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Changes in protein patterns of *Staphylococcus aureus* and *Escherichia coli* by silver nanoparticles capped with poly (4-styrenesulfonic acid-co-maleic acid) polymer

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Abstract

Background: While silver nanoparticles (AgNPs) are increasingly attractive as an antibacterial agent in many applications, the effect of AgNPs on bacterial protein profiles, especially AgNPs stabilized by polymeric molecules, is not well understood.

Objectives: To investigate the changes in bacterial protein patterns by AgNPs capped with poly (4-styrenesulfonic acid-co-maleic acid) (AgNPs-PSSMA) polymer toward *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922.

Methods: The growth of bacteria after incubated with AgNPs-PSSMA for different time intervals was determined by optical density at 600 nm. Their protein patterns were observed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteomic analysis of extracted proteins was determined by liquid chromatography-tandem mass spectrometry (LC–MS/MS).

Results: AgNPs-PSSMA was able to inhibit the growth of both *S. aureus* and *E. coli* cells. The treated bacterial cells expressed more proteins than the untreated cells as seen from SDS-PAGE study. Nanosilver (NS) caused the upregulation of metabolic gene, waaA, in *S. aureus* cells. For *E. coli* cells, the upregulated proteins were metabolic genes (srlB, fliE, murD) and other genes dealt with DNA replication (dinG), DNA–RNA transcription (yrdD), RNA–protein translation (rplD), molecular transport (sapF), and signal transduction (tdcF).

Conclusions: The antibacterial effect of AgNPs-PSSMA may arise by changing the bacterial proteins and thus interfering with the normal cell function.

Keywords: *Escherichia coli*; nanoparticles; nanomedicine; proteomics; *Staphylococcus aureus*

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The high surface area per mass and small size of the nanoparticles make silver nanoparticles (AgNPs) an effective antibacterial agent [1–3]. For this reason, AgNPs have been applied in diverse medical applications ranging from silver-based dressings to silver-coated medicinal devices and on numerous consumer products [4]. Although AgNPs have been widely used for antimicrobials, their mechanism is still not well understood. Possible antimicrobial mechanisms proposed include (1) interference with cell wall synthesis [5–8], (2) inhibition of protein synthesis [6, 8], (3) interference with nucleic acid synthesis [6, 8], and (4) inhibition of a metabolic pathway [6, 8, 9]. To get a better insight on how AgNPs affect bacterial growth, the proteomic approach is concerned as an effective tool for protein analysis. Recently, only few studies have been reported on bacterial proteomes after AgNP exposure [10–17], and most of the AgNPs previously described were stabilized with anionic citrate.

It is noteworthy that the stability of AgNPs synthesized can be increased by coating the nanoparticles with polymers, which can prevent particle agglomeration by steric hindrance. The differences in type of stabilizers could lead to different properties of AgNPs including the release or distribution of silver ions related to perhaps the protein activity [11, 12]. Thus, to study of how AgNPs affect the protein function, the capping agent of synthesized AgNPs should be mentioned. Poly (4-styrenesulfonic acid-co-maleic acid) or PSSMA is a polyelectrolyte copolymer of styrenesulfonic acid and maleic acid, which is widely used to develop multilayer thin films for biomaterial or controlled drug release [18].

In this paper, AgNPs capped with anionic polystyrene-*o*-maleic acid polymer (AgNPs-PSSMA) were studied for antibacterial activity against Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, and Gram-negative bacteria, *Escherichia coli* ATCC 25922. Bacterial proteins were identified by proteomic approach. Our study is the first report on changes in protein expression of bacteria after the antibacterial (AgNPs-PSSMA) treatment.

Materials and methods

Preparation and characterization of AgNPs

Nanoscale silver particles (3.33 mM) stabilized by PSSMA (Aldrich, USA) were prepared using a chemical reduction and characterized as detailed in Tamiyakul et al. [19]. Briefly, 10 mM sodium borohydride ($NaBH_4$; Fisher Scientific, USA) was rapidly added into a mixture of 10 mM silver nitrate ($AgNO_3$; Carlo Erba Reagents, Italy) and 1 mM PSSMA in order to reduce silver ions to form nanoparticles. A dark brown solution of PSSMA-stabilized nanosilver (NS) showed a characteristic peak at a maximum wavelength (λ_{max}) of 395 nm, as determined by Specord S 100 UV spectrophotometer (Analytikjena, Germany). The spherical shape of NS was seen from transmission electron microscope (TEM; JEM-2100, Jeol, Japan). The average paricle size of 5.21 ± 4.43 nm (n = 250) was obtained by further TEM analysis using equipped SemAfore v.5.21 program. The value of surface charge of NS measured by Zetasizer NanoZS (Malvern Instruments, UK) was –35.5 ± 0.96 mV.

