Antibacterial Activity and Mechanism Action of Silver Nanoparticles against *Burkhoderia pseudomallei* isolates

ประสิทธิภาพและกลไกการงานของอนุภาคเงินนาโนในต่อการยับยั้งเชื้อ *Burkhoderia pseudomallei*

Pawinee Siritongsuk (ภาวินี ศิริทองสุข)* Dr.Rina Patramanon (ดร.รินา ภัทรมานนท์)**

ABSTRACT

Burkholderia pseudomallei is a causative agent of melioidosis, the disease endemic in north and northeast Thailand and northern Australia. *B. pseudomallei* is intrinsically resistant to many antibiotics. Ceftazidime (CAZ) is currently used in treatment regimen but increasing incidence of CAZ resistance has been reported in recent years in Thai major hospitals. Silver nanoparticles (AgNPs) are attractive antimicrobial agents against drug-resistant pathogenic bacteria. AgNPs are alternative agents for cope with drug-resistance bacterial cell. In this study, we investigated the antibacterial activity and action mechanism of AgNPs against five clinical isolates of *B. pseudomallei*. The results showed that AgNPs (10-20 nm) at 32-48 μg/mL completely inhibited the growth of cells and the inhibitory concentrations were similar in all *B. pseudomallei* isolates. Importantly, the inhibitory concentrations of AgNPs were not toxic to human red blood cells. AgNPs killed cells within 5 min due to AgNPs directly acted on the membrane of bacterial cell. In addition, TEM was used to examine the ultrastructural changes in bacteria cells. The action of AgNPs at MBC led to severely damage of cell membrane, DNA, and biomolecules.

บทคัดย่อ

โรคเมลิออยโดสิส (Meliodosis) เป็นโรคที่เกิดจากการติดเชื้อแบคทีเรีย *B. pseudomallei* ซึ่งพบอัตราการเสียชีวิตสูงในภาคตะวันออกเฉียงเหนือของประเทศไทย และในปัจจุบันพบอัตราการการดื้อต่อยาปฏิชีวนะเซฟดาซิดิมซึ่งเป็นยาที่ใช้ในการรักษาโรคนี้สูงขึ้น ดังนั้นอนุภาคเงินนาโนจึงเป็นสารทางเลือกใหม่เพื่อแก้ไขปัญหาดังกล่าว ซึ่งในงานวิจัยครั้งนี้จึงทำการสังเคราะห์อนุภาคเงินนาโนด้วยวิธีทางเคมี ศึกษาประสิทธิภาพในการยับยั้งเชื้อ *B. pseudomallei* จำนวน 5 isolate และทำการศึกษากลไกของอนุภาคเงินนาโนต่อเชื้อดังกล่าวอีกด้วย จากผลการทดลองพบว่าอนุภาคเงินนาโนขนาด 10-20 นาโนเมตรสามารถยับยั้งเชื้อทั้ง 5 isolate ได้อย่างมีประสิทธิภาพโดยพบค่า MIC เฉลี่ยเท่ากับ 32-48 μg/mLและที่ความเข้มข้นดังกล่าวยังพบว่าไม่มีพิษต่อเซลล์เม็ดเลือดแดงของมนุษย์อีกด้วยนอกจากนี้ยังพบว่าอนุภาคเงินนาโนสามารถฆ่าเชื้อได้อย่างรวดเร็วภายในเวลา 5 นาที และที่ความเข้มข้นของอนุภาคเงินนาโนที่สามารถฆ่าเชื้อได้ 99.99% (MBC) เมื่อใช้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านแสดงให้เห็นการฉีกขาดอย่างรุนแรงของเยื่อหุ้มเซลล์และการเปลี่ยนแปลงของโครงสร้างในระดับนาโน เช่น สารพันธุกรรม และสารชีวโมเลกุล ซึ่งนำไปสู่การตายของเซลล์ในที่สุด

Key Words: Silver nanoparticles (AgNPs), Ceftazidime (CAZ), *Burkholderia pseudomallei*

คำสำคัญ: อนุภาคเงินนาโน ยาปฏิชีวนะเซฟตาซิดิม เชื้อแบคทีเรีย *Burkholderia pseudomallei*

