Combating the melioidosis pathogen using antibiotics in combination with silver nanoparticles

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Research

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Abstract

Background

Melioidosis is an infectious disease caused by the Gram-negative bacillus bacterium, *Burkholderia pseudomallei*. Due to the emerging resistance of *B. pseudomallei* to antibiotics, including ceftazidime (CAZ), the development of novel antibiotics and alternative modes of treatment has become an urgent issue. Here, we demonstrate an ability to synergistically increase the efficiency of antibiotics through their combination with silver nanoparticles (AgNPs).

Method:

Combinations of four conventional antibiotics, including CAZ, imipenem (IMI), meropenem (MER), or gentamicin sulfate (GENT), with AgNPs were tested for their bactericidal effects against three isolates of *B. pseudomallei*, including 1026b, H777, and 316c, using the microdilution checkerboard method of antibiotic and AgNPs mixing. Morphological changes in the bacteria after treatment with the combined antibiotic-AgNPs was observed using scanning electron microscopy (SEM).

Result

The combination of four antibiotics with AgNPs gave fractional inhibitory concentration (FIC) index values and fractional bactericidal concentration (FBC) index values ranging from 0.312 to 0.75 μg/mL and 0.252 to 0.625 μg/mL, respectively, against the three isolates of *B. pseudomallei*. SEM imaging revealed damage to the bacterial cell structure at the minimal inhibitory concentration (MIC) and FIC levels, while extreme severe cellular damage was observed at the FBC level. Surprisingly, at the FBC level, the bacteria produced large amounts of fibers that are the components of biofilm.

Conclusion

The study clearly shows that most of the combinatorial treatments exhibited synergistic antimicrobial effects against all three isolates of *B. pseudomallei*. The highest enhancing effect was observed for GENT with AgNPs. We also found that the combination of these antibiotics with AgNPs restored their bactericidal potency in the bacterial strains previously shown to be resistant to the antibiotic. The observed synergistic activities of conventional antibiotics with AgNPs suggest that it might also be possible to achieve equivalent or higher levels of bacterial cell death with lower concentrations of antibiotics using the combined treatments. These results support the use of the antibiotic/AgNPs combination as an alternative design strategy for new therapeutics to more effectively combat melioidosis.

Background

Melioidosis is an infectious disease caused by the Gram-negative bacillus bacterium, *Burkholderia pseudomallei*. This organism is an important causative agent of septicemia and is community acquired. It is believed to be vastly underreported, with ~ 165,000 cases worldwide and a fatality rate of approximately 89,000 per year (1). The incidence of melioidosis is highest in Southeast Asia and northern Australia, with a case fatality rate of 40% in northeast Thailand and 19% in Australia (2, 3). Currently, no licensed vaccine against melioidosis has been established in clinical use. Melioidosis has been dubbed “the great imitator” as it presents with great clinical diversity, and several of its symptoms are often confounded with those of other diseases, such as tuberculosis (4). Furthermore, melioidosis can also be asymptomatic sometimes. Altogether, these features make the disease difficult to diagnose.

The selection of antibiotics for the treatment of melioidosis is limited due to the bacteria’s resistance to several commonly prescribed antibiotics, including aminoglycosides, fluoroquinolone compounds, and many β-lactam antibiotics (5, 6). Ceftazidime (CAZ), a third-generation antibiotic of the cephalosporin family, is recommended as a first-line therapy for the treatment of severe melioidosis (7–9). CAZ works by interfering with bacterial cell wall synthesis. Carbapenem antibiotics, such as imipenem and meropenem, have also shown potent activity against *B. pseudomallei* (10, 11). Although the resistance of *B. pseudomallei* to CAZ is rare, it has been demonstrated to exist both in vitro and in vivo (6, 12, 13). Moreover, as with any antibiotic, repeated exposure to the drug through increased use elevates the risk of developing bacterial resistance over time. The increasing prevalence of antibiotic resistance has become a serious public health problem worldwide, and alternative therapies that can overcome resistance and prevent future resistance are urgently needed. One approach to controlling bacterial resistance is through using a combination of antibiotics with other agents that increase the efficacy of the antibiotic. These agents include other antibiotics (14–16), antimicrobial peptides (17, 18), plant extract (19, 20), and nanoparticles (21, 22).

Nanoparticles are of great interest for researchers as they have unique physical, chemical, and electrical properties that differ from bulk materials. Such properties are the result of the shape and size of the nanoparticles, which have a high surface-area-to-volume ratio due to their small size. Among the nanoparticles, silver nanoparticles (AgNPs) have attracted the most attention because they have broad-spectrum efficacy against several microorganisms, including bacteria, fungi, and viruses, with low cytotoxicity to mammalian cells (23, 24). AgNPs have multiple modes of action that lead to bacterial cell killing, including the rupture of the bacterial cell membrane through AgNP adherence and the penetration of the AgNPs into the cell and nucleus, resulting in binding interactions with proteins and DNA and leading to ROS production and subsequently cell death (25, 26). Due to the nonspecific nature of these mechanisms, AgNPs do not place selective pressure on the bacteria and have a much lower risk for the development of resistance compared to conventional antibiotics.

