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Thesis for the Degree of Master of Engineering

Inhibition of Biofilm Formation and Regulation of Virulence Properties by Iron Nanoparticles in *Pseudomonas aeruginosa*

by

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Department of Food Science & Technology

The Graduate School

Pukyong National University

February 2020

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(철 금속 나노입자에 의한

녹농균의 생물막 생성 억제 및 병원성 인자 생성 조절)

Advisor: Prof. Young-Mog Kim

by

Pham Nguyen Thuy Dung

A thesis submitted in partial fulfillment of the requirements for the degree of

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in Department of Food Science and Technology, The Graduate School,
Pukyong National University

February 2020

Inhibition of Biofilm Formation and Regulation of Virulence Properties by Iron Nanoparticles in *Pseudomonas aeruginosa*

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Inhibition of Biofilm Formation and Regulation of Virulence Properties by Iron Nanoparticles in *Pseudomonas aeruginosa*

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Abstract

Biofilm formation is one of the resistance mechanisms of *Pseudomonas aeruginosa* against antimicrobial compounds. Biofilm formation also characterizes for the infection and pathogenesis of *P. aeruginosa*, along with production of various virulence factors. With recent development of nanotechnology, the present study evaluated the synthetic iron nanoparticle (FeOOH-NP) as an active agent to inhibit the formation of *P. aeruginosa* biofilm. The FeOOH-NP was synthesized and subjected to characterization using UV-visible spectroscopy, fourier transform infrared spectroscopy (FTIR), field emission transmission electron microscopy (FE-TEM), dynamic light scattering (DLS), and energy dispersive X-ray diffraction (EDX). The synthesized FeOOH-NP was characterized with rod shape and average size of 40 nm. Subsequently, the FeOOH-NP was evaluated for their inhibitory effects against *P. aeruginosa* bacterial growth, biofilm formation, virulence factor production, and motility. Results have shown that *P. aeruginosa* biofilm inhibited by FeOOH-NP was in a concentration-dependent manner, with inhibition of the bacterial biofilm

formation increased as FeOOH-NP concentration increased. Microscopic observations as well as viable cell counts also confirmed the disruption in the biofilm structure in the presence of FeOOH-NP. In addition, the presence of FeOOH-NP was also found to modulate some important virulence factors produced simultaneously with biofilm formation such as pyocyanin, pyoverdine, rhamnolipid, proteolytic and hemolytic activities. Besides, the bacterial motility, including swimming and swarming, was affected by FeOOH-NP treatments. These findings provide insights to anti-biofilm effect of a new iron NP, contributing to the search for an effective anti-biofilm agent to combat *P. aeruginosa* infections associated with biofilm formation.

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Introduction

Pseudomonas aeruginosa is a common opportunistic Gram-negative pathogen that causes a vast range of infections to the global population. Infection severity and source are dependent on the bacterial genotype and colonization lifestyle (Valentini et al. 2018). The ability to form biofilm structure (1) characterizes for the bacterial pathogenesis and antimicrobial resistance, (2) protects from the host immune system and environmental stressors and (3) enables survival on artificial surfaces compared to the planktonic counterpart (Lee et al. 2014; Maurice et al. 2018; Rasamiravaka et al. 2015). Thus, the search for effective therapeutic strategies has become more challenging. Along with biofilm formation, the mortality of *P. aeruginosa* infections is enhanced by the production of various virulence factors, which are extremely diverse in functions and regulation. For instance, pyocyanin, which is regulated by quorum sensing, characterizes for the unique blue-green pigment of *P. aeruginosa* cells and induces oxidative stress in several human systems (Hall et al. 2016; Lau et al. 2004; Little et al. 2018). Pyoverdine and pyochelin, which are regulated by Fur signal receptor and sigma factors, are the two siderophores playing an essential role for the bacterial iron uptake (Maura et al. 2016; Oglesby-Sherrouse et al. 2014; Ringel and Bruser 2018). Rhamnolipid, which is controlled by *rhlR* and *rhlI* quorum sensing signaling system, is associated with fatal cystic fibrosis in

human and is a promising biosurfactant for bioremediation (Kharami et al. 1989; Maier and Soberon-Chavez 2000; Murray et al. 2003; Ochsner and Reiser 1995; Vatsa et al. 2010). Furthermore, protease enzymes such as elastase B and protease IV controlled by LasI quorum sensing system, are the crucial constituent of *P. aeruginosa*-caused cystic fibrosis, wound infection and eye infection (Hoge et al. 2010; Pesci et al. 1997). In addition, *P. aeruginosa* performs several types of motilities such as swimming in liquid environment, and swarming in semi-solid environment, in order to properly attach and colonize on a desired surface, priming for biofilm formation (Rasamiravaka et al. 2015; Yeung et al. 2009).