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

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

Introduction

Burkholderia pseudomallei is a causative agent of melioidosis, a disease endemic in Southeast Asia and Northern Australia but also increasingly reported in other tropical areas throughout the world (Leelarasamee, Bovornkitti, 1989; Currie et al., 2000). There is no vaccine for melioidosis and it can be fatal if a specific antibiotic regimen is not delivered (Peacock et al., 2012). Standard administering antibiotics include the third-generation cephalosporin and ceftazidime (CAZ). *B. pseudomallei* is intrinsically resistant to many antibiotics. Prolong nature of melioidosis treatment increases the probability that acquired resistance can develop (Sarovich et al., 2012). Especially, mono therapy is used or the same type of antibiotic is used for several times in the same patient for treatment of infection relapses. In fact, emerging resistance of some *B. pseudomallei* isolates to CAZ has recently been reported. This serious problem, along with the paucity of alternate treatment options for melioidosis, has encouraged us to seek for novel candidate agents to overcome bacterial resistance in melioidosis (Schweizer, 2012; Kager et al., 2014)

In recent years, many reports have found that silver nanoparticles (AgNPs) have strong inhibitory and bactericidal effect against bacteria, fungi, and virus (Lok et al., 2006; Cho et al., 2005; Li et al. 2011). In addition, AgNPs exhibit high toxicity to microorganism while it exhibits low toxicity to mammalian cells (Zhao, Stevens, 1998). Antibacterial activity and mechanism are dependent on size, shape, dispersion, agglomeration and dissolution rate (Tiede et al., 2008). Hence, AgNPs are used for developing nanotechnology and the wide-range of healthcare products such as cosmetic product, washing compounds, and medical devices/agents (Kim et al., 2007; Thomus et al., 2007). Nevertheless, evidence on AgNPs action mechanism is still in infantry stage. The killing action of AgNPs was propose to involve with interaction of silver ions with components on bacterial membrane such as lipopolysaccharide leading to membrane, DNA, proteins and especially enzymes damage (Chaloupka et al., 2010; Li et al., 2010). Moreover, AgNPs induce high level of reactive oxygen species (ROS) in intracellular compartment of bacteria resulting in damage of organic compounds within the cell (Xu et al., 2011). There are very few reports on alternative agents against *B. pseudomallei*, an no report on the effect of AgNPs against *B. pseudomallei*. In this study, we aim to find the antibacterial activity and mechanism of AgNPs on *B. pseudomallei.*

Objective of the study

To determine the antibacterial activity and insight mechanism of AgNPs against *B. pseudomallei* at the ultrastructure level and to investigate toxicity of AgNPs in mammalian cells.

Methods

Synthesis and characterization of AgNPs

AgNPs were prepared using silver nitrate as metal precursor and sodium citrate as well as sodium borohydride as reducing agents and polyvinylpyrrolidone as stabilizing agents. The morphologies and structures of the nanoparticles were characterized by UV-visible spectroscopy and transmission electron microscopy. The equation of AgNPs synthesis: $AgNO_3 + NaBH_4 \rightarrow Ag^0 + 1/2B_2H_6 + NaNO_3$ (Guzman et al., 2012).

Bacterial strains and growth conditions

Burkhoderia pseudomallei five isolates were kind gifts from Melioidosis Research Center, Khon Kaen University. The following isolates were used in this study: *B. pseudomallei* K96243, NF10/38, H777, 979b and 316C. All isolates were stored at -70°C in 20% glycerol in an micro centrifuge tubes. The bacteria was streaked on NA and then cultured at 37°C overnight. Colonies were picked and cultured in Luria-Bertani (LB) broth at 37°C in an incubator overnight and then subcultured at 37°C in a 200 rpm shaker-incubator for 2-3 h to yield a mid-logarithmic growth phase culture (Madhongsa et al., 2013).

MIC and MBC determination

MIC and MBC were determined by a micro dilution method (Re, using Luria–Bertani broth and an inoculum of $1x10^7$ CFU/mL. Ten isolates of *B. pseudomallei* were incubated with 2-256 µg/mL of AgNPs or 2-1024 µg/mL of CAZ, then the antibacterial activity was measured after 24 h. The MIC value corresponded to the concentration that inhibited 99% of bacterial growth and the MBC value corresponded to the concentration where 100% of the bacterial growth was inhibited, compared to the positive control (no treatment). Inhibition of bacterial cells was measured with serially dilution plate count method assay. The percentage of inhibition was calculated using the formula $[1-(CFU_{sample}/CFU_{control})]\times100$ (Lara et al., 2010).