The combination of AgNPs with antibiotics (e.g., ampicillin, gentamicin [GENT], and vancomycin) has been reported to have synergistic antibacterial effects toward both nonresistant and resistant strains (27,

28). In addition, several studies examining the synergistic activity of AgNPs in combination with other antibiotics have been reported: a combination of AgNPs and cefotaxime or CAZ, MER, ciprofloxacin, or GENT strongly enhanced antibacterial activity against multi-drug resistant, β-lactamase, and carbapenemase-producing *Enterobacteriaceae* (29). AgNPs with enoxacin, kanamycin, neomycin, or tetracycline showed greater bactericidal efficiency toward the drug-resistant bacteria *Salmonella typhimurium* (30). A combination of AgNPs with chloramphenicol or kanamycin resulted in synergistic bactericidal activity toward *Pseudomonas aeruginosa*, a virulent species sharing a common ancestry with *B. pseudomallei* (31, 32). In this study, we investigated the synergistic antimicrobial effects of antibiotics and AgNPs against *B. pseudomallei*, which has not been reported before. The AgNPs were combined with four types of antibiotics against *B. pseudomallei* from three clinical isolates. The synergistic antibacterial effects were evaluated by measuring the FIC indices and the FBC indices, which were obtained by plate counts from the microdilution checkerboard method. Moreover, we compared the changes in the bacterial cell morphology between the treatment with individual and combination therapies using scanning electron microscopy (SEM).

Materials And Cell Culture

The antibiotics were purchased from their respective manufacturers and dissolved according to the recommendations. The antibiotics tested were CAZ (Reyoung Pharmaceutical Co., Ltd.), IMI (JW Pharmaceutical Corporation), MER (Siam Bheasach Co., Ltd.), and GENT (Sigma-Aldrich). The following isolates were used in this study: *B. pseudomallei* 1026b (CAZ non-resistant isolate), *B. pseudomallei* H777 (CAZ moderately resistant isolate), and *B. pseudomallei* 316c (CAZ highly resistant isolate). They were provided by the Melioidosis Research Center, Khon Kaen University. All the strains were isolated from the blood of a patient (33–35). These bacteria were stored at − 70 °C in 20% glycerol in microcentrifuge tubes until used. The bacteria were streaked on Ashdown's medium (a selective culture medium for the isolation and characterization of *B. pseudomallei*) and then cultured at 37 °C for 48–72 h. The colonies were picked and inoculated in 5 mL of Mueller Hinton broth (MHB) at 37 °C overnight and then subcultured in 5 mL of the same medium at 37 °C in a 180 rpm shaker-incubator for 3 h to yield a mid-logarithmic growth phase culture (36).

Preparation And Characterization Of Silver Nanoparticles (agnps)

The AgNPs with diameters of 10–20 nm were obtained from Prime Nanotechnology, Bangkok, Thailand. The samples were resuspended in deionized water at a concentration of 1 mg/mL. The UV-vis spectra of the AgNPs were recorded using a SpectraMax M5 fluorescence microplate reader. The dimensions of the AgNPs were confirmed using a transmission electron microscope (FEI/TECNAI G220) operating at 200 kV. The sizes of the silver nanoparticles were directly obtained from the TEM image using Image J software, a Java program developed by the National Institute of Mental Health, Bethesda, Maryland, USA.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MICs and MBCs of the antibiotics (CAZ, IMI, MER, and GENT) and AgNPs were determined by the plate-counting method measured by serial dilutions, as previously described (37). Briefly, a range of concentrations of AgNPs (4–64 µg/mL) and antibiotics (0.25–1024 µg/mL) were prepared in a 96-well plate by serial dilution. The solutions were then added to an equal volume of bacterial suspension (100 µL) in each well of a 96-well plate with the final cell concentration ranging from 10^6–10^7 CFU/mL. The plates were incubated at 37 °C for 24 h. Then, 50 µL of each treated condition was collected for a serial tenfold dilution plate count with sterile water, and 10 µL of each dilution was dropped on Mueller Hinton agar in triplicate and incubated overnight at 37 °C to count the bacterial colonies formed. Bacteria with no treatment were used as a control. The MIC value was defined as the lowest concentration that inhibits 99% of bacterial growth, and the MBC value was defined as the lowest concentration that inhibits 100% of the bacterial growth. The percent inhibition was calculated using the formula [1-(CFU sample/CFU control)] × 100.

Determination Of Synergistic Antibacterial Effects

The synergistic antibacterial effects were evaluated using the FIC index and the FBC index, which were obtained by plate counting measured by the microdilution checkerboard method. Briefly, a range of concentrations of AgNPs (4–64 µg/mL) and antibiotics (0.5–1024 µg/mL) were prepared by serial dilution, and then 50 µL of each sample of AgNP and antibiotic concentration were transferred to each well of a 96-well plate (total 100 µL of AgNPs-antibiotic combination). After that, 100 µL of cell suspension of each bacterial isolate (final cell concentration range between 10^6–10^7 cells/mL) were added in each well of the 96-well plate containing the AgNPs-antibiotic mixture. The plates were incubated at 37 °C for 24 h. Due to the inherent absorbance of the silver solution, we needed to determine antimicrobial activity using a plate-counting method. After 24 h of incubation, 50 µL of each treated condition was collected for a serial 10-fold dilution plate count with sterile water in triplicate for the determination of MIC and MBC.