Up to the present, the approaches targeting *P. aeruginosa* biofilm inhibition have targeted three directions: (1) to prevent the initial adhesion of planktonic bacterial cells, (2) to disrupt the internal cell-to-cell signaling pathways within the biofilm community and their down-regulated production of the aforementioned virulence factors and adhesion factors and (3) to eradicate the pre-existing matured biofilm and subsequently disperse the biofilm-released sessile cells (Blackledge et al. 2013; Maurice et al. 2018). However, high complexity of the biofilm structure and regulation network remain a challenge to understand about the effectiveness as well as underlying mechanisms.

With recent advanced development in nanotechnology, nanoparticles (NPs) have been exploited as an effective antimicrobial agent due to several advantages derived from its nanometric size (<100 nm) such as increased surface area-to-volume ratio, controlled release and rapid penetration through

bacterial cell membrane (Javaid et al. 2018; Khan et al. 2018; Neethirajan et al. 2014; Ramasamy and Lee 2016; Wang et al. 2017). NPs morphology, structure, size and optical properties, which majorly determine their bioactivities, can be studied by characterization work using transmittance electron microscopy (TEM) and scanning electron microscopy (SEM) (Joo and Aggarwal 2018; Khan et al. 2017). NPs can be synthesized from a wide variety of materials, one of which is metal. The distinct optical and mechanical properties of metal ions combined with nano-scaled size of the NPs have given the metal-based NPs numerous applications (Khan et al. 2017). Recently, significant number of metal NPs have been recognized for antimicrobial and anti-biofilm activities (Bandara et al. 2015; Borcherding et al. 2014; Ghasemian et al. 2015; Gurunathan et al. 2014; Lee et al. 2014; Yu et al. 2016). In particular, iron-based nanoparticles were previously found to attenuate biofilm formation of major pathogenic bacteria such as *Staphylococcus epidermidis* and *Staphylococcus aureus* (Naha et al. 2019; Subbiahdoss et al. 2012; Taylor et al. 2012). Besides, using iron-based nanoparticles was considered beneficial for antibiotic delivery by simply applying magnetic field (Taylor and Webster 2009). However, to our updated knowledge, NP synthesized from iron oxyhydroxide (FeOOH) has not yet been characterized and its effects on *P. aeruginosa* biofilm formation as well as virulence factors also remain unknown. For these reasons, the aim of the present study is to synthesize and characterize FeOOH-NP, which in turn evaluates the biofilm inhibition and virulence modulation of FeOOH-NP in *P. aeruginosa*.

Materials and Methods

1. Bacterial strains, chemicals and growth conditions

The culture media for the growth of *P. aeruginosa* include tryptic soy broth (TSB; Difco Laboratory Inc., Detroit, MI) and tryptic soy agar (TSA; Difco La boratory Inc.). *P. aeruginosa* PAO1 KCTC 1637 was obtained from Korean Co llection for Type Cultures (KCTC, Daejeon, Korea). To perform the experimen t, a seed culture was prepared by taking a single colony from the TSA plate an d inoculated in TSB (30 mL) and incubated at 35ºC in shaking incubator (250 rpm). For each experiment, the cultures were grown overnight and re-inoculated at 1:100 dilution in fresh TSB. The cell growth measurements indic ated by optical density (OD) was measured at 600 nm wavelength using the sp ectrometer. Chemicals and reagents used in the entire experiment were of anal ytical grade.

2. Synthesis and characterization of FeOOH-NP

FeOOH-NP was synthesized as reported previously with minor modifications (Ozaki et al. 1984). Briefly, $FeCl_3.6H_2O$ (0.5406 g) and NaH_2PO_4 (5.4 mg) were mixed with distilled water at room temperature for 30 min until the yellow color was shown. The solution was collected in a glass bottle and kept in the preheated oven at 100°C for 24 h. The solution color was

changed to brown yellow, indicating the formation of FeOOH-NP. The product was subjected to centrifugation (15,000 rpm for 15 min), then was washed thrice with distilled water.

Field-emission transmission electron microscopy (FE-TEM) images were acquired by JEM-2010 microscope (JEOL, Milpitas, CA). Dynamic light scattering (DLS) analysis was recorded by an electrophoretic light scattering spectrophotometer ELS-8000 (OTSUKA Electronics Co. Ltd., Tokyo, Japan). Fourier transform infrared spectroscopy (FTIR; Perkin Elmer Inc., Waltham, MA) was used to check the functional groups of the prepared nanoparticles over the wavelength range of 400-4000/cm. UV-Visible absorption spectrum was determined using a UV-Visible spectroscopy (Thermo Biomate 5, Waltham, MA). Thermogravimetric (TG) analysis was conducted on a thermal analyzer (TGA 7; Perkin Elmer, Waltham, MA) within the temperature range from 50°C to 800°C with a heating rate of 10ºC per min.

3. Biofilm formation assays

The procedure for biofilm assay used in this study was adopted from the previous study (Lee et al. 2011). Briefly, the overnight grown cell culture was diluted in TSB (1:100) and added to a 96-well microtiter plate. FeOOH-NP solution at various concentrations ranging from 0.16875 to 1.35 mM was added to the diluted bacterial culture and incubated at 35ºC for 24 h in static condition. After incubation period, planktonic cells had been discarded and the attached cells were washed with distilled water, followed by staining with

aqueous crystal violet (0.1%) and incubation at room temperature for 20 min. Following the dye removal, the plate was washed, mixed with 95% ethanol and the OD value was measured at 570 nm wavelength. Simultaneously, the growth of bacterial cells within the biofilm in the presence of FeOOH-NP was estimated by OD value at 600 nm.