Killing kinetic assay

Killing kinetics was determined using a culture of *B. pseudomallei* NF10 and 316C re-suspended in 10 mM Potassium phosphate buffer (1x107 CFU/mL). AgNPs or CAZ were added to the bacterial suspension to a final concentration of MIC and MBC of each isolates. Suspension cells were incubated in a 180 rpm shaker-incubator at 37 ˚C. At the indicated times (0, 1, 2, 4, 6 and 24 h), samples were taken, serially diluted, plated in triplicate on NA and incubated for 24 h to allow colony counting. The percentage killing or inhibiting effects of AgNPs was calculated using the formula same MIC and MBC experiment. A bactericidal effect was defined as a $\geq$3 log10 reduction in CFU/mL compared with the initial inoculum (Kanthawong et al. 2012).

The LIVE/DEAD bacterial viability assay

Bacterial viability was determined using LIVE/DEAD BacLight™ bacterial-viability kit (Invitrogen). Kit provides a two-color fluorescence cell viability assay that is based on the simultaneous determination of live and dead cells with two probes as sytox® green and propidium iodide dye, respectively. Cell suspension of *B. pseudomallei* NF10 or 316C in 10 mM PPB ($1x10^5$ CFU/mL) were incubated with AgNPs at MIC each isolates in a 180 rpm shaker-incubator at 37 ˚C. At indicate times (0, 5, 30 and 60 min), cell suspension was incubated with fluorescent dye from LIVE/DEAD kit for 15 min in dark for fluorescence microscope by Ex 494, Em 515 nm for live cell and Ex 528 and Em 617 nm for dead cell (Mei et al., 2013).

Hemolytic activity assay

Human red blood cells (hRBCs) were collected via centrifugation, washed three times with PBS, and then resuspended to 4% (v/v) in PBS. 100 µL hRBC suspensions were added into each well of a sterilized 96-well plate. 100 µL AgNPs, at 2-1024 µg/mL, was added concentration in each well, and the plates were incubated for 1 h at 37°C and centrifuged at 1000 g for 5 min. 100 mL aliquots of supernatant were transferred to fresh 96-well plates and hemoglobin release was monitored with a SpectraMax M5

fluorescence microplate reader by measuring the absorbance at 405 nm. The 0% and 100% hemolysis was determined in PBS and 0.1% Triton X-100, respectively (Golubeva et al., 2010).

Transmission electron microscopy (TEM)

In order to find out and elucidate the visual effect of antibacterial mechanism of AgNPs at MIC concentration, transmission electron microscopy technique was used. Cells of *B. pseudomallei* 316C before and after treatment with AgNPs were fixed overnight with 2.5% glutaraldehyde. Samples were post fixed in 2% osmium tetroxide, dehydrated in a series of graded ethanol, infiltrated and embedded in spur resin. Then, ultra-thin sections (60 nm thicknesses) were cut, stained with uranyl acetate and counter stained with 4% lead citrate. These sections were mounted on carbon coated copper grids and observed under TEM (Hitachi HT7700) (Kora, Arunachalam, 2011).

Results

Synthesis and characterization of AgNPs

In this study, AgNPs were synthesized by chemistry method and were characterized with a spectrometer coupled with a DH-2000 deuterium/halogen light source and TEM. The reaction between $AgNO_3$ with $NaBH_4$ to produce AgNPs was indicated by the appearance of yellow color in the reaction mixture (Fig.1A). A single strong peak with maximum at around 400-410 nm indicated the presence of AgNPs (Fig.1B). Moreover, the solution of AgNPs is highly stable, without any color change and visual aggregation. TEM micrographs show that AgNPs have spherical shape and average size as 10-20 nm (Fig.1C).

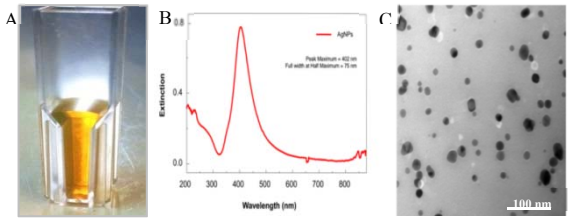

Figure 1 Characterization of AgNPs (A); photograph of AgNPs (B); UV-vis spectra of AgNPs and (C) show TEM micrograph.