The FIC or FBC index was calculated to evaluate the combined antimicrobial effect of the antibiotics and the AgNPs:

FICI = MIC of drug A in the combination + MIC of drug B in the combination

MIC of drug A alone MIC of drug B alone

FBCI = MBC of drug A in the combination + MBC of drug B in the combination

MBC of drug A alone MBC of drug B alone

“Synergy, Antagonism, and Indifference were defined as FIC or FBC indices ≤ 0.5, > 4, and

> 0.5 – ≤ 4, respectively” (38).

Evaluating The Morphological Changes Of The Bacterial Cells

The morphological changes of the treated bacterial cells were observed by scanning electron microscopy (SEM). The colonies of *B. pseudomallei* 1026b were grown in MHB for 24 h at 37 °C and then subcultured in 10 mL of the same medium for 3 h to yield a mid-logarithmic growth phase culture. Subsequently, the bacteria were washed three times with deionized water and resuspended in the same solution to a final concentration of 1×10^7 CFU/mL. The cells were treated with AgNPs or antibiotics alone or AgNPs/antibiotic combinations at the MIC and FIC concentrations for 5 h and at the FBC level for 1 h, respectively. All the cells were washed two times with deionized water and then fixed with 2.5% glutaraldehyde for 1 h and dehydrated in a gradient of ethanol (30%, 50%, 70%, and 90%) for 10 min followed by rinsing in 100% ethanol twice. The cells were coated with gold and observed by scanning electron microscopy (LEO 1450VP) (39).

Results And Discussion

Characterization of silver nanoparticles

The colloid AgNPs used in this study were purchased from a commercial source at a concentration of 1 mg/mL. The solution was diluted to 100 µg/mL in deionized water, resulting in a yellowish solution (Fig. 1a). The AgNPs were characterized by UV-vis spectroscopy and TEM to observe the size, shape, and homogeneity of the AgNPs. The absorbance spectra showed a single strong peak at 404 nm, which indicated the presence of spherical AgNPs (Fig. 1b). TEM micrographs confirmed that the particles had a spherical shape and demonstrated monodispersity (Fig. 1c). The AgNPs had an average size of 15.20 ± 9.08 nm in diameter as calculated using Image J software (Fig. 1d).

Antimicrobial Susceptibility

The individual antimicrobial activities of CAZ, IMI, MER, GENT, and AgNPs against *B. pseudomallei* were determined. The first three are conventional antibiotic used in melioidosis treatment, while *B. pseudomallei* is normally resistant to the GENT antibiotic. As shown in Table 1, the MICs of CAZ, IMI, MER, and GENT were in the ranges of 4–128 µg/mL, 0.5–1 µg/mL, 2 µg/mL, and 16–64 µg/mL, respectively. As expected, *B. pseudomallei* 1026b and H777, but not *B. pseudomallei* 316c, were susceptible to CAZ. All three isolates were susceptible to IMI and MER but were all completely resistant to GENT. According to a previous report, the *B. pseudomallei* antibiotic breakpoint used for in vitro

susceptibility testing of CAZ was 32 µg/mL, and that of IMI, MER, and GENT were 8 µg/mL (6). Of these, IMI and MER were most effective for treatment of the three isolates of *B. pseudomallei*.

Table 1
Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of antibiotics and AgNPs against *B. pseudomallei* in three clinical isolates by the serial dilution plate-count method.

Bacteria (isolates)	MIC (µg/mL)					MBC (µg/mL)				
	CAZ	IMI	MER	GENT	AgNPs	CAZ	IMI	MER	GENT	AgNPs
*1026b	4	1	2	32	8	64	2	4	512	16
**H777	8	1	2	16	16	32	2	4	256	32
***316c	128	0.5	2	64	16	512	1	4	512	32
The MIC value is the lowest concentration that inhibited ≥ 99% of bacterial growth, and the MBC value is the lowest concentration that inhibited 100% of the bacterial growth.										
AgNPs: silver nanoparticles; CAZ: ceftazidime; IMI: imipenem; MER: meropenem; GENT: gentamicin.										
*(CAZ non-resistant isolates)										
**(CAZ moderately resistant isolates)										
***(CAZ highly resistant isolates)										

CAZ, IMI, MER, and GENT had MBCs in the ranges of 64–512 µg/mL, 1–2 µg/mL, 4 µg/mL, and 256–512 µg/mL, respectively (Table 1). The MBCs of CAZ and GENT were much higher than the antibiotic breakpoints used for *B. pseudomallei* susceptibility testing. This indicates that all three isolates of *B. pseudomallei* are difficult to kill by CAZ and GENT, but not by IMI and MER.