Similar methodology was applied to examine the disrupting effect of FeOOH-NP on *P. aeruginosa* pre-existing matured biofilm. The overnight grown bacterial culture (1:100 dilution in TSB) was allowed to form biofilm in a 96-well microtiter plate and incubated at 35ºC for 24 h without agitation. After planktonic cells were removed, fresh TSB media along with different concentrations of FeOOH-NP ranging from 0.16875 to 1.35 mM was added to the wells containing attached cells and incubated further at 35ºC for 24 h. The quantity of cells eradicated from the matured biofilm was determined by the OD value at 570 nm after staining with crystal violet dye. For both biofilm and growth assays, each FeOOH-NP concentration was replicated three times and average value was calculated.

4. Survivability assay of planktonic and biofilm cells

The impact of FeOOH-NP on the viability of planktonic and biofilm cells were evaluated by performing bacterial colony count. Briefly, the overnight *P. aeruginosa* grown cell culture (1:100 dilution in TSB) was added with FeOOH-NP of different concentrations (from 0.16875 mM to 1.35 mM) in a 96-well titer plate and was incubated at 35ºC for 24 h under static conditions.

The planktonic cells were discarded while the remaining biofilm cells attached to the titer plate surface were washed two times with fresh TSB media. The planktonic cells (50 µL) were serially diluted up to 10^{-7} dilution factor. The serial-diluted planktonic cells (100 µL) were spread plated on TSA agar plates and incubated at 35ºC for 24 h. The attached biofilm cells after washing with TSB were then added with 300 µL fresh TSB and were scrapped off using sterile tips. The biofilm cells suspension (50 µL) was serially diluted up to 10^{-7} dilution factor, then 100 µL of serially diluted biofilm cell suspension was spread plated on TSA agar plates and incubated at 35ºC for 24 h. The bacterial colonies appeared on the plate after 24 h of incubation were counted. All the experiments were performed in triplicate.

The impact of FeOOH-NP to eradicate the established matured biofilm was also performed by bacterial colony count. The overnight *P. aeruginosa* grown cell culture (1:100 dilution in TSB) was allowed to form biofilm in a microtiter plate for 24 h at 35ºC under static condition. After incubation, the planktonic cells were removed and the remaining attached cells were washed twice with fresh TSB. After washing, fresh TSB (300 µL) was added to the plate and incubated along with different concentrations of FeOOH-NP (from 0.16875 mM to 1.35 mM) at 35ºC for 24 h under static condition. After incubation, the supernatant containing residual planktonic cells was removed and the remaining attached biofilm cells were washed two times with fresh TSB. After washing, the biofilm cells were added with TSB (300 µL) and were scrapped off using sterile tips. The cells were serially diluted to a 10^{-7} dilution

factor, then were spread plated on TSA agar plate and the plates were incubated for 24 h at 35°C. After 24 h of incubation, the bacterial colonies appeared on the agar plate were counted. All experiments were carried out in triplicate.

5. Growth kinetics assays

P. aeruginosa cell culture (overnight grown) was diluted (1:100 with TSB) and was used for the determination of growth kinetics in TSB broth medium in the presence and absence (control) of different concentrations of FeOOH-NP (ranging from 0.16875 to 1.35 mM) in the 96-well titer plate. The plate was incubated at 35°C under orbital shaking (120 rpm) in the microtiter plate reader for 30 h. The bacterial cell mass was determined by measuring OD value at 600 nm at every 2 h time interval. The experiment was performed in triplicates. Different concentrations of FeOOH-NP in TSB without bacterial cell culture were used as blank. The OD values of the test samples were subtracted to the OD value of the blank.

6. Microscopic observations

6.1. Scanning electron microscopy (SEM)

Changes of *P. aeruginosa* biofilm architecture and cellular morphology upon exposure to FeOOH-NP were examined by SEM analysis which was previously described by Lee et al. (Lee et al. 2011). Briefly, the nylon filter membrane (0.5 × 0.5 cm) was placed in a 24-well microreader plate for the *P.*

aeruginosa cell culture (1:100 diluted in TSB) (1 mL) to grow in the presence and absence of FeOOH-NP (1.35 mM). The microtiter plate containing membrane was incubated at 35ºC for 24 h under static condition. The biofilm-forming cells grown on the nylon filters were directly fixed with formaldehyde (2%) and glutaraldehyde (2.5%) and kept at 4ºC overnight. The samples were washed thrice with phosphate buffer saline (PBS; pH 7.2) for 20 min, post-fixed with Osmium tetroxide for 90 min, followed by dehydration with ethanol (50, 70, 80, 90, 95 and 100%), each concentration for 20 min. Each filter was freeze-dried by using a freeze dryer (Ilshin BioBase Co. Ltd., Seoul, Korea). Then, the dried nylon filters were fixed to SEM stubs and followed by coating with white gold using an Ion-sputter (Hitachi, Tokyo, Japan) for 120 s. The fixed biofilm cells were investigated using SEM (JEOL, Milpitas, CA) at magnifications at ×5000 and a voltage of 15 kV.