MIC and MBC determination

The MIC and MBC of AgNPs against five clinical isolates of *B. pseudomallei* (Table 1) were in the range of 32-48 µg/mL and 96-128 µg/mL respectively. CAZ had higher concentrations in MIC and MBC, with the range of 128-512 µg/mL and 512-1024 µg/mL, respectively. The results indicated that AgNPs have better antibacterial activity than CAZ.

Table 1 Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs and CAZ against five isolates of *B. pseudomallei*

Isolates of *B. pseudomallei*	**AgNPs (µg/mL)**		**CAZ (µg/mL)**	
		MBC	**MIC**	**MBC**
K96243	48	96	128	512
NF10/38	48	128	128	512
H777	48	128	256	1024
316c	32	96	512	1024
979b	32	96	512	1024

The MIC value corresponded to the concentration that inhibited >99% of bacterial growth

The MBC value corresponded to the concentration that killed 100% of the bacterial growth

Analysis of the killing kinetics and bacterial viability of *B. pseudomallei* cells

We investigated killing kinetic of AgNPs against *B. pseudomallei* NF10 and *B. pseudomallei* 316C. At MIC and MBC, AgNPs exerted a rapid bactericidal activity against both isolates of *B. pseudomallei*, reducing the number of visible bacterial cells to ≥3-log with in 1 hour of exposure, while CAZ could reduce bacterial cells within 6 hour (Fig. 2A and 2B). AgNPs appeared to better inhibit cell growth than CAZ. Moreover, the fluorescence micrographs show that AgNPs killed bacterial cell within 5 minutes (Fig. 3). Also, the dead cells has smaller size than live cells, indicating that AgNPs cause cell shrinkage to change conformation of bacterial cell for apply to therapeutic.

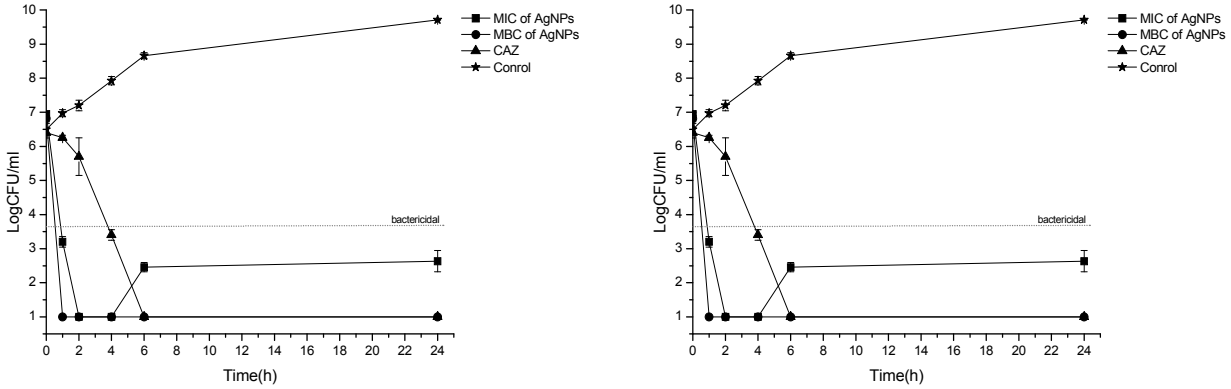

Figure 2 Killing kinetics of AgNPs against 2 isolates of *B. pseudomallei*. Bacterial suspensions of *B. pseudomallei* NF10 (A) and *B. pseudomallei*316C (B) were incubated with AgNPs and CAZ at concentrations of MIC (black square), MBC (black circle), CAZ as control (black up triangle), 0 as control (black down triangle) and samples were taken at 1, 2, 4, 6 and 24 h. Error bar showed the data are the mean of two independent experiments performed in triplicate.