Bacteria, culture media, and cultivation

The strains of Gram-positive *S. aureus* ATCC 25923 and Gram-negative *E. coli* ATCC 25922 were purchased from American Type Culture Collection (ATCC). Tryptic soy broth (TSB; Difco, USA) and tryptic soy agar (TSA; Difco, USA) media were used for bacterial cultivation at 37°C. To prepare bacteria for investigation of bacterial growth, a loop of *S. aureus* and *E. coli* in glycerol stock was streaked on TSA agar plate and then incubated at 37°C for 24 h. Then, a bacterial colony was inoculated into 10 mL TSB broth and further incubated at 37°C for 24 h. The bacteria were then centrifuged at 5000 rpm for 3 min. The supernatant was discarded, and the pellet was re-suspended with TSB media for further study. For the TSB media, it was prepared by dissolving 30 g TSB powder in 1 L water or NS and then sterilized by membrane filtration, Acrodisc syringe filter with 0.2 μm Supor membrane (Pall Science, USA).

Growth of bacteria exposed to AgNPs

The bacterial growth experiment was performed using *S. aureus* and *E. coli* (1.5×10^8 CFU/mL, determined at OD of 0.1 at 600-nm wavelength (OD_{600}). Bacteria incubated either in an absence (control) or in a presence of NS were collected at different incubation times (0–5 h). The bacterial growth curves were determined in quadruplicate from OD_{600} values.

Bacterial protein extraction

Bacteria collected at incubation times of 1, 15, 30, 45, 60, 120, and 180 min were centrifuged at 5000 rpm for 5 min, and the supernatant was discarded. The pellet was re-suspended with 0.5% w/v sodium dodecyl sulfate (SDS; Sigma-Aldrich, USA) solution, vortexed vigorously, and frozen in a freezer at –80°C

overnight. After thawing, the suspension was centrifuged at 5000 rpm for 5 min at room temperature, and the supernatant was determined for the extracted protein by a method of Lowry using bovine serum albumin (BSA) as a standard [20].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The extracted proteins were mixed with a loading buffer (0.125 M Tris-HCl pH 6.8, 20% w/v glycerol, 5% w/v SDS, 0.2 M dithiothreitol [DTT], 0.02% w/v bromophenol blue) before loading onto polyacrylamide gels (12.5% separating gel, 5% stacking gel), which were prepared following the method of Laemmli [21]. An electrophoresis system was run at 50 V for stacking gel and 70 V for separating gel until the dye front reached approximately 1 cm from the edge of the gel. All gels were visualized by silver and Coomassie brilliant blue G staining and scanned with Image Scanner (Bio-Rad, USA).

Liquid chromatography-tandem mass spectrometry

In order to analyze the bacterial peptides by liquid chromatography-tandem mass spectrometry (LC–MS/MS), each lane of the gel was sectioned horizontally in order to acquire the entire population of proteins in the lane. Gel slices were excised to obtain gel plug with 1 mm^3 in size. For in-gel digestion, the gel plugs were dehydrated with 100% acetonitrile (RCI Labscan, Thailand) and reduced with 10 mM DTT (USB Co. Ltd., USA) for 1 h at room temperature. Alkylation was further done in the dark using 100 mM iodoacetamide (GE Healthcare, UK) for 1 h at room temperature before dehydrated twice with 100% acetonitrile for 5 min. Finally, 10 ng of trypsin solution (Promega, USA) was added to the gels followed by overnight incubation at 37°C so as to digest proteins. Peptides were extracted, collected, and kept at −80°C prior to mass spectrometry analysis [22].