The MICs and MBCs of the AgNPs against the three isolates of *B. pseudomallei* tested were in the ranges of 8–16 µg/mL and 16–32 µg/mL, respectively. We found that the MICs and MBCs of AgNPs were lower than those observed for *B. pseudomallei* in a previous study, which reported the MICs and MBCs of AgNPs against *B. pseudomallei* in these three isolates in the ranges of 32–48 µg/mL and 96–128 µg/mL, respectively (37). This was because some properties of the AgNPs, such as size and shape, were slightly different. The smaller size show more antimicrobial activity than the larger size. These differences can cause uncertainty in biological activity (40).

It can be seen that these antibiotics and AgNPs have different antimicrobial efficiencies against *B. pseudomallei*. The bacteria were susceptible to IMI, MER, and AgNPs but resisted CAZ and GENT. Therefore, CAZ and GENT could be combined with AgNPs to observe any improved antimicrobial efficiency of these antibiotics. To determine the combination effect of these agents, we combined the antibiotics and AgNPs to explore their synergistic effect in the following section.

Synergistic Antibacterial Effects

FIC and FBC indices are commonly used to define the synergistic effects of agents for the inhibition of bacterial growth (41). Synergism is defined as FIC or FBC index values ≤ 0.5, and indifference is defined as FIC or FBC index values > 0.5 – ≤ 4. The FIC indices of CAZ, MER, and GENT in combination with AgNPs against the three isolates of *B. pseudomallei* are shown in Fig. 2a. The blue and light blue bars on the graph represent combinations with a FIC index ≤ 0.5, while the black bars on the graph represent combinations with a FIC index > 0.5. The FIC indices of CAZ, MER, and GENT in combination with AgNPs indicate that these antibiotics have a synergistic effect (FIC index ≤ 0.5) with AgNPs against *B. pseudomallei*. The combination of IMI with AgNPs, however, showed no synergism against *B. pseudomallei* 1026b and 316c (black bar graph, FIC index > 0.5). This indicates that co-treatment with IMI and AgNPs does not improve the bactericidal efficacy against *B. pseudomallei* 1026b and 316c.

From these results, we found that the combinations of most of the antibiotics tested with AgNPs have strongly synergistic effects on inhibiting the growth and killing *B. pseudomallei* in the three isolates. Mixing together different combinations of antibiotics/AgNPs can kill bacteria with different mechanisms. Therefore, the synergistic effect can act as a powerful tool against resistant bacteria. The mechanisms of synergy are often not fully understood, but feasible explanations exist for some antibiotics.

For the β-lactam antibiotics (CAZ, IMI, and MER), the interaction of antibiotics with AgNPs was the purposed mechanism. A previous study indicated that the synergistic effect of amoxicillin (a β-lactam antibiotic) may be caused by a bonding reaction (van der Waals interaction and other weak bonds) between the antibiotic and the AgNPs. This suggests that the concentration of antimicrobial groups at particular points on the cell surface may increase the severity of damage to the bacterial cell. Another study demonstrated that the synergistic effect may be the action of the “AgNPs’s drug carrier.” Moreover, membrane phospholipids and glycoprotein have been targeted by hydrophobic AgNPs, with the amoxicillin being transported to the cell surface to damage the cell (42, 43). The combination of GENT with AgNPs against these bacteria has not been demonstrated in other studies, although one previous report suggested that *Staphylococcus aureus* can be killed through the interaction of GENT and AgNPs. Hydroxyl and amide groups of GENT easily react with AgNPs, and they can then deliver the drug to the cell. Thus, it is necessary to perform further investigations on the efficiency of antibiotics/AgNPs combinations against *B. pseudomallei* to illustrate the mechanism of this synergistic antibacterial effect.

However, among all the conditions tested, only one combination showed no synergistic effect: IMI in combination with AgNPs against *B. pseudomallei* 1026b and 316c. This result could imply that the action of antibiotics with AgNPs depends on the bacterial isolates because of the cell membrane component of each *B. pseudomallei* isolate (44, 45). In a similar study, a combination of antibiotics including ampicillin, chloramphenicol, or kanamycin with AgNPs showed differences in activity between the two isolates of *Escherichia coli* tested. The combination showed synergism and partial synergism in inhibiting and killing *E. coli* ATCC 43895 and *E. coli* ATCC 25922, respectively (31).

Tables 2 and 3 show the lowest antibiotic concentrations that have a synergistic effect with AgNPs. The MICs of antibiotics alone, AgNPs alone, or of the combinations of antibiotics with AgNPs are presented in Table 2. The concentrations of CAZ, IMI, MER, and GENT in combination with AgNPs that inhibited bacterial growth were in the ranges of 1–16 µg/mL, 0.25–0.5 µg/mL, 0.5 µg/mL, and 2–16 µg/mL, respectively. We demonstrated that the combination of antibiotics and AgNPs allows the use of lower antibiotic concentrations to achieve equivalent antimicrobial efficiency. The MIC concentrations of CAZ decreased by up to 4–8 fold, IMI decreased up to 2–4 fold, MER decreased up to 4–fold, and GENT decreased up to 4–16 fold when compared with antibiotics alone.