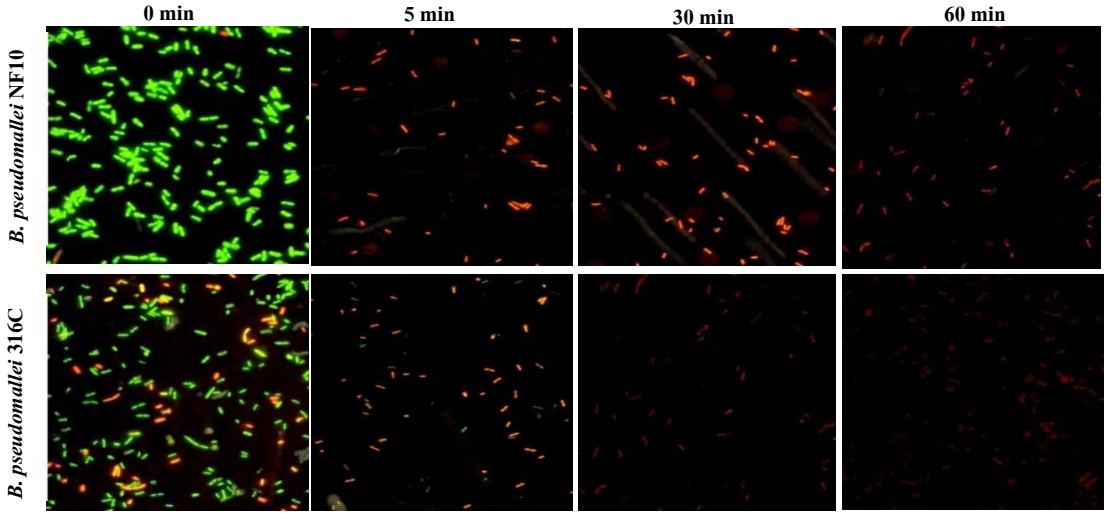

Figure 3 Fluorescence micrographs of *B. pseudomallei* NF10 and *B. pseudomallei* 316C after treated with AgNPs for 0, 5, 30 and 60 min, respectively.

Hemolytic activity determination

The cytotoxicity of AgNPs against normal mammalian cells was assessed by hemolysis. As shown in Fig. 4, AgNPs did not cause hemolysis at the concentration range of MIC and MBC (<10%) but at higher concentration (256 µg/mL) led to hemolysis of more than 10%. This result indicated that at MIC and use.

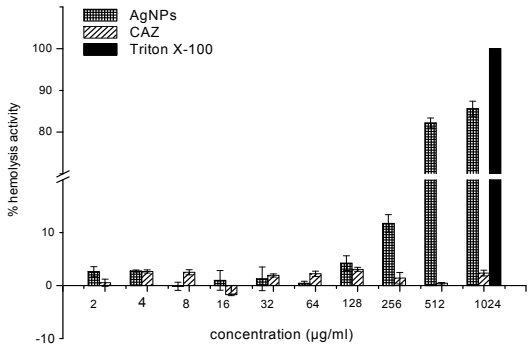

Figure 4 Hemolytic activity of AgNPs. Human erythrocytes were incubated in PBS with various concentrations of AgNPs for 1 h at 37 °C. Error bar showed the data are the mean of two independent experiments performed in triplicate.

Action of AgNPs on the ultrastructure of B. pseudomallei cells

The TEM micrographs of *B. pseudomallei* 316C cell untreated with AgNPs showed the smooth and intact membrane surface as typical characters of rod shape. The electron-dense area indicated normal genetic material of cell (Fig. 5A). Cells treated with the MBC of AgNPs (96 µg/mL) had severe damage including cell burst and debris of membrane tearing (Fig. 5B). In addition, electron-dense particle or precipitates were also observed around damaged bacterial cell (Fig. 5C)

Discussions and Conclusions

In this study, we found that the MIC and MBC values of AgNPs against all isolates of *B. pseudomallei* were higher than those observed in other strains of bacteria reported before (Li et al., 2011; Xu et al., 2011). This could be due to the difference in shape and size of AgNPs. Also, different bacterial species can eliminate metal nanoparticles via efflux pump on membrane with different extent (Bolla et al., 2011). *B. pseudomallei* is known to have exceptional activity of efflux pump, making it an intrinsic resistant species to most antibiotics. During antibacterial action, silver ions were released from AgNPs and were reduced to silver atom by nitrate reductase (Kalimuthu et al., 2008). In this study, with MIC of AgNPs, the free silver ions resulted in severe damage of membrane and biomolecule within 5 minutes (Fig. 3 and Fig. 5B and 5C). Moreover, at MIC not toxic to mammalian cell .The visual results could be better observed if we reduce the concentration of AgNPs into sub-MIC, to allow the visible cell shape and interaction of Ag to the intracellular target and destruction of the membrane.

