

**Synergistic Interaction of Silver Nanoparticle Combined with Ceftazidime  
against *Burkholderia pseudomallei***

การเสริมฤทธิ์ระหว่างอนุภาคเงินนาโนกับยาเซฟตาซิดิมในการยับยั้งเชื้อ *Burkholderia pseudomallei*

Nuttaya Hongsing (ณัฐชยานันท์ หงษ์สิงห์)\* Dr. Suwimol Taweechaisupapong

(ดร.สุวิมล ทวีชัยศุกพงษ์)\*\* Dr. Rina Patramanon (ดร.รินา ภัทรมานนท์)\*\*\*

**ABSTRACT**

*Burkholderia pseudomallei* is causative agent of melioidosis, a severe disease of human and animals that is endemic in areas of Southeast Asia and Northern Australia. Currently, some clinical isolates of *B. pseudomallei* exhibit resistance to antibiotics, allowing limited choice of classical antibiotics for the treatment such as ceftazidime (CAZ). However, emerging resistance of some *B. pseudomallei* isolates to CAZ has recently been reported. In this study, we synthesized and structurally characterized silver nanoparticles (AgNPs) and tested for combined activity with CAZ against *B. pseudomallei*. The synthesized AgNPs were characterized using UV-vis spectroscopy and transmission electron microscopy (TEM). The size of AgNPs were 5-30 nm. The combination of AgNPs with CAZ in various concentrations was examined for the synergistic inhibitory effect against *B. pseudomallei*. The IC<sub>50</sub> of AgNPs-CAZ combination was much lower than AgNPs or CAZ alone. Moreover, the hemolytic impact of AgNPs-CAZ on red blood cells shown the less potent cytotoxic effect than AgNPs alone at IC<sub>50</sub> concentration. This findings could lead to development of clinically-relevant synergistic antibiotics for providing new direction in drug discovery.

**บทคัดย่อ**

เชื้อ *Burkholderia pseudomallei* (*B. pseudomallei*) เป็นเชื้อก่อโรคมะลิวอดิโอซิส โดยพบการติดเชื้อในมนุษย์และสัตว์ในแถบเอเชียตะวันออกเฉียงใต้และตอนเหนือของออสเตรเลีย ปัจจุบันพบว่า *B. pseudomallei* บางสายพันธุ์เกิดการพัฒนาดื้อยาให้ดื้อต่อรักษาโรค จึงเป็นข้อจำกัดในการรักษาโรค โดยยาปฏิชีวนะที่ใช้รักษาโรคในปัจจุบัน เช่น เซฟตาซิดิม (CAZ) ถึงอย่างไรก็ตามพบการรายงานว่าเชื้อ *B. pseudomallei* บางสายพันธุ์เกิดการพัฒนาดื้อยาให้ดื้อต่อยาเซฟตาซิดิม ในงานวิจัยนี้ผู้วิจัยจึงต้องการสังเคราะห์และศึกษาคุณลักษณะของอนุภาคเงินนาโน (AgNPs) ซึ่งพบว่าอนุภาคเงินนาโนที่สังเคราะห์ได้มีขนาด 5-30 นาโนเมตร ในการศึกษาการเสริมฤทธิ์ระหว่างอนุภาคเงินกับเซฟตาซิดิมได้จากการหาค่าความเข้มข้นของสารทั้งสองชนิดที่ความเข้มข้นที่เหมาะสม ซึ่งจากการทดลองพบว่าเมื่อมีการทำงานร่วมกันของอนุภาคเงินกับเซฟตาซิดิมสามารถยับยั้งการเจริญของเชื้อแบคทีเรียได้ดีกว่าการใช้อนุภาคเงินนาโนหรือเซฟตาซิดิมอย่างเดียว ในด้านการเหนี่ยวนำให้เกิดการแตกของเซลล์เม็ดเลือดแดงจากการทำงานร่วมกันของอนุภาคเงินนาโนและเซฟตาซิดิม พบว่ามีความเป็นพิษต่อเซลล์เม็ดเลือดแดงอ่อนข้างต่ำ ซึ่งผลจากการทดลองสามารถพัฒนาเพื่อเสริมฤทธิ์ยาปฏิชีวนะต่างๆ ในทางคลินิกสำหรับพัฒนาเป็นยาชนิดใหม่ในการรักษาโรค