Table 2
Minimum inhibitory concentrations (MICs; µg/mL) of antibiotic alone or in combination with AgNPs. Fold change in MIC combination compared to MIC alone of antibiotics.

Antibiotics	*B. pseudomallei* isolations	MIC (µg/mL)		Reduction in concentration (folds)	FICI	Type of interaction
		Alone	In combination			
Ceftazidime	1026b	4	1	4	0.5	S
	H777	8	2	4	0.375	S
	316c	128	16	8	0.375	S
Imipenem	1026b	1	0.5	2	0.75	I
	H777	1	0.25	4	0.5	S
	316c	0.5	0.25	2	0.75	I
Meropenem	1026b	2	0.5	4	0.5	S
	H777	2	0.5	4	0.375	S
	316c	2	0.5	4	0.375	S
Gentamicin	1026b	32	2	16	0.312	S
	H777	16	4	4	0.5	S
	316c	64	16	4	0.375	S

Synergy (S) was defined as an FIC index ≤ 0.5; antagonism (A) was defined as an FIC index > 4; and indifference (I) was defined as an FIC index > 0.5 – ≤ 4. The FIC index is the fractional inhibitory concentration.

Likewise, the MBCs of the antibiotics alone or AgNPs alone or antibiotic with AgNPs are presented in Table 3. The bactericidal concentrations of CAZ, IMI, MER, and GENT in combination with AgNPs are in the ranges of 4–16 µg/mL, 0.25–0.5 µg/mL, 1 µg/mL, and 1–16 µg/mL, respectively. The greatly reduced MBCs for the antibiotic/AgNP combinations demonstrate that we could use lower concentrations of antibiotic with the combined therapy in comparison to the antibiotic alone to achieve equivalent antimicrobial efficiency. The CAZ concentration reduced up to 4–32 fold, IMI up to 2–4 fold, MER up to

4–fold, and GENT up to 32–512 fold. As a result, the reduction amounts in the concentration of antibiotic needed to achieve the same inhibition activity in combination with AgNPs were in the following order: GENT > CAZ > MEM > IMI. Our results are similar to those previously reported for CAZ. In another study, the combination of CAZ and AgNPs also showed synergy in the inhibition of *P. aeruginosa* (23), a virulent bacterium that shares an ancestry with *B. pseudomallei*.

Table 3
Minimum bactericidal concentrations (MBCs; µg/mL) of antibiotic alone or in combination with AgNPs. Fold change in MBC combination compared to MBC alone of antibiotics.

Antibiotics	*B. pseudomallei* isolations	MBC (µg/mL)		Reduction in concentration (folds)	FBCI	Type of interaction
		Alone	In combination			
Ceftazidime	1026b	64	4	16	0.313	S
	H777	32	8	4	0.375	S
	316c	512	16	32	0.281	S
Imipenem	1026b	2	0.5	4	0.5	S
	H777	2	0.5	4	0.5	S
	316c	1	0.25	2	0.625	I
Meropenem	1026b	4	1	4	0.375	S
	H777	4	1	4	0.375	S
	316c	4	1	4	0.375	S
Gentamicin	1026b	512	1	512	0.252	S
	H777	256	4	64	0.265	S
	316c	512	16	32	0.289	S

Synergy (S) was defined as an FBC index ≤ 0.5; antagonism (A) was defined as an FBC index > 4; and indifference (I) was defined as an FBC index > 0.5 – ≤ 4. The FBC index is the fractional bactericidal concentration.

The highest synergistic effect for antibiotic/AgNP combinations was observed with GENT. Because no previous reports have been made on the use of combinations of CAZ, IMI, MER, and GENT with AgNPs against *B. pseudomallei*, we can only compare the combinations with the results reported for other Gram-negative bacteria. In a previous report studying the effects of combining GENT with AgNPs against *S. aureus*, *E. coli*, and GENT-resistant *E. coli*, a dual role for GENT was found in which it increased the dissolution of the AgNPs and facilitated the attachment of AgNPs onto the surface of bacteria, thereby enhancing the antibacterial activity of the AgNPs (46).

In addition, the results obtained here are similar to those found in previous reports on the use of combinations of CAZ, IMI, MER, GENT, and AgNPs against other Gram-negative bacteria. Those results have shown strongly enhanced bactericidal activity and the restored bactericidal activity of inactive antibiotics against bacteria (29, 47). Our analysis presented here showed that the antibacterial activities of most of the antibiotics increased in the presence of AgNPs, indicating the effectiveness of the combinations against *B. pseudomallei*. Above all, we demonstrated that the concentrations of four antibiotics when combined with AgNPs were reduced to below the antibiotic breakpoints used for *B. pseudomallei* susceptibility testing of these antibiotics (6).