In conclusion, AgNPs may provide as new antimicrobial agent in solving drug resistance problem of *B. pseudomallei.*

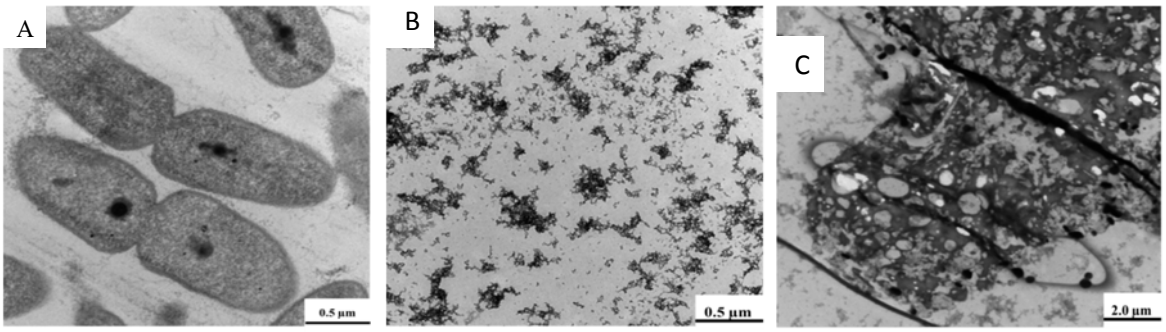

Figure 5 TEM micrographs show ultra-structural features of AgNPs with cells of *B. pseudomallei*316C, 1 h of incubation.(A) cell wasn't treated with AgNPs as control and (B and C) cell was treated with AgNPs at MBC.

Acknowledgements

The student scholarship is supported by Protein and Proteomics Research Center for Commercial and Industrial Purposes, Khon Kaen University. The instrument service (fluorescence microplate reader) was provided by Research Instrument Center, Khon Kaen University, Thailand. *B. pseudomallei* strains were kind gifts from Melioidosis Research Center, Khon Kaen University.

References

Bolla, J. M., S. Alibert-Franco, J. Handzlik, J. Chevalier, A. Mahamoud, G. Boyer, K. Kiec- Kononowicz and J. M. Pages (2011). "Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria." FEBS Lett 585(11): 1682-1690.

Chaloupka, K., Y. Malam and A. M. Seifalian (2010). "Nanosilver as a new generation of nanoproduct in biomedical applications." Trends Biotechnol 28(11): 580-588.

Cho, K.-H., J.-E. Park, T. Osaka and S.-G. Park (2005). "The study of antimicrobial activity and preservative effects of nanosilver ingredient." Electrochimica Acta 51(5): 956-960.

Currie, J. B., Fisher. A.D., Howard. M.D., Burrow. C. N. J., Lo. D., Selva-nayagam. S., Anstey. M. N., Huffam. E. S., Snelling. L. P., Marks. J. P., Stephens. P. D., Lum. D. G., Jacups. P. S. and Krause. L. V (2000). "Endemic Melioidosis in Tropical Northern Australia: A 10-Year Prospective Study and Review of the Literature." Clin Infect Dis. 31(4): 981-986.

Golubeva, O. Y., O. V. Shamova, D. S. Orlov, T. Y. Pazina, A. S. Boldina and V. N. Kokryakov (2010). "Study of antimicrobial and hemolytic activities of silver nanoparticles prepared by chemical reduction." Glass Physics and Chemistry 36(5): 628-634.

Guzman, M., J. Dille and S. Godet (2012). "Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria." Nanomedicine 8(1): 37-45.

Kager, M. L., Poll. V. T. and Wiersinga. J. W (2014). "The coagulation system in melioidosis: from pathogenesis to new treatment strategies." Expert Rev Anti Infect Ther.12(8):993-1002.

Kalimuthu, K., R. Suresh Babu, D. Venkataraman, M. Bilal and S. Gurunathan (2008). Biosynthesis of silver nanocrystals by *Bacillus licheniformis*." Colloids Surf B Biointerfaces 65(1): 150-153.

Kanthawong, S., J. G. Bolscher, E. C. Veerman, J. van Marle, H. J. de Soet, K. Nazmi, S. Wongratanacheewin and S. Taweechaisupapong (2012). "Antimicrobial and antibiofilm activity of LL-37 and its truncated variants against *Burkholderia pseudomallei*." Int J Antimicrob Agents 39(1): 39-44.

