



# Investigation of functional selenium nanoparticles as potent antimicrobial agents against superbugs



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## ABSTRACT

Developing highly effective antibacterial agents is important for a wide range of applications. However, the emergence of multiple antibiotic-resistant bacteria poses a public health threat. Many developed agents have limited practical application due to chemical instability, low biocompatibility, and poor long-term antibacterial efficiency. In the following study, we synthesize a synergistic nanocomposite by conjugating quercetin (Qu) and acetylcholine (Ach) to the surface of Se nanoparticles (Qu–Ach@SeNPs). Quercetin has been reported to exhibit a wide range of biological activities related to their antibacterial activity and acetylcholine as a neurotransmitter, which can combine with the receptor on the bacterial cell. Arrows indicate NPs and arrowheads indicate compromised cell walls. The study demonstrated how Qu–Ach@SeNPs exhibit a synergistically enhanced antibacterial performance against the multidrug-resistant superbugs (MDRs) compared to Qu@SeNPs and Ach@SeNPs alone. Qu–Ach@SeNPs are effective against MDRs, such as Methicillin-resistant *Staphylococcus aureus* (MRSA), at a low dose. The mechanistic studies showed that Qu–Ach@SeNPs attach to the bacterial cell wall, causing irreversible damage to the membrane, and thereby achieving a remarkable synergistic antibacterial effect to inhibit MRSA. The findings suggested that the synergistic properties of quercetin and acetylcholine enhance the antibacterial activity of SeNPs. In this way, Qu–Ach@SeNPs comprise a new class of inorganic nano-antibacterial agents that can be used as useful applications in biomedical devices.

### Statement of significance

The Qu–Ach@SeNPs have low cytotoxicity when tested on normal human cells *in vitro*. Qu–Ach@SeNPs are effective against MDRs, such as Methicillin-resistant *S. aureus* (MRSA), at a low dose. Importantly, Qu–Ach@SeNPs showed no emergence of resistance. These results suggest that Qu–Ach@SeNPs have excellent antibacterial activities. These agents can serve as good antibacterial agents against superbugs. Our data suggest that these antibacterial agents may have widespread application in the field of medicine for combating infectious diseases caused by MDRs, as well as other infectious diseases.

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## 1. Introduction

Infectious diseases caused by bacteria have attracted wide spread attention during the past few decades, afflicting millions of people worldwide each year [1,2]. Multidrug-resistant superbugs (MDRs) have emerged worldwide due to the abuse of antibiotics. MDRs, including *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [3–5],

are becoming a major public health problem [6]. In the few past years, numerous non-antibiotic drugs have been developed, such as cationic polymers [7–12], antimicrobial peptides [13–15], photothermal/photodynamic agents [16,17]. While these non-antibiotic drugs show promising antibacterial activity, there are still shortcomings associated with these methods. For example, cationic polymers showed a certain degree of hemolysis *in vivo*, proving a barrier for utilization as an antibacterial agent during infection [18]. In addition, antimicrobial peptides are natural antimicrobial agents but are easily degraded under physiological conditions. Due to these problems, finding safe and effective non-antibiotic antibacterial agents has become a major goal in medical research. Recently, there has been renewed interest in developing

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nanoparticle-based antibacterial agents, due to their antibacterial efficiency performances and relatively low toxicity in human cells. It has been reported that Ag nanoparticles exhibit excellent antibacterial activity by binding the thiol groups in enzymes, generating reactive oxygen species (ROS), and disrupting the conserved bacterial respiratory chain in different kinds of bacteria. However, further studies showed that AgNPs caused potential adverse responses by the immune system, both *in vitro* and *in vivo* [19,20].

Due to the antibacterial potential of metal nanoparticles, and the potential toxic disadvantage of silver nanoparticles, we focused our research on the widely studied selenium nanoparticle. Selenium nanomaterials, including nanoparticles, nanorods, nanowires, and nanotubes, have been widely explored and used in many fields, owing to the excellent anticancer activities and low toxicity [21–23]. At present, there are a few studies concerning the antibacterial properties of selenium nanomaterials. For example, in one study *S. aureus* growth was inhibited in the presence of selenium nanoparticles *in vitro* [24]. In another study Yip et al. studied the effects of fabric padded with highly stable selenium nanoparticles (PSP-SeNPs) as antifungal and antibacterial materials [25]. These prior studies used selenium nanoparticles that were not targeted to bacteria. Using compounds with targeting ability to bacteria on the surface of selenium nanoparticles will improve the antibacterial ability of selenium nanoparticles. Our results showed that, when compared with the selenium nanoparticles without targeting ability to bacteria, the antibacterial activities of the bacterial-targeting selenium nanoparticles were significantly improved. This suggests that selenium nanoparticles may be useful in biomedical devices.