For the peptide analysis, the extracted peptides were re-suspended with 0.1% v/v formic acid (FA, [AppliChem, Germany]) and centrifuged at 10,000 rpm for 5 min. The supernatant was injected into Ultimate 3000 LC system (Dionex) coupled with ESI-ion Trap MS (HCT Ultra PTM Discovery System, BrukerDaltonics Ltd., UK). DeCyder MS Differential Analysis software (DeCyderMS, GE Healthcare) was used for protein quantitation [23, 24]. Mascot software (Matrix Science Ltd., London, UK) was used for the protein identification based on the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) [25]. For Mascot MS/MS ion search, the peptide taxonomy was set up as bacteria (eubacteria), fixed modifications as carbamidomethyl (C), and variable modifications as oxidation (M). The peptide charge was 1+, 2+, and 3+ with MS/MS ion mass tolerance of ±1.2 Da, fragment mass tolerance of ±0.6 Da, and allowance for one miscleavage [26]. The protein functions of the identified proteins were further searched based on the UniProt (http://www.uniprot.org) for enabling the gene ontology (GO) prediction. The association or interaction network of protein was clarified through the Search Tool for the Retrieval of Interacting Genes/Proteins or String database (http://string-db.org). The String database analyzes the data based on the known and predicted protein interactions [27].

Results

Bacterial growth and extracted protein from gel electrophoresis

The proliferation of *E. coli* cells and *S. aureus* cells after exposed to AgNPs-PSSMA was determined by measuring OD_{600} at various incubation times. The bacterial proteins of *E. coli* and *S. aureus* cells treated with NS for incubation times of 15, 30, 45, 60, 120, and 180 min were determined by double staining SDS-polyacrylamide gel electrophoresis (PAGE). The results indicated that growths of the bacteria were inhibited, and no exponential phase was clearly observed in the NS-treated bacteria (**Figure 1A,B**). In contrary, for the untreated (control) bacterial cells, the growth pattern was in a lag phase for 1 h and then continued to an exponential phase. From the SDS-PAGE study, NS-treated *S. aureus* and *E.coli* cells showed more protein expression than the corresponding untreated cells (**Figure 1C,D**).

In order to maintain the optimal level of bacterial protein prior to proteomic analysis, the cells cultured in the medium were collected at time intervals covering the early exponential phase, which were 15, 30, 45, 60, 120, and 180 min.

Bacterial proteomic analysis

The extracted proteins of bacteria gathered from every incubation time were digested prior to analysis by LC/MS–MS. The proteins of NS-treated *E. coli* cells, untreated *E. coli* cells, NS-treated *S. aureus* cells, and untreated *S. aureus* cells were identified based on the NCBI database. There were a total number of 860 bacterial proteins matched with proteins deposited in the database. However, 26 proteins belonged to *E. coli* (treated