Kim, J. S., E. Kuk, K. N. Yu, J. H. Kim, S. J. Park, H. J. Lee, S. H. Kim, Y. K. Park, Y. H. Park, C. Y. Hwang, Y. K. Kim, Y. S. Lee, D. H. Jeong and M. H. Cho (2007). "Antimicrobial effects of silver nanoparticles." Nanomedicine 3(1): 95-101.

Kora, A. J. and J. Arunachalam (2010). "Assessment of antibacterial activity of silver nanoparticles on *Pseudomonas aeruginosa* and its mechanism of action." World Journal of Microbiology and Biotechnology 27(5): 1209-1216.

Lara, H. H., N. V. Ayala-Núñez, L. d. C. Ixtepan Turrent and C. Rodríguez Padilla (2009). "Bactericidal effect of silver nanoparticles against multidrug-resistant bacteria." World Journal of Microbiology and Biotechnology 26(4): 615-621.

Leelarasamee, A. and Bovornkitti, S (1989). "Melioidosis: Review and Update."Clinical Infectious Diseases 11 (3):413-425.

Li, W. R., X. B. Xie, Q. S. Shi, S. S. Duan, Y. S. Ouyang and Y. B. Chen (2011). "Antibacterial effect of silver nanoparticles on *Staphylococcus aureus*." Biometals 24(1):135-141.

Li, W. R., X. B. Xie, Q. S. Shi, H. Y. Zeng, Y. S. Ou-Yang and Y. B. Chen (2010). "Antibacterial activity and mechanism of silver nanoparticles on *Escherichia coli*." Appl Microbiol Biotechnol 85(4): 1115-1122.

Lok, N-C., Ho. M-C., Chen. R., He. Y-Q., Yu. Y-W., Sun. H., Tam. K-H. P., Chiu. F-J. and Che. M-C (2006). "Proteomic Analysis of the Mode of Antibacterial Action of Silver Nanoparticles." J Proteome Res. 5(4): 916-24.

Madhongsa, K., S. Pasan, O. Phophetleb, S. Nasompag, S. Thammasirirak, S. Daduang, S. Taweechaisupapong, A. L. Lomize and R. Patramanon (2013). "Antimicrobial action of the cyclic peptide bactenecin on *Burkholderia pseudomallei* correlates with efficient membrane permeabilization." PLoS Negl Trop Dis 7(6): e2267.

Mei, L., Z. Lu, W. Zhang, Z. Wu, X. Zhang, Y. Wang, Y. Luo, C. Li and Y. Jia (2013). "Bioconjugated nanoparticles for attachment and penetration into pathogenic bacteria." Biomaterials 34(38): 10328-10337.

Peacock, S. J., D. Limmathurotsakul, Y. Lubell, G. C. Koh, L. J. White, N. P. Day and R. W. Titball (2012). "Melioidosis vaccines: a systematic review and appraisal of the potential to exploit biodefense vaccines for public health purposes." PLoS Negl Trop Dis 6(1): e1488.

Sarovich, D. S., E. P. Price, D. Limmathurotsakul, J. M. Cook, A. T. Von Schulze, S. R. Wolken, P. Keim, S. J. Peacock and T. Pearson (2012). "Development of ceftazidime resistance in an acute *Burkholderia pseudomallei* infection." Infect Drug Resist 5: 129-132.

Schweizer, P. H (2012). “Mechanisms of antibiotic resistance in *Burkholderia pseudomallei*: implications for treatment of melioidosis.” Future Microbiol. 7(12): 1389–1399.

Thomas, V., M. M. Yallapu, B. Sreedhar and S. K. Bajpai (2007). "A versatile strategy to fabricate hydrogel-silver nanocomposites and investigation of their antimicrobial activity." J Colloid Interface Sci. 315(1): 389-395

Tiede, K., A. B. Boxall, S. P. Tear, J. Lewis, H. David and M. Hassellov (2008). "Detection and characterization of engineered nanoparticles in food and the environment." Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25(7): 795-821.

Xu, H., F. Qu, H. Xu, W. Lai, Y. Andrew Wang, Z. P. Aguilar and H. Wei (2012). "Role of reactive oxygen species in the antibacterial mechanism of silver nanoparticles on *Escherichia coli* O157:H7." Biometals 25(1): 45-53.

Zhao, G. and Stevens. E. S (1998). “Multiple parameters for the comprehensive evaluation of the susceptibility of *Escherichia coli* to the silver ion.” Biometals. 11(1): 27-32.