Cell Morphological Change

To evaluate the morphology of bacterial cells under different treatment conditions, we observed the morphological changes of *B. pseudomallei* 1026b treated with CAZ, IMI, MER, or GENT, alone or in combination with AgNPs using SEM (Fig. 3). The SEM images showed that the untreated control cells appeared intact, plump, and typically rod-shaped with a smooth exterior (Fig. 3a). In contrast, the bacterial cells exposed to antibiotics alone or in combination with AgNPs at the MIC and FIC levels showed losses of membrane integrity. The cell walls became loose and porous, distorted from their normal shape, or even ruptured (Fig. 3b to 3j). Furthermore, we noticed that the treatment of bacteria with the antibiotics alone and in combination with AgNPs resulted in more elongated cells compared to the control. The shape change was likely caused by CAZ, IMI, and MER interference with the cell wall synthesis. The inhibition of protein synthesis by GENT may have also led to the observed shape change. The mechanism of the AgNPs' inhibition of bacterial growth is not clearly known, but several studies have suggested that they function through damaging the bacterial cell wall (26, 48–50).

We further observed the morphology of bacteria treated with combinations of antibiotics with AgNPs at the concentrations of FBC (Fig. 3k to 3n). The results clearly showed that the bacterial cells were more severely damaged in this combination than in those at MIC and FIC levels. At the FBC level, a microscopic analysis of the bacterial cells revealed gross leakage and holes on the outer surface, with a bulgy, disfigured, and fragmented shape. Surprisingly, under these conditions, the bacterial cells produced a substantial amount of fibers that appeared within 1 h.

The fibers produced under such harsh conditions at the FBC might be exopolysaccharides or EPS (also known as extracellular polysaccharides), which are part of the biofilm found in the extracellular medium surrounding the bacteria (51). Normally, *B. pseudomallei* can produce a biofilm to protect it from proximal unsuitable environments, but it can also produce large biofilms in severe condition (52, 53). The EPS in biofilms are a variety of macromolecules, including proteins, DNA, lipids, and polysaccharides (the main structural component). Polysaccharides are produced first during biofilm production to allow the bacterial cell to adhere to the surface. Subsequently, the bacteria will then proceed to create suitable conditions for survival that protect them against the dangerous environment (54, 55).

We demonstrated that the FBC is a severely stressful level that causes *B. pseudomallei* to produce large amounts of fibers to protect the cells. Previous studies have indicated that the biofilm of *B. pseudomallei* is not a virulence factor, but is associated with melioidosis relapse because of its facilitation of antibiotic resistance development (56, 57). Importantly, high levels of EPS in biofilms have been demonstrated to inversely correlate with the ability of antibiotics and nanoparticles to penetrate *B. pseudomallei* (58). It is possible that the fiber production observed at the level of FBC may be a relapsing factor that causes bacterial resistance to a combination of antibiotics and AgNPs. To confirm this, more elaborate experimental evidence will be required in future work.

Conclusion

Due to the increasing problem of antibiotic resistance, *B. pseudomallei* infections have become harder to treat. To address this problem, we offer alternative ways to potentially combat the bacteria. In this study, we evaluated the synergism of antibiotics with AgNPs against *B. pseudomallei*, which has not been previously reported on. *B. pseudomallei* was susceptible to IMI, MER, and AgNPs but was completely resistant to GENT. For CAZ, an antibiotic recommended as a first-line therapy for severe melioidosis, we found that only *B. pseudomallei* 316c was resistant to CAZ. We then combined CAZ, IMI, MER, or GENT with AgNPs and found that the combinations revealed synergistic or indifferent effects, but no antagonism was found against all three isolates of the *B. pseudomallei* tested. In correlation with the results provided in this work, we concluded that certain combinations of antibiotics with AgNPs are able to enhance the antimicrobial effect of antibiotics by reducing the antibiotic dose that is needed for bacterial growth inhibition. However, more experimental evidence will be required in future work to elucidate the mechanisms of action in the antibiotics/AgNPs combinations. Our research points to a way to fight antibiotic-resistant bacteria. These findings support the use of antibiotic/AgNP combinations as an alternative design strategy for new therapeutics to more effectively combat melioidosis.

Abbreviations

B. pseudomallei: Burkholderia pseudomallei; CAZ: ceftazidime; IMI: imipenem; MER: meropenem; GENT: gentamicin; AgNPs: silver nanoparticles; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; FICI: the fractional inhibitory concentration index; FBCI; the fractional bactericidal concentration index, CFU: colony forming unit; EPS: exopolysaccharides or extracellular polysaccharides; SEM: scanning electron microscopy; TEM: transmission electron microscopy; MHB: Mueller Hinton broth; CFU: colony forming unit.

Declarations

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SM planned and conducted most of the experiments; ST analyzed the data and drafted the manuscript; PS and SN contributed to the study design and data collection; SD and SK reviewed the study design and contributed to the interpretation of the data; PTW reviewed and revised the manuscript; RP directed the research, analyzed the results, and provided feedback on the experimental strategies. All the authors have accepted the final version of the manuscript.