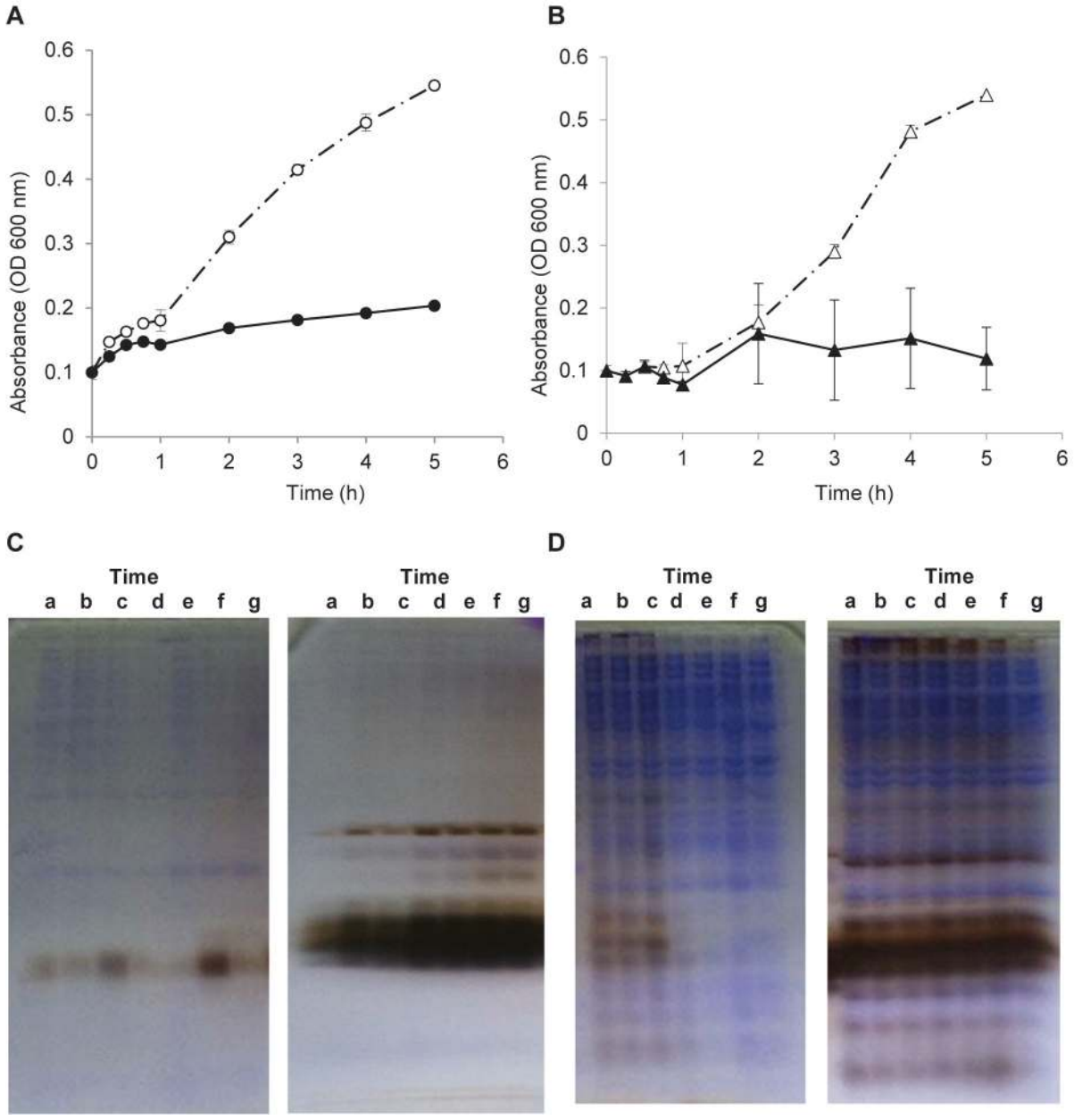

Figure 1. (**A**) Growth curves of *Staphylococcus aureus* ATCC 25923 cells and (**B**) those of *Escherichia coli* ATCC 25922 cells after exposed (solid line) and unexposed (dashed line) to nanosilver. Error bars indicate standard deviations of the means of $n = 4$. (**C**) Patterns of total protein extracted from *S. aureus* ATCC 25923 and (**D**) those of *E. coli* ATCC 25922 after (left) unexposed and (right) exposed to nanosilver at different incubation times (a: 0 min, b: 15 min, c: 30 min, d: 45 min, e: 60 min, f: 120 min, and g: 180 min).

or untreated) and 3 proteins belonged to *S. aureus* (treated or untreated) were further identified for protein functions as shown in **Table 1**.

From proteomes analysis, AgNPs-PSSMA were able to downregulate long-chain fatty acid transport protein (fadL) of *S. aureus* and quinolinate synthase A (nadA) and protein YhgF (yhgF) of *E. coli*. For *S. aureus*, only 3-deoxy-D-manno-octulosonic-acid transferase protein (waaA) was upregulated after exposed to NS (**Table 1**). In contrast, eight proteins of *E. coli* were expressed after exposed to NS. The *E. coli* proteins mentioned were functioned with metabolism (srlB, fliE, murD), DNA replication (dinG), DNA–RNA transcription (yrdD), RNA–protein translation (rplD), molecular transport (sapF), and signal transduction (tdcF).

