Software). *p* < 0.05 was considered statistically significant.

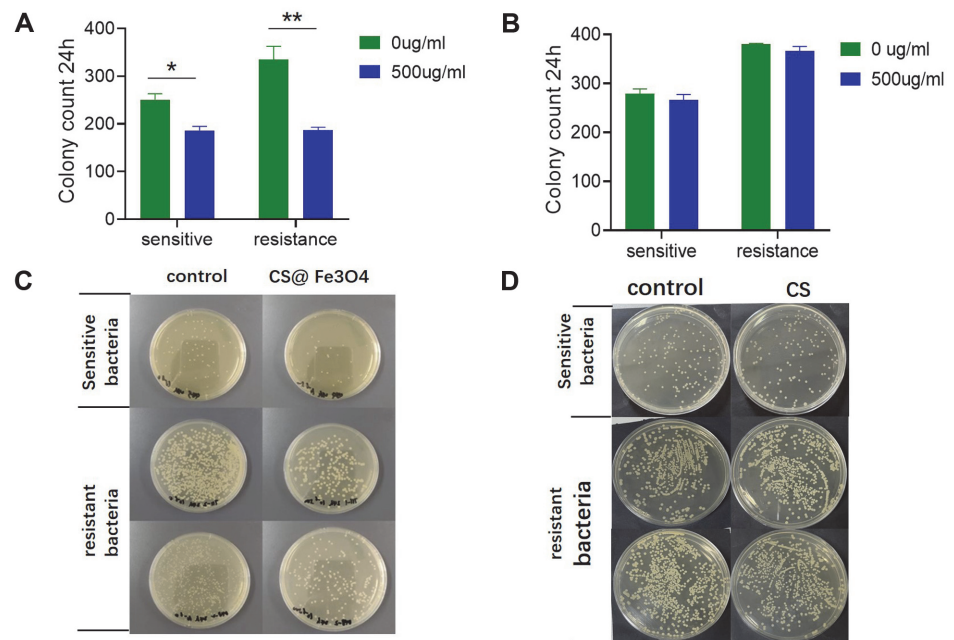
## Results

### CS@Fe<sub>3</sub>O<sub>4</sub> Nanoparticles Inhibit *A. baumannii*

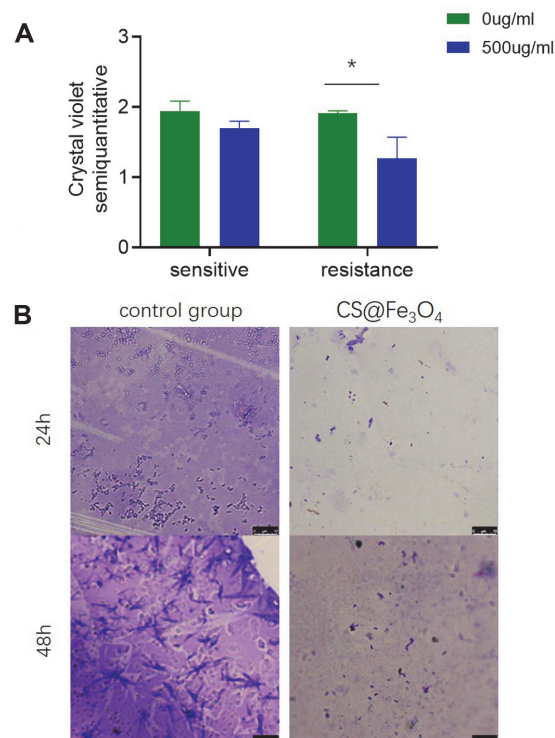
Based on our drug sensitivity test, drug-sensitive and drug-resistant bacteria were selected (Table S1). Next, we performed in vitro bacteriostatic experiments and found that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles had a significant inhibitory effect on *A. baumannii* compared with the control group in both drug-sensitive (*p* < 0.05) and drug-resistant (*p* < 0.01) bacteria (Fig. 1A). The number of colonies growing in the experimental group was significantly less than in the control group (Fig. 1C). Importantly, chitosan alone (at the same concentration) had no antibacterial effect on the number of colonies. (*p* > 0.05) (Fig. 1B), It also had no effect on the colony formation of *A. baumannii* (Fig. 1D).

### CS@Fe<sub>3</sub>O<sub>4</sub> Nanoparticles Inhibit the Biofilm Formation of Drug-Resistant *A. baumannii*

The crystal violet quantitative assay revealed that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles had an inhibitory effect on the biofilm formation of drug-resistant *A. baumannii* (*p* < 0.05), but not drug-sensitive bacteria (Fig. 2A). Microscopic

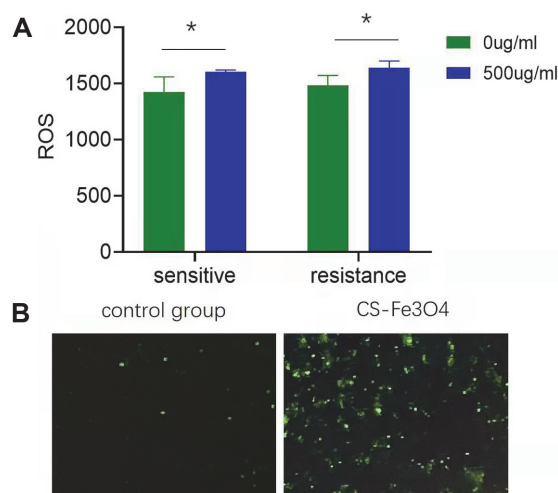


**Fig. 1.** The inhibition rate of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles (A) and chitosan (B) on *A. baumannii* was detected by the colony formation assay. Data are shown as the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ . The broth dilution method was used to determine the inhibition rate of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles (C) and chitosan (D) on *A. baumannii*.