6.2. Fluorescence microscopy

Effect of FeOOH-NP on *P. aeruginosa* biofilm architecture was also investigated using the fluorescence microscopic assay using the acridine orange dye (Sigma-Aldrich, St. Louis, MO). Briefly, the cell culture (overnight grown in TSB) was diluted at 1:100 in TSB with and without the presence of FeOOH-NP (1.35 mM) on glass pieces (1 × 1 cm) placed in 12-well micro-titter plate and incubated at 35ºC for 24 h under static condition. The embedded cells were thrice with PBS and allowed to air-dry. Acridine orange (10 µg/mL) was used to stain the attached cells and the plates were then

incubated at 35ºC for 10 min. After dye removal and three-time washing with PBS, embedded biofilm cells were visualized under Leica DMI300B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at 40 × objective lens.

7. Virulence factors assays of *P. aeruginosa*

Hemolytic activity of FeOOH-NP was studied by using different concentrations ranging from 0.16875 to 1.35 mM following the method described by Khan et al. (Khan et al. 2019) with slight modifications. In the tubes containing 950 µL of sheep red blood cells (RBCs) per tube, a volume of 50 µL of cell culture of *P. aeruginosa* (grown overnight in TSB) which was treated with different concentrations of FeOOH-NP were mixed. The tubes containing RBCs and culture of *P. aeruginosa* were incubated at 35ºC for 1 h in shaking incubator at 250 rpm. After incubation, the tube was centrifuged (16,600 × *g* in 10 min) and the supernatants were measured for OD values at 543 nm wavelength. The experiment was performed in triplicates using two independent cell cultures.

For the effect of FeOOH-NP on the production of pyocyanin virulence factor by *P. aeruginosa*, the following concentrations were used: 0.16875 mM, 0.3375 mM, 0.675 mM and 1.35 mM and the method was adopted from Essar et al. (Essar et al. 1990). The culture supernatant was extracted with chloroform and 0.2 N HCl to give a deep pink colored solution eventually. The pyocyanin assay was performed in triplicate and production level was

measured by measuring OD value at 520 nm wavelength.

For the effect of FeOOH-NP on the production of pyoverdine by *P. aeruginosa*, the following concentrations were used: 0.3375 mM, 0.675 mM and 1.35 mM. The minimal salt medium (MSM) plus 2% sodium succinate formulation was used as medium for the assay as described previously (Khan et al. 2019). *P. aeruginosa* cell culture (overnight grown in TSB) was diluted at 1:100 with MSM plus 2% sodium succinate and was incubated along with different concentrations of FeOOH-NP at 35ºC for 12 h in shaking incubator (250 rpm). Pyoverdine inhibitory activity of each FeOOH-NP concentration reflected by corresponding OD at 405 nm is the average of two replicates.

For the effect of FeOOH-NP on the production of rhamnolipid by *P. aeruginosa*, the following concentrations were used: 0.16875 mM, 0.3375 mM, 0.675 mM and 1.35 mM. TSB was used as the medium for the assay. *P. aeruginosa* cell culture (overnight grown in TSB) was diluted at 1:100 dilution with TSB and was incubated along with different concentrations of FeOOH-NP at 35ºC for 12 h in shaking incubator (250 rpm). After extraction with diethyl ether and centrifugation, the supernatant was let dried completely and mixed sequentially with distilled water, 1.6% orcinol and 60% sulfuric acid and heating at 80ºC for 30 min under shaking condition (Wilhelm et al. 2007). Rhamnolipid inhibitory activity of each FeOOH-NP concentration reflected by corresponding OD at wavelength 421 nm is the average of three replicates.

Skim milk agar plates were used to determine the effect of FeOOH-NP on *P.*

aeruginosa protease activity. Briefly, the supernatants were collected from the overnight grown cell culture of *P. aeruginosa* (grown in 1:100 diluted TSB) which was treated with different concentrations of FeOOH-NP ranging from 0.16875 to 1.35 mM. The supernatants were then filtered using 0.2 μm filter (Agilent, Santa Clara, CA, USA) and 25 μL supernatant were added to the holes in milk agar plates. The plate was incubated at 35°C for 24 h and protein digestion was indicated by the diameter (cm) of the clear zone surrounding the holes. The experiment was performed in triplicate and the diameter of each clear zone represented as the average value.