The upregulated genes and downregulated genes of the bacteria were further studied for protein–protein interaction data via String database. The bioinformatics result was shown as a network of predicted functional associations for

Table 1. The identified proteins by LC–MS/MS with known functions of *Staphylococcus aureus* and *Escherichia coli* with untreated (control) and nanosilver (NS)-treated conditions

Protein accession number	Protein name	Protein symbol	Function
***S. aureus* (control)**			
gi|170081960	Long-chain fatty acid transport protein	fadL	Molecular transport
***S. aureus* (NS treated)**			
gi|326798469	3-Deoxy-D-manno-octulosonic-acid transferase	waaA	Metabolic process
***S. aureus* (control and NS treated)**			
gi|1786970	2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase	gpmA	Metabolic process
***E. coli* (control)**			
gi|1786964	Quinolinate synthase A	nadA	Metabolic process
gi|153844091	Protein YhgF	yhgF	Metabolic process
***E. coli* (NS treated)**			
gi|146319043	Threonine dehydrogenase and related Zn-dependent dehydrogenase	srlB	Metabolic process
gi|308050024	Flagellar hook-basal body protein	fliE	Metabolic process
gi|156973219	UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase	murD	Metabolic process
gi|1279404	Peptide transport system ATP-binding protein SapF	sapF	Molecular transport
gi|323524861	Enoyl-CoA hydratase/isomerase	tdcF	Signal transduction
gi|1787018	Probable ATP-dependent helicase DinG	dinG	DNA replication
gi|283778629	DNA topoisomerase type IA Zn finger domain-containing protein	yrdD	Transcription
gi|1789715	50S ribosomal protein L4	rplD	Translation
***E. coli* (control and NS treated)**			
gi|227357988	Siderophore biosynthesis IucA/IucC family protein	iucA	Metabolic process
gi|226943115	Isopropylmalate isomerase large subunit	leuC	Metabolic process
gi|225010785	PhoH family protein	phoH	Metabolic process
gi|261854682	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	purH	Metabolic process
gi|319762019	Haloacid dehalogenase (HAD)-superfamily hydrolase	yqaB	Metabolic process
gi|331007050	Colicin V production protein	cvpA	Molecular transport
gi|325067458	Oligopeptide ATP-biding cassette (ABC) transporter periplasmic protein	oppA	Molecular transport
gi|320353100	OmpA/MotB domain-containing protein	motB	Molecular transport
gi|257459944	Putative TonB-dependent receptor	tonB	Signal transduction
gi|333984533	TonB-dependent siderophore receptor	yncD	Signal transduction
gi|312196358	IclR family transcriptional regulator	iclR	Transcription
gi|384412003	NusA antitermination factor	nusA	Transcription
gi|85860692	ATP-dependent RNA helicase	rhlB	Transcription
gi|260893293	Alanyl-tRNA synthetase	alaS	Translation
gi|227495901	Leucine—tRNA ligase	leuS	Translation
gi|29653579	50S ribosomal protein L1	rplA	Translation

a group of proteins. The network nodes represent the proteins while the edges (lines) are the predicted associations of protein (**Figure 2**). The connection among proteins in *S. aureus* represented genes mainly involved in metabolic process, while those found in *E. coli* were genes mostly implicated mainly in the transporter system, metabolic activity, transcription, and translation. Interestingly, for *E. coli*, there were four unmatched proteins, such as family transcriptional regulator (iclR), HAD-superfamily hydrolase (yqaB), threonine dehydrogenase, and related Zn-dependent dehydrogenase (srlB) and probable ATP-dependent helicase DinG (dinG). As srlB and dinG genes were expressed in NS-treated *E. coli*, they were represented as biomarkers for antibacterial effect of AgNPs-PSSMA.

Discussion

In this study, we investigate the role of AgNPs-PSSMA on the changes in protein patterns of Gram-positive *S. aureus* and Gram-negative *E. coli*, which eventually lead to bacterial cell death. A decrease in the cell survival of the bacteria treated with NS was observed while the untreated control cells grew exponentially after an hour of incubation (**Figure 1A,B**). From