We designed and synthesized Qu@SeNPs, Ach@SeNPs and Qu–Ach@SeNPs to test the antimicrobial activity of these nanomaterials. Quercetin (Qu), a bactericidal compound found in plants, has been shown to have antibacterial action. Its activity is limited to certain bacterial species, such as *S. aureus* [26]. Acetylcholine, a neurotransmitter, has the ability to interact with the acetylcholine-receptor and increase the permeability of cell membranes. Acetylcholine is also used as a permeability-active unit in antibacterial compounds [27,28]. Acetylcholine, as a drug, for treating vasospastic disease, such as Raynaud's disease, embolism angitis and treatment of tinnitus, deaf, ear vertigo et al. (0.1 g at a time) Nederberg et al. have reported that acetylcholine did not induce significant toxicity in mice [27]. Taking advantage of the antimicrobial properties of quercetin and the cell membrane targeting of acetylcholine, we attempted to prepare a synergistic material of acetylcholine chloride and quercetin on Selenium nanoparticles for use against superbugs. The following experiments show that Qu–Ach@SeNPs has efficient antibacterial and bactericidal activities, proving they are good antimicrobial alternatives for the development of antimicrobial agents. The minimal inhibition concentration (MIC) of Qu–Ach@SeNPs showed high antibacterial and bactericidal activities against superbugs. Zone of inhibition tests, and LIVE/DEAD bacterial viability assays were used to characterize the antibacterial activity of Qu–Ach@SeNPs. In addition, the specific antimicrobial mechanism of Qu–Ach@SeNPs action for has been clarified. Furthermore, a cytotoxicity assay showed low toxicity to human embryonic kidney cells (HEK 293T) *in vitro*.

## 2. Materials and methods

### 2.1. Materials and reagents

Na<sub>2</sub>SeO<sub>3</sub>, quercetin, acetylcholine chloride (Ach) and sodium borohydride (NaBH<sub>4</sub>, 98% min) were purchased from Sigma–Aldrich Chemical Co. *Escherichia coli* ATCC 8739, *S. aureus* ATCC

6538, *P. aeruginosa* ATCC 27853, *A. baumannii* ATCC11038 were from Guangdong Microbiology Culture Center. MDR *E. coli* E76227, MDR *P. aeruginosa* BJ915, MDR *K. pneumoniae* R12K2637, MDR *S. aureus* ATCC 25213, MDR *A. baumannii* GIM 1.650 were from domestic hospitals in China. Ultrapure MilliQ water (18.2 MW) was used in all experiments and all solutions were stored in the refrigerator at 4 °C.

### 2.2. Synthesis and characterization of SeNPs

A mixture of quercetin (C<sub>15</sub>H<sub>10</sub>O<sub>7</sub>·2H<sub>2</sub>O) (1 mmol/L, dissolved in methanol), Na<sub>2</sub>SeO<sub>3</sub> (0.5 mmol/L, dissolved in water) and acetylcholine chloride (1 mmol/L, dissolved in water) was stirred in the presence of acetic acid for 10 min in an ice-water bath, then an aqueous solution of NaBH<sub>4</sub> (1 mL 0.01 mol/L) was added dropwise with vigorous stirring [29]. After half an hour of gentle stirring, the solution turned red. Finally, NPs were centrifuged at 8000 rpm for 10 min and the red precipitate was collected. The particles were washed three times with PBS. Ach@SeNPs were synthesized using Na<sub>2</sub>SeO<sub>3</sub> (0.5 mmol/L) and acetylcholine chloride (2 mmol/L) without the addition of quercetin. Qu@SeNPs were synthesized using Na<sub>2</sub>SeO<sub>3</sub> (0.5 mmol/L) and quercetin (2 mmol/L). The morphologies of SeNPs were observed with transmission electron microscopy (TEM, Hitachi H-7650). The Zeta potential was tested by Nano-ZS instrument (Malvern Instruments Limited).

### 2.3. Antibacterial activity test of SeNPs

A 96-well microtiter plate dilution was applied to determine the minimum inhibitory concentration (MIC). Logarithmic-phase bacteria (OD<sub>600nm</sub> 0.5) were diluted with nutrient broth 100 folds and then a sterile pipette was used to add 190 µL of the logarithmic-phase bacterial suspension (the final density of bacteria was 2.5 × 10<sup>7</sup> CFU/mL) and 10 µL of both varied concentrations of SeNPs. Then added 100 µL turn draw down, decreasing as double dilution, and finally every hole filled 200 µL, the concentration of each sample set of three parallel samples, incubated them at 37 °C for 24 h. The MIC was the concentration at which no significant increased.

The zone of inhibition test was employed to observe the inhibitory effects of quercetin (15.1 mg/mL), acetylcholine chloride (9.1 mg/mL), mixture of quercetin and acetylcholine chloride (15.1/9.1 mg/mL) and SeNPs (25 mg/mL) intuitive. Logarithmic-phase bacteria (OD<sub>600nm</sub> 0.5) were diluted to approximately 1.0 × 10<sup>7</sup> CFU/mL with LB broth. Then, 20 µL of the bacterial suspension was inoculated on LB agar plates evenly. Subsequently, the sample disk containing the antimicrobial agent solution was gently placed at the center of the LB agar plates and cultured overnight at 37 °C (at least three times each group). The diameter of the zone of inhibition around the disk is the standard of measure of the antibacterial activity.

### 2.4. Fluorescence microscopic observation (live/dead)

Logarithmic-phase bacteria (1.5 mL OD<sub>600nm</sub> 0.5) were centrifuged at 5000 rpm for 5 min and washed by phosphate buffer solution (PBS, 0.01 mol/L, pH 7.4) three times. The supernatant was discarded and the remaining precipitates, which were bacteria, were resuspended in 1.5 mL of PBS. Bacteria were treated with 100 µL of 25 µg/mL SeNPs. Then 100 µL of fluorescent dye was added and stained in the dark for 15 min. The fluorescent dyes were mixed, by mixing 10 mg of acridine orange (AO, Fluk) and 10 mg of ethidium bromide (EB, Fluk) in 10 mL of PBS (0.01 mol/L, pH 7.4). After rinsing with PBS three times, the bacterial cells were imaged using laser scanning confocal microscope (LSM, Leica

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