**Fig. 2.** A. The semi-quantitative crystal violet staining assay was used to determine the amount of bacterial biofilm. OD values are given as the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ . B. Microscopic examination of the effects of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on biofilm formation were observed by crystal violet staining after 24 and 48 h.

examination of the biofilms revealed that the biofilm of the control group was large, dense and dark purple, whereas the biofilm of the experimental group was significantly smaller with light purple staining (Fig. 2B).



**Fig. 3. A.** Quantitative analysis of the effects of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on bacterial oxidative stress-activated oxygen content were determined using a commercially available bacterial ROS fluorescence detection kit. Data are shown as the mean  $\pm$  SD of three independent experiments. \*  $p < 0.05$ . **B.** Examination of the effects of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on ROS levels in *A. baumannii* by fluorescent microscopy.

#### CS@Fe<sub>3</sub>O<sub>4</sub> Nanoparticles Stimulate *A. baumannii* to Produce ROS

A bacterial ROS kit was used to quantify ROS production in the bacteria. ROS levels were significantly increased in the experimental group for both drug-sensitive and drug-resistant ( $p < 0.05$ ) bacteria (Fig. 3A). Microscopic examination revealed an increase in both the number of ROS-positive bacteria, as well as the staining intensity in the experimental group compared to the control group (Fig. 3B).

## Discussion

*A. baumannii* has become one of the main sources of infection in intensive care unit patients, and a large number of strains are now resistant to common antibiotics [15]. Thus, the development of a new drug for multidrug-resistant *A. baumannii* is critical. One of the pathogenic properties of *A. baumannii* is the production of a large amount of biofilm. Bacterial biofilms have a protective effect on bacteria, and thus, inhibition of biofilm function or formation could reduce bacterial resistance [16]. Many chronic wound infections have been shown to be associated with biofilms [17]. *A. baumannii* can form biofilms that attach to the surface of ventilators and tracheal intubations [18], and can also occur in the respiratory tract and skin of patients [19]. The formation of biofilms not only enhances the resistance of bacteria to antibiotics [20], but also stimulates the host immune system to release a large number of cytokines to trigger an immune response.

Fe<sub>3</sub>O<sub>4</sub> nanozymes exhibit triple enzyme-like activities including peroxidase, catalase, and superoxide dismutase [21], and are a typical metal oxide nanoenzyme that exhibits catalytic properties similar to HRP. Their catalytic activity is associated not only with the pH, reaction temperature and H<sub>2</sub>O<sub>2</sub> concentration of the solution, but also with the nanoparticle size, with smaller particles leading to higher catalytic activity [22]. The Fe<sub>3</sub>O<sub>4</sub> nanozyme was shown to catalyze H<sub>2</sub>O<sub>2</sub> to destroy the biofilm matrix of *Pseudomonas aeruginosa*, and its bactericidal effect was more than 10 times higher than that of using H<sub>2</sub>O<sub>2</sub> alone [23]. Recently, iron sulfide and Fe<sub>3</sub>O<sub>4</sub> nanozymes were shown to not only destroy the biofilm formed by *Salmonella typhimurium*, but also prevent its formation [24]. In addition, nanomaterials based on cerium ions have been shown to inhibit biofilm formation [25].

ROS are an important component of the immune response, and play a crucial role in eliminating invading pathogens, as well as promoting oxidative stress and damaging cellular proteins and lipids [26]. Mammalian macrophages and neutrophils can directly internalize foreign pathogens and degrade them in lysosomes in a ROS-dependent manner. Many lysosomal enzymes catalyze the production of ROS in an acidic environment, leading to the inactivation of biological macromolecules such as nucleic acids and proteins [27]. Nanozymes have the ability to regulate ROS levels [21, 28], which may account for the antibacterial activity of nanozymes [29]. Here, we found that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles can stimulate ROS production in both drug-sensitive and drug-resistant *A. baumannii*, disrupting internal metabolism and exerting an antibacterial role.

We found that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles had a more significant inhibitory effect on drug-resistant bacteria than drug-sensitive bacteria. Thus, we next sought to determine the mechanisms mediating the antibacterial effect of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles. We found that CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles could inhibit the formation and function of drug-resistant *A. baumannii* biofilms, and significantly increase the ROS content in the drug-resistant *A. baumannii*. Furthermore, we found that in drug-sensitive *A. baumannii*, CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles stimulated the production of ROS, but had no significant inhibitory effects on the biofilm. These findings may explain the differential antibacterial effects of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on drug-sensitive and drug-resistant *A. baumannii*, and highlight the need to develop new antibiotics for the treatment of drug-resistant bacteria [30].

In conclusion, our study is the first description of the antibacterial effects of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles on drug-resistant and drug-sensitive strains of *A. baumannii*. In addition, the antibacterial mechanism of CS@Fe<sub>3</sub>O<sub>4</sub> nanoparticles was preliminarily explored. Future studies will examine the molecular pathways and targets within the bacteria. This study provides a solid foundation for the development of antimicrobial agents to treat drug-resistant *A. baumannii*.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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