**Key Words:** Synthesis silver nanoparticles, *Burkholderia pseudomallei*, Synergistic effect

**คำสำคัญ:** การสังเคราะห์อนุภาคเงิน เชื้อ *Burkholderia pseudomallei* และการเสริมฤทธิ์

\* Student, Master of Science Program in Biochemistry, Department of Biochemistry, Faculty of Science, Khon Kaen University

\*\*Associate Professor, Department of Oral Diagnosis, Faculty of Dentistry, Khon Kaen University

\*\*\* Assistant Professor, Department of Biochemistry, Faculty of Science, Khon Kaen University

## Introduction

*Burkholderia pseudomallei* (*B. pseudomallei*) is a Gram negative bacteria and is causative agent of melioidosis. This disease is endemic in Southeast Asia and Northern Australia. Importantly, in northeastern Thailand, melioidosis accounts for 20% of all community acquired septicemias and causes death toll of 40% in treated patients (Dance et al. 1989; Dance 1991). *B. pseudomallei* can be found in wet soil and water. Most infections invade the lung through inhalation and skin via wounded skin. *B. pseudomallei* can escape host defense, enable bacteria to progress in an invasion into epithelial and macrophage cells, survive freely, replication and spread to other cells. Moreover, virulence factors such as capsule, LPS, flagella, pili and quorum sensing from *B. pseudomallei* easily enhance pathogenesis (Lazar Adler et al. 2009). Recently, some clinical isolates of *B. pseudomallei* exhibit resistance to antibiotics, allowing limited choice of classical antibiotics for the treatment such as ceftazidime (CAZ). The mechanism of resistance *B. pseudomallei* can produce  $\beta$ -lactamase, the enzyme able to degrade cephalosporins including cephalothin and cefuroxime (Tribuddharat et al. 2003). In addition, emerging resistance of some *B. pseudomallei* isolates to CAZ has recently been reported (Mittal et al. 2007).

Silver nanoparticle (AgNPs) are the most effective agents against bacteria, viruses, and other eukaryotic microorganisms. AgNPs can increase the permeability of bacterial cell membrane, penetrate into the cytoplasm (Xu et al. 2004) and inhibit

essential enzymes and proteins responsible for RNA and DNA replication, leading to bacterial death (Jose Ruben et al. 2005). Unfortunately, these antibacterial actions of AgNPs are often dependent on high concentration because of the random physical collision of AgNPs with the bacterial surface, leading to penetration of AgNPs into the cytoplasm (Lara et al. 2011). So, we aim to synthesis and structurally characterize of AgNPs and examine for combined activity with CAZ against *B. pseudomallei*. The recent research shown that combining nanoparticles with antibiotics not only reduces the toxicity of both agents towards human cell but also decreases the requirement for high dosages and enhances their bactericidal properties. Combining antibiotics with nanoparticles also restores their ability to destroy bacteria that have acquired resistance to them (Kumar et al. 2012).

## Objectives of the study

The aim of this study was 1) to synthesize and characterize the AgNPs; 2) to determine the combination effect of AgNPs and CAZ (combined AgNPs-CAZ) at various concentrations against *B. pseudomallei* by reporting the inhibitory concentration (IC<sub>50</sub>) and zone of inhibition; 3) to determine the biocompatibility of combined AgNPs-CAZ using red blood cell.

## Materials and method

### Bacterial test

To examine the bactericidal effect of AgNPs on bacteria, approximately  $10^5$  colony

forming units (CFU) of *Burkholderia pseudomallei* isolate H777 on NA agar plates supplemented with nanosized silver particles in concentrations of *Burkholderia pseudomallei* isolate H777 was used in this study. The media used in this study were Mueller Hinton Broth (MHB) and nutrient agar (NA). The bacteria was streaked on NA and then cultured at 37 °C overnight. Colonies were picked and cultured in MHB at 37 °C in an incubator overnight and then subcultured at 37 °C in a 200 rpm shaker-incubator for 1.5 h to yield a mid-logarithmic growth phase culture.