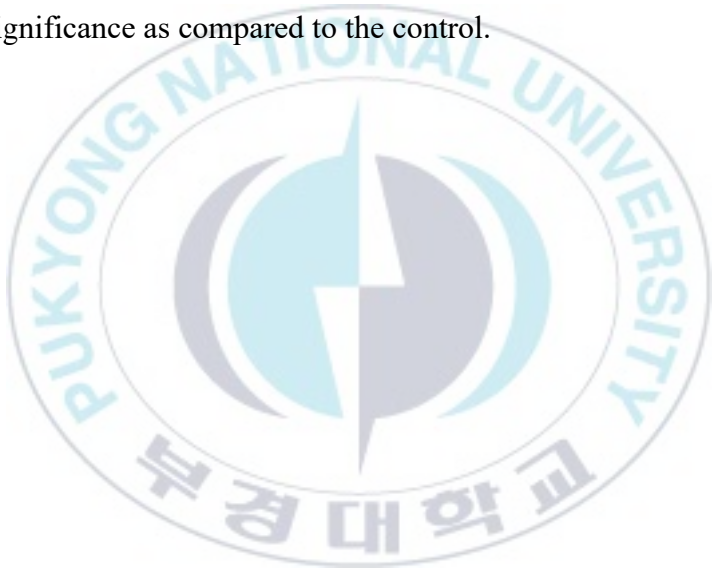
8. Motility assay-swimming and swarming

Effect of FeOOH-NP on *P. aeruginosa* swimming and swarming motilities was performed on overnight *P. aeruginosa* grown culture at 35°C. The swimming medium was prepared from 0.2% (w/v) casamino acids, 30 mM glucose and 0.3% (w/v) Bacto agar and the procedure was performed according to previous study (Luo et al. 2017). The medium used for swarming motility was prepared from Luria Britani (LB) supplemented with 0.5% (w/v) casamino acid, 0.4% (w/v) Bacto agar and 0.5% (w/v) glucose and the procedure was performed according to the previous study (Luo et al. 2017). Different concentrations of FeOOH NP (1.35 mM and 0.3375 mM) were added to the agar media and then the agar media was poured in the plate and allowed to solidify for 30 min. The overnight grown cell culture (10 μL) was loaded in the swimming and swarming agar plates and incubated at 35°C for

24 h. The migration zone appeared on the agar plates were monitored. All the motility experiments were performed in triplicates.

9. Statistical analysis

All graphs were constructed by using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). The statistical analysis of data was carried out by performing one-way ANOVA followed by Dunnett's posttest and the results were presented as means ± SD. $*p < 0.05$ and $**p < 0.01$ were selected as statistical significance as compared to the control.



Results

1. Characterization of FeOOH-NP

The TEM images showed that FeOOH-NP had the rod-like shape with fairly uniform size of about 40 nm (**Fig. 1A, B and C**). The FTIR spectrum with the band record at 847 and 696 cm was ascribed to the -OH bending modes in β-FeOOH (**Fig. 1D**). **Fig. 1E** illustrated the UV-visible absorption spectrum of FeOOH-NP. FeOOH-NP had the absorbing peak at 345 nm. The TGA revealed that the FeOOH-NP had high thermal stability in the temperature range of 50°C-800°C, and approximately 20% mass was lost at 800°C.

2. Effects of various FeOOH-NP concentrations on *P. aeruginosa* biofilm formation

FeOOH-NP at various concentrations ranging from 0.16875 to 1.35 mM was examined for anti-biofilm activity of *P. aeruginosa*. The percentage of biofilm inhibition was compared to the control sample at 570 nm wavelength. FeOOH-NP inhibited the biofilm formation of *P. aeruginosa* in a concentration-dependent manner. FeOOH-NP at the concentration of 1.35 mM showed the most effective biofilm inhibition (93%) (**Fig. 2A**). Supportive evidence was obtained by performing viable counts of planktonic and biofilm cells in the absence and presence of FeOOH-NP at different concentrations

ranging from 0.16875 mM to 1.35 mM (**Fig. 3A and B**). High concentrations of FeOOH-NP (1.35 and 0.675 mM) reduced the viability of the sessile cells by ~1.4 log CFU as compared to the control (**Fig. 3B**); whereas the presence of FeOOH-NP at all concentrations did not cause viability changes to the planktonic cells (**Fig. 3A**). Combining the results from crystal violet assay and bacterial cell count assay, it can be concluded that the FeOOH-NP exhibited inhibition to the attachment of biofilm cells to the titer plate surface, thereby inhibiting the biofilm formation by *P. aeruginosa*.

The impact of FeOOH-NP on the growth of *P. aeruginosa* under static condition was also determined by the OD value at 600 nm wavelength obtained after 24 h. The results showed that at all tested concentrations of FeOOH-NP, no lethal effect on the growth properties was shown. Moreover, the bacterial growth rates at all FeOOH-NP concentrations were increased by approximately 20.5% compared to control (**Fig. 2B**).