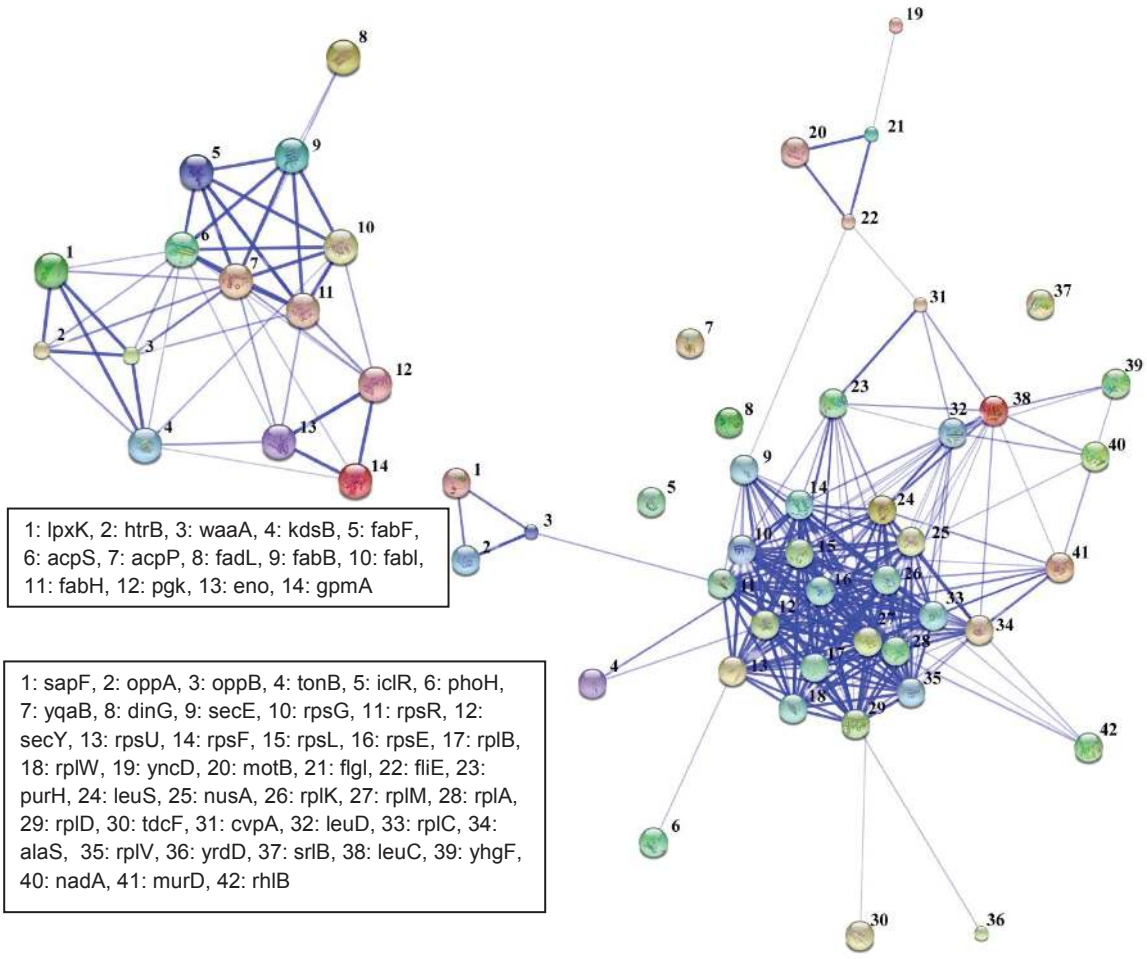

Figure 2. Interaction network of matched categories for (left) *Staphylococcus aureus* ATCC 25923 and (right) *Escherichia coli* ATCC 25922. Thicker lines indicate higher confidence and higher number of interactions described by String database.

the previous reports on the antimicrobial activity of NS [28, 29], it is anticipated that AgNPs-PSSMA when penetrating into the cells make cell death by causing structural changes and damage, which could disturb vital cell functions. From our study, the changes in cellular protein after exposed to AgNPs-PSSMA were obviously seen from gel electrophoresis results, especially the *E.coli* cells (**Figure 1C,D**). Protein pattern modification leads to bacterial death by interference with metabolic function and synthesis of cell wall, nucleic acid, and protein [6].

We used the proteomic approach as a tool to explore the bacterial proteins of the NS-treated cells. For *S. aureus*, protein fadL was downregulated by NS. Protein fadL is associated with protein receptor and translocation of long-chain fatty acids across the outer membrane, the first part to confront with NS [30]. AgNPs-PSSMA might affect the protein receptors leading to lipid transport deficiency and ultimately cell death [2, 31, 32]. In contrast, 3-deoxy-D-manno-octulosonic acid (Kdo) transferase or waaA, a protein locating at an inner cell membrane and involved in lipopolysaccharide (LPS) biosynthesis [33, 34], was upregulated in NS-treated *S. aureus*. Cellular oxidative response has been reported to promote the function of LPS biosynthesis [35], and NS is evident to cause oxidative stress. It was then not unexpected that AgNPs-PSSMA induce waaA gene.