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Figures

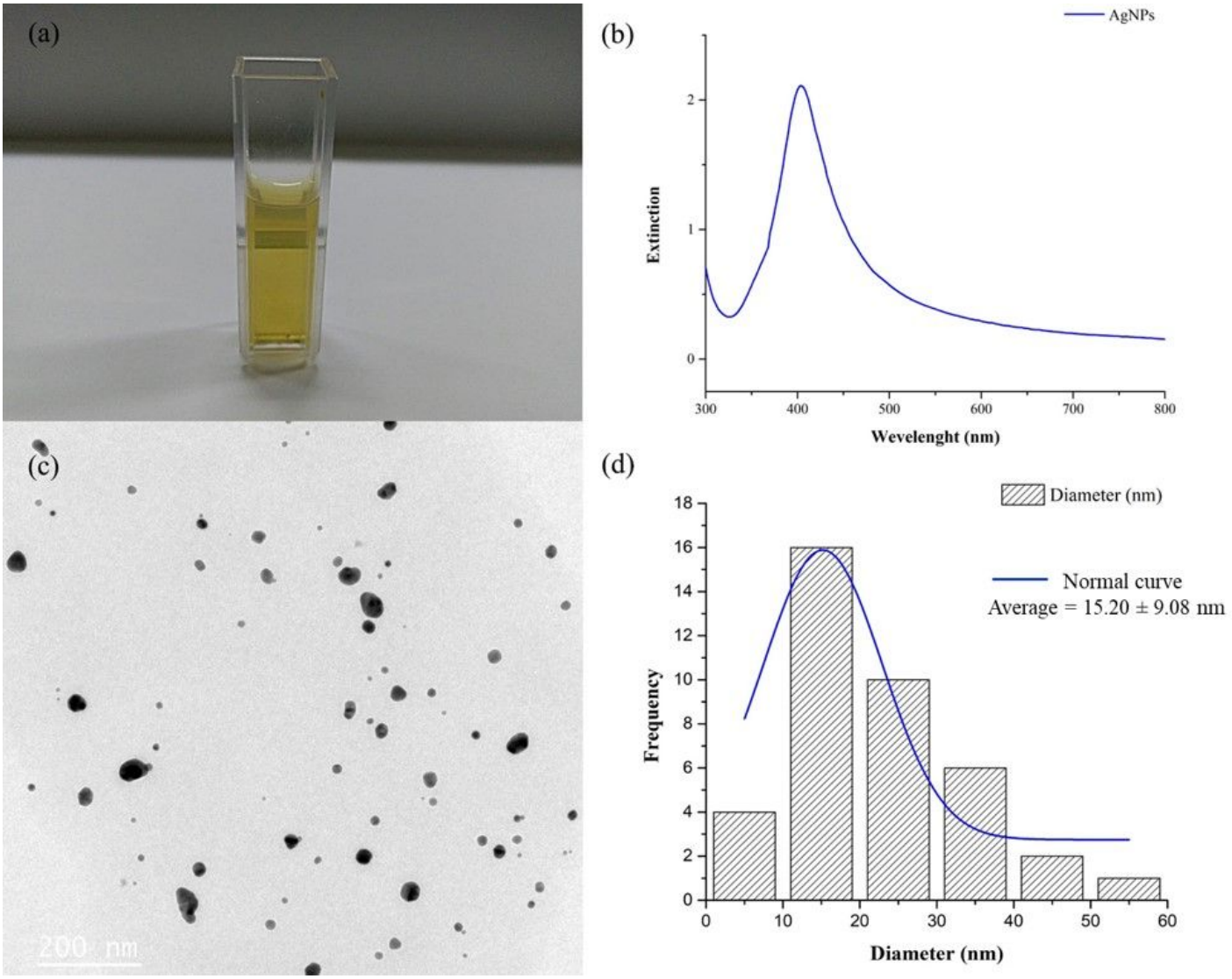

Figure 1

Physicochemical characteristics of AgNPs: (a) AgNPs solution, (b) UV-vis spectrum absorption of AgNPs, (c) TEM image of AgNPs, and (d) size distribution of AgNPs based on a TEM image.