#### Synthesis and characterization of AgNPs

AgNPs were prepared from the  $\text{NaBH}_4$  reduction of  $\text{AgNO}_3$ . Briefly, A 10 mL volume of 1.0 mM silver nitrate was added dropwise to 30 mL of 2.0 mM sodium borohydride solution stirred on ice (Mulfinger et al. 2007). The rapid formation of AgNPs was indicated by color change of mixture solution from colorless to yellow. All solutions were stored at 4 °C

Characterization of AgNPs was performed using UV-vis absorption spectra of the samples were recorded on a UV-vis spectrophotometer using quartz cell with a path length of 1.0 cm. The morphology of the obtained nanoparticles was observed using a transmission electron microscope (TEM) AgNPs were examined after synthesized and subsequently deposition onto copper coated carbon grids. TEM software was calibrated to measure the size of the AgNPs (Shervani et al. 2008).

#### Inhibitory concentration (IC50) and Synergistic interaction

The inhibitory concentration (IC50) of the AgNPs and CAZ against *B. pseudomallei* was determined by the broth microdilution method.

Briefly, a range of concentrations (0.313, 0.625, 1.25, 2.5, 5, 10 and 20  $\mu\text{g/ml}$ ) 100  $\mu\text{L}$  of AgNPs and CAZ was prepared by serial dilution and added to  $1 \times 10^7$  CFU/mL of *B. pseudomallei* (100  $\mu\text{L}$ ) in each well of a 96-well plate. The plates were incubated at 37 °C and read after 36 h. Each assay was carried out in triplicate. Growth media containing only microbial cells was used as the negative control (Wang et al. 2010). The MIC was defined as the concentration at which no microbial growth was observed visually by readings in triplicates of optical density (OD) at 600 nm. Percentage inhibition of bacterial was calculated by  $[1 - \text{OD}_{\text{test}} / \text{OD}_{\text{control}}] \times 100$ ,  $\text{OD}_{\text{test}}$  was defined as OD of bacteria treated with AgNPs, CAZ, the combined AgNPs-CAZ and  $\text{OD}_{\text{control}}$  was defined as OD of bacteria only.

Synergistic interaction of AgNPs-CAZ were prepared from IC50 at which no microbial growth was observed visually by readings of optical density (OD) at 600 nm and readings in triplicates. Percentage inhibition of bacterial was calculated by  $[1 - \text{OD}_{\text{test}} / \text{OD}_{\text{control}}] \times 100$ ,  $\text{OD}_{\text{test}}$  was defined as OD of bacteria treated with AgNPs, CAZ, the combined AgNPs-CAZ and  $\text{OD}_{\text{control}}$  was defined as OD of bacteria only.

#### Inhibition growth of the synergistic effect

The inhibition growth of *B. pseudomallei* studies were determine from Synergistic interaction method. The plates were incubated at 37 °C and read after 1, 2, 4, 12, 24, 36 and 48 h and measured optical density of microbial growth at 600 nm (Sopirala et al. 2010).

#### Zone of inhibition

The zone of inhibition was determined to evaluate the synergistic effect of combined AgNPs-CAZ. The bacteria were incubated in Nutrient agar

(NA) at 37°C for overnight. The bacterial suspension was diluted to approximate  $1.0 \times 10^7$  CFU/mL *B. pseudomallei* and spread in NA plate. Subsequently, 500  $\mu$ L of the bacterial suspension were inoculated evenly on NA plates. Then, the sample disk containing the antimicrobial agent solution (AgNPs, CAZ, AgNPs-CAZ) 30  $\mu$ L was gently placed at the center of the NA plates and incubated overnight at 37°C. The antibacterial activity was measured by evaluating the diameter of the zone of inhibition around the disk.