For NS-treated *E. coli*, quinolinate synthase A (nadA) and protein YhgF (yhgF) were downregulated. Protein nadA is in a cytoplasm involved in the cell metabolism. It catalyzes the condensation of iminoaspartate with dihydroxyacetone phosphate to form quinolinate (pyridine nucleotide biosynthesis) [36–38]. As the appearance of hydrogen peroxide (H_2O_2) has known to depress quinolinate synthetase, which is used for quinolinate production [39], a release of H_2O_2 from NS [40] could possibly lead to inhibition of nadA protein. Protein yhgF

is the respiratory protein involved in cyclic di-GMP signaling [41]. Accordingly, NS could interfere with the respiratory process of the bacteria, finally leading to cell death.

In contrary to the downregulation, NS triggered the expression of approximately eight proteins in *E. coli* cells (**Table 1)**. It seems that when *E. coli* cells are perturbed, a higher number of genes dealt with many cell functions are expressed [42]. In this study, the upregulated proteins found were involved in the metabolic function (srlB, fliE, and murD proteins), signal transduction (tdcF protein), and signal transcription (yrdD protein).

Interestingly, we have found the upregulation of proteins dinG, rplD, and sapF, which are suggested to play a role in antimicrobial resistance. The ATP-dependent helicase dinG (dinG) is related to DNA repair and replication [43]. The process of DNA repair after exposure to NS might be consequences of cell resistance to antibacterials. Protein rplD is the 50S ribosomal protein functions as ribosome-mediated translation of mRNA into a polypeptide [44]. Protein sapF is an ATP-binding protein in plasma membrane involved in a peptide transport system [45]. The upregulation of proteins rplD and sapF of *E. coli* perhaps indicates a change in protein synthesis after confront with antibacterial agent.

The previous proteomic study in *E. coli* cells was usually focused on the membrane protein. The expression of outer membrane precursors (OmpA, OmpC, OmpF, OppA) of *E. coli* cells was reported to be enhanced by NS [10]. The *rfa* gene necessary for outer member lipopolysaccharide was expressed in *E. coli* mutant strain [12]. The bacterial response to NS could be different and perhaps dependent on a type of stabilizer. For example, genes functioning in quinone binding and ubiquinone biosynthesis were found in *E. coli* mutant strain treated with AgNPs stabilized by polyethylene imine polymer but not found in those treated with polyvinylpyrrolidone polymer-capped AgNPs [12]. Herein, we reported the additional information about the effect of AgNPs stabilized by PSSMA polymer on gene expression of *S. aureus* and *E. coli* cells. Finally, the association of bacterial proteins was determined via String database. We have found that srlB and dinG proteins expressed in *E. coli* after confronted with NS were not found for any protein association (**Figure 2**). The finding makes proteins srlB and dinG be considered as novel biomarkers for antibacterial effect of such NS.

Conclusion

The stable AgNPs-PSSMA could be used as antibacterial agents to Gram-positive and Gram-negative bacteria. NS caused both downregulation and upregulation of proteins in bacterial cells with the latter was more obviously found in *E. coli* cells.

In this study, the NS without capping agent added was not performed due to the reasons dealing with the stability of AgNPs. The data obtained from the present study were the antibacterial effect AgNPs capped with PSSMA. The AgNPs could be further applied clinically as materials for antibacterial purposes. As the bacterial proteins change after AgNPs exposure seemed to be depend on the type of stabilizers [12], the further study should be on the comparison of bacterial protein change of AgNPs capped with different types of polymer. The information could be used to select the appropriate stabilizing polymer for the targeted bacterial proteins.

Author contributions. SR, ST, and WW contributed substantially to the conception and design of this study. HT, JJ, and NP contributed substantially to the acquisition of data. HT, SR, ST, and WW analyzed and interpreted the data. HT and WW drafted the manuscript. SR, JJ, NP, ST, and WW contributed substantially to its critical revision. All the authors approved the final version submitted for publication and take responsibility for the statements made in the published article.

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Conflict of interest statement. The authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflicts of interest.

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