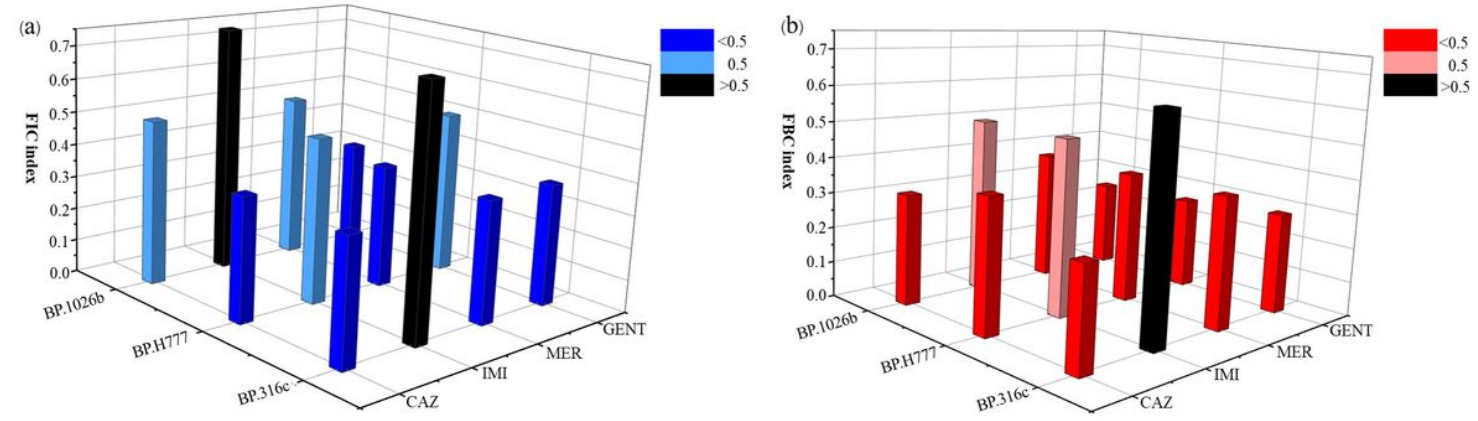

Figure 2

3D graphs showing the synergistic bactericidal activity of antibiotics with AgNPs combinations. Comparison of the FIC (a) and FBC (b) indexes for each of the antibiotics when combined with AgNPs against the three isolates of B. pseudomallei. Synergy was defined as FIC or FBC index values ≤ 0.5; antagonism was defined as FIC or FBC index values > 4; and indifference was defined as FIC or FBC index values > 0.5 – ≤ 4.

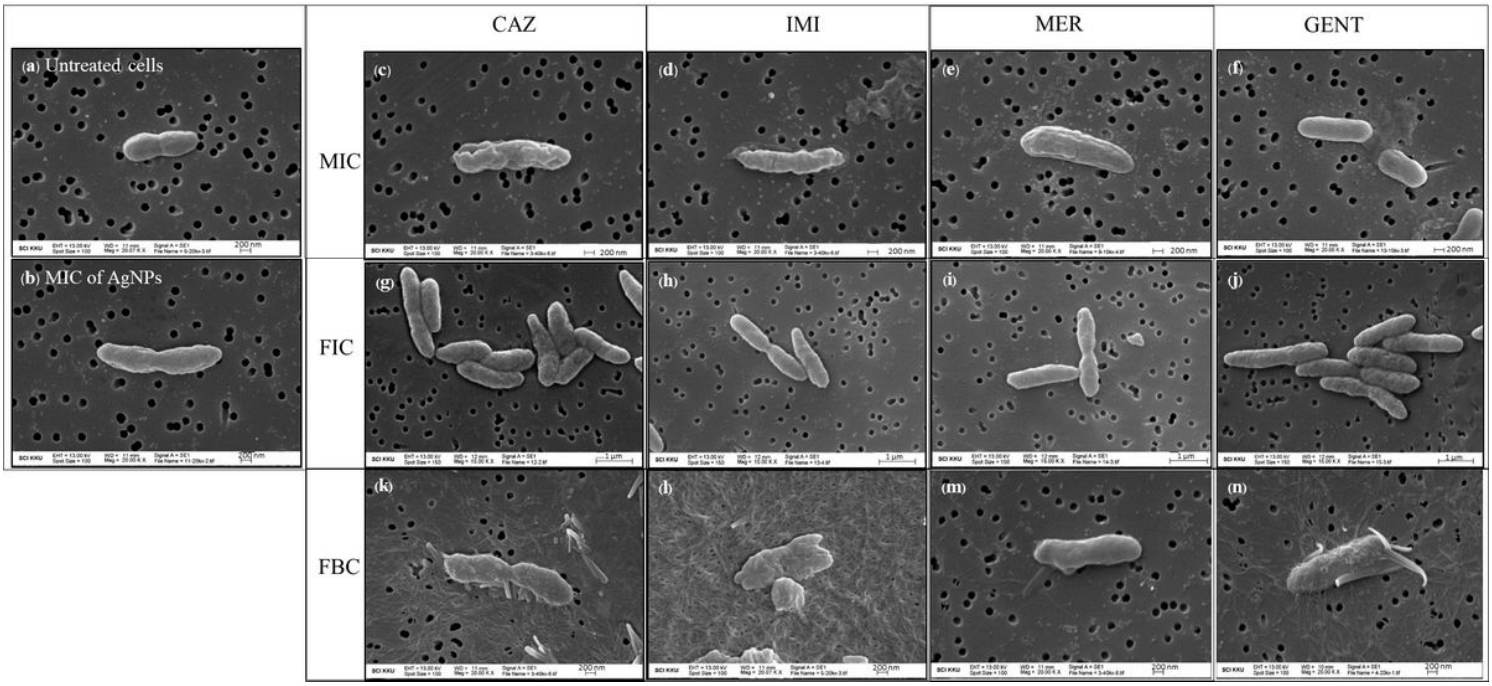

Figure 3

Scanning electron micrographs of B. pseudomallei 1026b; untreated (a), or treated with the concentration at MIC of AgNPs (b), CAZ (c), IMI (d), MER (e), or GENT (f). SEM images of 1026b treated at the FIC of AgNPs with CAZ (g), IMI (h), MER (i), or GENT (j) for 5 h. SEM images of 1026b treated at the FBC of AgNPs with CAZ (k), IMI (l), MER (m), or GENT (n) for 1 h.

Supplementary Files

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