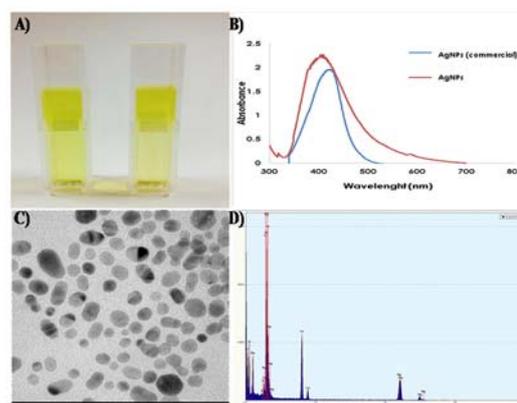
### Hemolytic activity

The hemolytic activity of the compounds was measured after exposure of human red blood cells to the combined AgNPs-CAZ at various concentration. Combined AgNPs-CAZ was diluted in phosphate buffer (PBS) for test. The red blood cells, obtained from a healthy donor was separated from plasma by centrifugation. Then they were washed three times in PBS, and re-suspended in PBS (final concentration of the red blood cells per sample was 2% (V/V)). The red blood cells were incubated with different concentrations of combined AgNPs-CAZ at 37°C for 1 h and centrifuged (3min, 3000 rpm). The supernatants were transferred to 96-well plates and measured by recording the absorbance at 550 nm. Each assay was carried out in triplicate. The percent hemolysis was obtained by dividing each sample's cell-free hemoglobin concentration by the total hemoglobin concentration. PBS buffer was used as a negative control and Triton X-100 was used as a positive control (Chen et al. 2015).

## Results

### Synthesis and characterization of AgNPs

In this study, we synthesized AgNPs via reduction of  $\text{AgNO}_3$  by  $\text{NaBH}_4$ . The formation of AgNPs can be observed by a change in color since small nanoparticles of silver are yellow in the reaction mixture (Fig. 1A). The synthesized AgNPs were analyzed using UV-vis absorbance spectroscopy. The absorption was clearly visible at  $\lambda_{\text{max}}$  400 nm while the commercial AgNPs had  $\lambda_{\text{max}}$  420 nm, indicating a slight difference in size. (Fig. 1B). Furthermore, the morphology of AgNPs was observed under TEM (Fig. 1C). The obtained AgNPs were mono-dispersed and mostly spherical in shape with an average diameter of 5-30 nm. Energy-dispersive spectroscopy of the AgNPs showed the presence of the elemental silver signal (Fig. 1D).

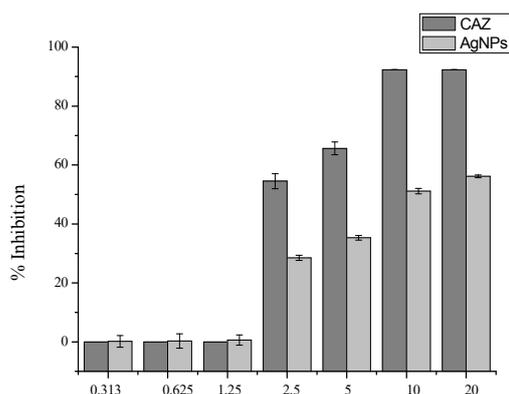


**Figure 1** Characterization of AgNPs. A photograph of AgNPs (A); UV-vis absorption spectrum of clear yellow colloidal AgNPs (B); TEM image of AgNPs (C); Energy-dispersive spectroscopy spectrum of silver nanoparticles (D).

### Inhibitory concentration (IC50) and Synergistic interaction

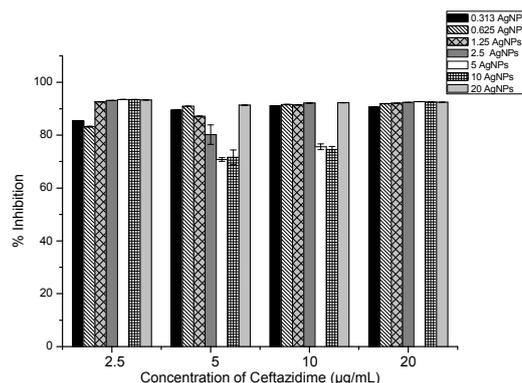
The IC50 is the concentration of AgNPs and ceftazidime can inhibits the visible growth 50% of *B. pseudomallei*. To provide an overall measurement of antimicrobial activity of AgNPs and CAZ, we

calculated the IC<sub>50</sub> required inhibiting growth of 50% of *B. pseudomallei* (IC<sub>50</sub>). For the inhibitory activities, at 10 µg/ml of AgNPs exhibited 52% inhibition (IC<sub>50</sub> of AgNPs), while 2.5 µg/ml of CAZ antibiotic exhibited about 56% inhibition (IC<sub>50</sub> of CAZ) (Fig.2).