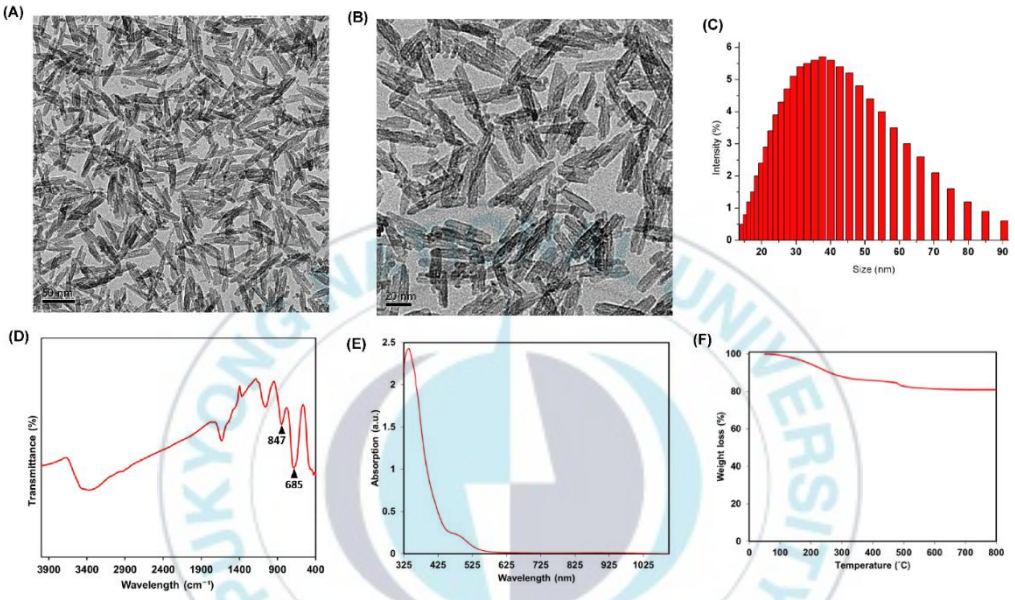


Fig. 1. Characterization of FeOOH-NP. TEM image of FeOOH-NP (A, B), size distribution (C), FT-IR spectrum (D), UV-visible absorption spectrum (E) and TGA graph (F).

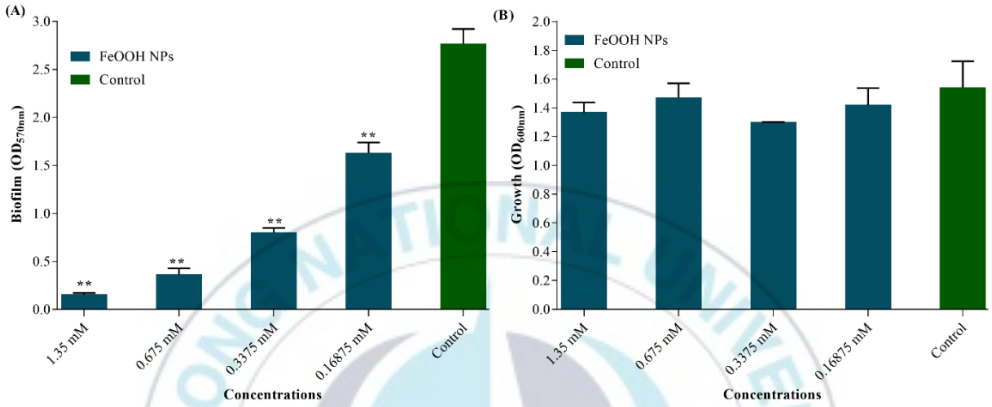


Fig. 2. Effects of FeOOH-NP on *Pseudomonas aeruginosa* biofilm formation (A) and biofilm cell growth (B). Each experiment was repeated two times using three independent cultures.

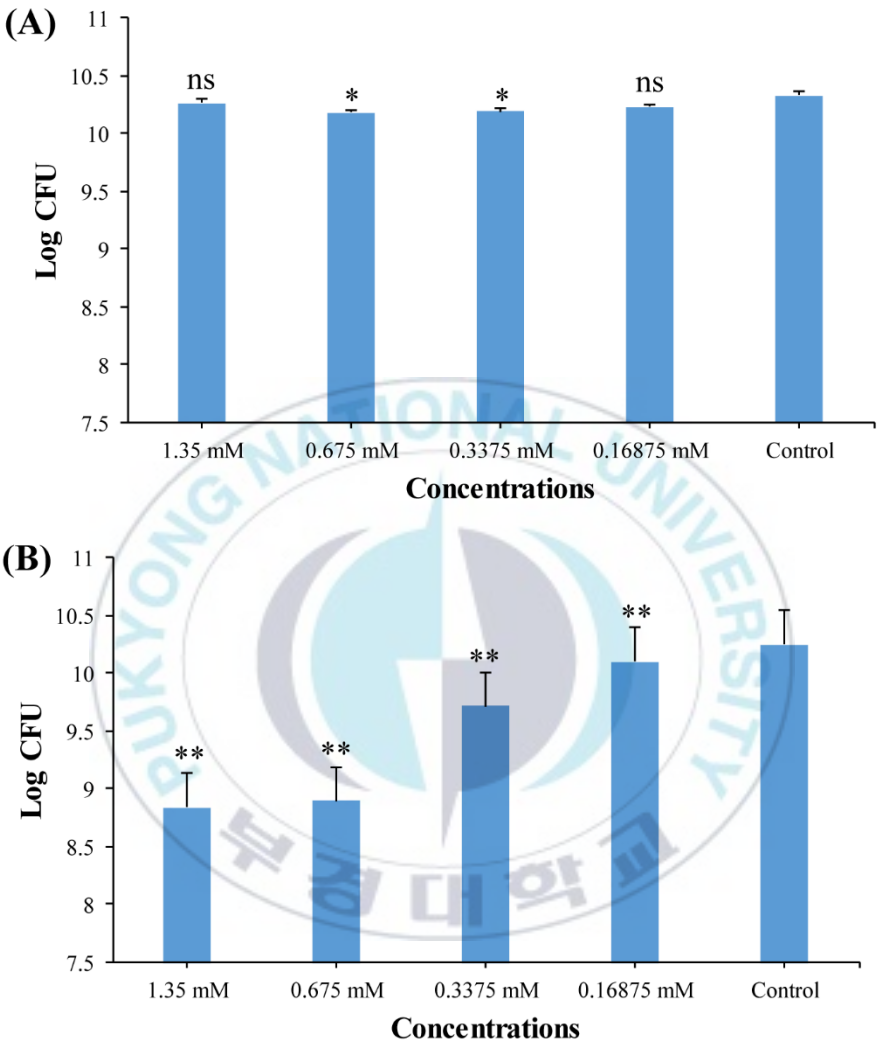


Fig. 3. Survivability effects of FeOOH-NP on *Pseudomonas aeruginosa* planktonic and biofilm cells. CFU of planktonic cells (A) and CFU of biofilm cells (B).

3. Microscopic analysis for biofilm architecture and bacterial cell morphology

The biofilm architecture and morphology of *P. aeruginosa* cells grown on nylon membrane under 24 h treatment of FeOOH-NP was investigated using SEM and fluorescence microscopy. Results from SEM indicated that in the presence of FeOOH-NP (1.35 mM), bacterial cells attached to the membrane were significantly restricted in comparison to the absence of FeOOH-NP (control) (**Fig. 4A**). Similar results were also found from the fluorescence microscopic analysis, where in the presence of FeOOH-NP (1.35 mM), less fluorescent intensity was visible as compare to the control (**Fig. 4B**). Microscopic analysis using SEM and fluorescence microscopy supported for the effectiveness of FeOOH-NP in inhibiting the attachment of the bacterial cells.

4. Eradication of preformed matured biofilm

FeOOH-NP at various concentrations ranging from 0.16875 mM to 1.35 mM was tested on mature *P. aeruginosa* biofilm. As shown in **Fig. 5A**, the eradication proportion of all concentrations were genuinely similar among all FeOOH-NP concentrations. Of all concentrations tested, 1.35 mM of FeOOH-NP caused the most significant effect on mature bacterial biofilm, in which 68.49% biofilm biomass was dispersed. The bacterial colony count was also performed on established matured biofilm cell culture in the presence of

FeOOH-NP at different concentrations from 0.16875 mM to 1.35 mM (**Fig. 5B**). At high concentration of FeOOH-NP (1.35 mM) there is significant reduction in log CFU (1.06 log) as compared to the control. This reduction in CFU of the pre-formed mature biofilm further confirmed the eradicating effectiveness of the FeOOH-NP treatment at high concentration (1.35 mM) to the established biofilm.

5. Effects of FeOOH-NP on *P. aeruginosa* hemolysis and virulence factors

An anti-hemolytic activity of FeOOH-NP on *P. aeruginosa* with regards to different concentrations ranging from 0.16875 to 1.35 mM were examined. Hemolytic property of *P. aeruginosa* was significantly reduced (67%) by the presence of FeOOH-NP at higher concentration (1.35 mM) in comparison with the control as well as FeOOH NP at lower concentrations (**Fig. 6A**).

Effects of FeOOH-NP on the production of *P. aeruginosa* virulence factors: rhamnolipid, pyocyanin and pyoverdine siderophore were then investigated (**Fig. 6**). The pyocyanin production level was also significantly reduced by the FeOOH-NP treatment in a concentration-dependent manner (**Fig. 6B**). The reduction was found as 61% at 1.35 mM of FeOOH-NP concentration. The inhibitory action of FeOOH-NP in pyocyanin production was also indicated by the change of the blue-green color of pyocyanin (**Fig. 9A**). Although no significant reduction was observed in the production of pyoverdine at high

FeOOH-NP concentration, slight production was observed at lower concentration (**Fig. 6C**). In contrast, an insignificant reduction in rhamnolipid production was observed at all FeOOH-NP concentrations (**Fig. 6D**).