**Figure 2** The percentage inhibition of Inhibitory Concentration required inhibiting growth of organisms about 50% (IC<sub>50</sub>) in *B. pseudomallei* of AgNPs and CAZ.

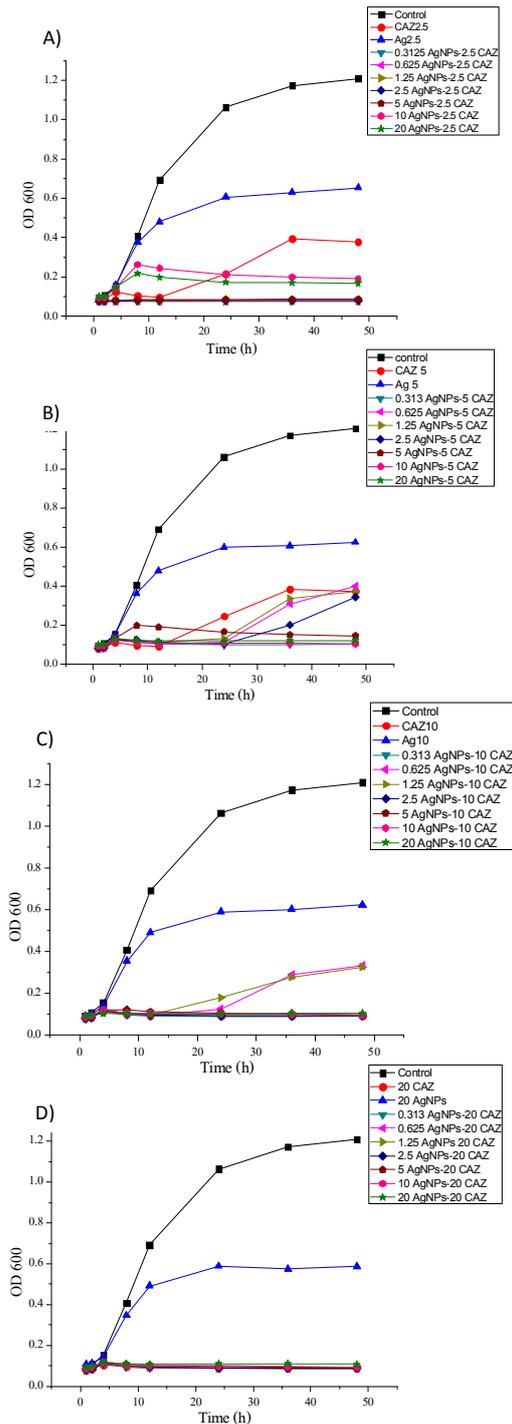
The combination of AgNPs with CAZ was investigated against *B. pseudomallei* using the micro dilution method following by calculation of percentage of inhibition. The result showed the combination effect of 2.5-20 µg/ml of CAZ and 0.313-20 µg/ml of AgNPs. The combined AgNPs-CAZ could inhibit the growth of *B. pseudomallei* much higher than AgNPs alone at all concentrations (Fig. 3).



**Figure 3** Effect of various concentration of AgNPs, and combined AgNPs-CAZ against the growth of *B. pseudomallei*.

#### Inhibition growth curve of combined AgNPs-CAZ

To assess the synergistic effect of combined AgNPs-CAZ, an in vitro inhibition growth experiment was carried out with *B. pseudomallei* (Fig. 4). Initial experiments with concentration of two agents showed that the combination exhibited the inhibitory activity within a minute of the start of the treatment (no viable cells detected within one minute of the treatment). Therefore, to follow the growth inhibition, the experiment at different concentrations of the combined AgNPs-CAZ against *B. pseudomallei* was performed. The inhibiting curves of combined AgNPs-CAZ with 2.5 or 5 µg/ml of CAZ are more effective than AgNPs or CAZ alone (Figure 4A and 4B). In contrast, the killing curves of combined AgNPs-CAZ with 10 or 20 µg/ml of CAZ (Fig. 4C and 4D, respectively) showed no difference from AgNPs and CAZ alone.

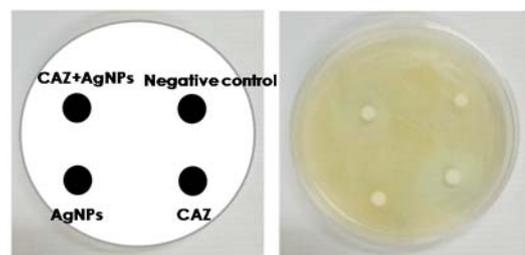


**Figure 4** Inhibition growth of AgNPs, CAZ and combined AgNPs-CAZ in difference concentration.

**The zone of inhibition**

The zone of inhibition was investigated to evaluate the synergistic effects of combined AgNPs-

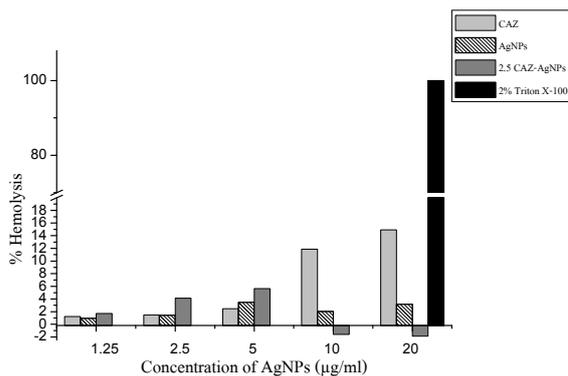
CAZ against *B. pseudomallei*. As shown in Fig. 5, the diameters of zone of inhibition of CAZ was 13 mm and the combination of AgNPs-CAZ was 13 mm. In contrast, the diameter zone of inhibition of AgNPs was not found because AgNPs aggregation. We concluded that the inhibition zone assay could not be used to assess AgNPs inhibitory activity.



**Figure 5** Inhibition zones of AgNPs, CAZ and combined AgNPs-CAZ in different concentration.

**Hemolytic activity**

In This study assessed the *in vitro* hemolytic potential of AgNPs, CAZ and combined AgNPs-CAZ in human blood to determine the cytotoxic effect toward human red blood cells (hRBC). As shown in Fig. 6, AgNPs alone, CAZ alone, and combined AgNPs-CAZ exhibited low hemolytic activity even up to 20 µg/ml. Increasing concentration of AgNPs to 10 and 20 µg/ml found aggregation of AgNPs.



**Figure 6** Hemolytic activity of AgNPs, CAZ and CAZ combination with 1.25, 2.5, 5, 19 and 20 µg/ml of AgNPs.

### Discussion and Conclusions

In the present research, we synthesized and characterized AgNPs. The formation of AgNPs can be observed by a change from colorless to yellow of the small nanoparticles of silver using UV-vis spectroscopy. It is well known that AgNPs show a yellowish brown color in aqueous solution (Henglein 1993). The particle size of AgNPs ranged 5-30 nm. When evaluating the antibacterial activity of AgNPs and CAZ, the IC<sub>50</sub> was 10 and 2.5 µg/ml, respectively. The synergistic interaction of AgNPs and CAZ showed that combined AgNPs-CAZ could inhibit growth of *B. pseudomallei* more than using AgNPs or CAZ alone. The antimicrobial activity of CAZ against *B. pseudomallei* increased in the presence of AgNPs. The increasing synergistic effect may result from the weak interaction between CAZ and AgNPs. The antibiotic molecules contain many active groups such as hydroxyl and amino groups, which can be easily chelated easily with AgNPs (Batarseh 2004). The AgNPs-CAZ complex may bring the AgNPs to localize nearby the bacterial surface, leading to higher chance in disrupting the membrane. We did not see the inhibitory effect of

AgNPs in the zone of inhibition assay. It appeared that the chloride ion in the culture medium agar could lead to aggregation or precipitation of AgNPs. Aggregation of AgNPs altered the size and shape of the nanoparticles, which greatly influenced the cell-particle interactions. Large aggregation of particles can significantly hinder the effects of individual particle size and shape on antimicrobial activity (Hussain et al. 2005) and the large aggregates of silver nanoparticles caused significantly less hemolytic toxicity than small aggregates (Zook et al. 2010). Taken together, the combination effect of CAZ and AgNPs may provide an alternative to melioidosis treatment with using lower dosage of both antibiotics and the metal nanoparticles.

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