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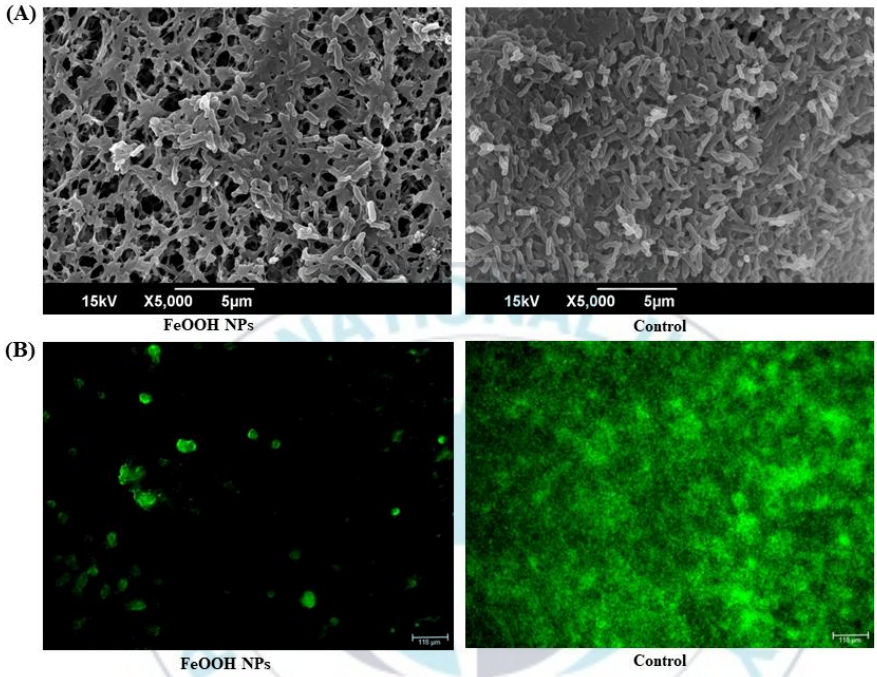

Fig. 4. Microscopic analysis of *Pseudomonas aeruginosa* biofilm architecture in the presence of FeOOH-NP (1.35 mM). SEM images (A) and fluorescence microscopy (B). Both microscopic observations were repeated two times with three independent culture per nanoparticle concentration.

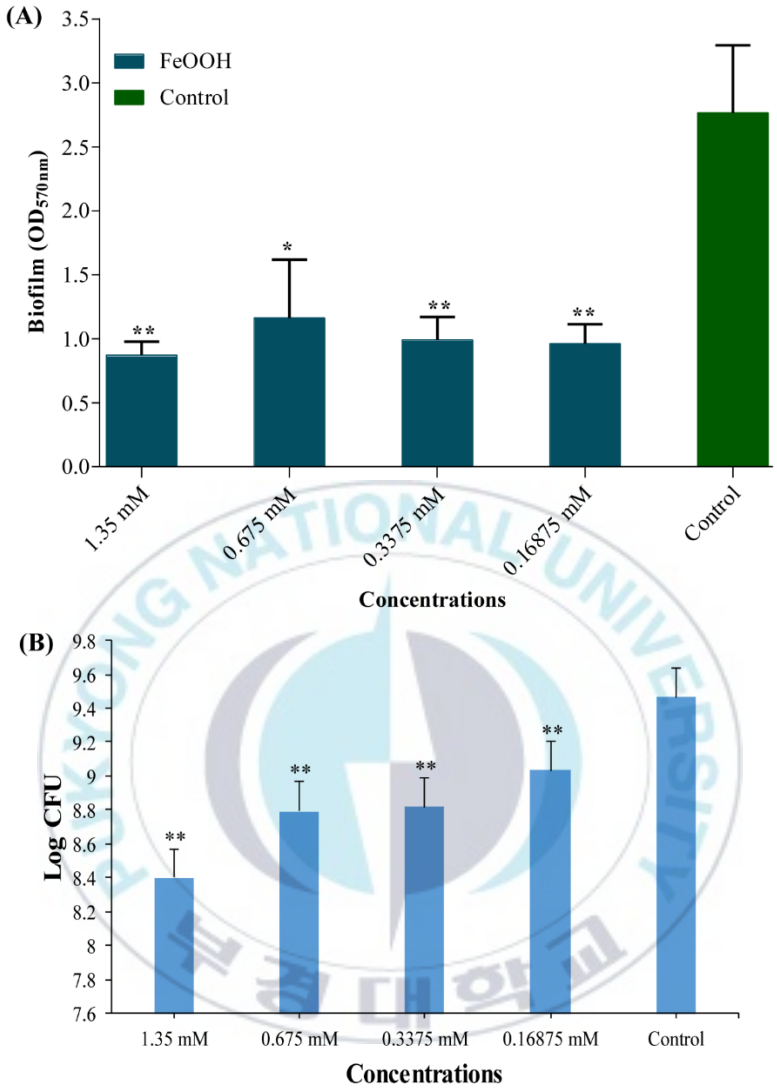

Fig. 5. Effects of FeOOH-NP on *Pseudomonas aeruginosa* pre-formed matured biofilm eradication (A) and CFU of the established (matured 24 h-old) biofilm cells which was further incubated for 24 h along with different concentrations of FeOOH-NP (B). Each experiment was repeated two times using three independent cultures.

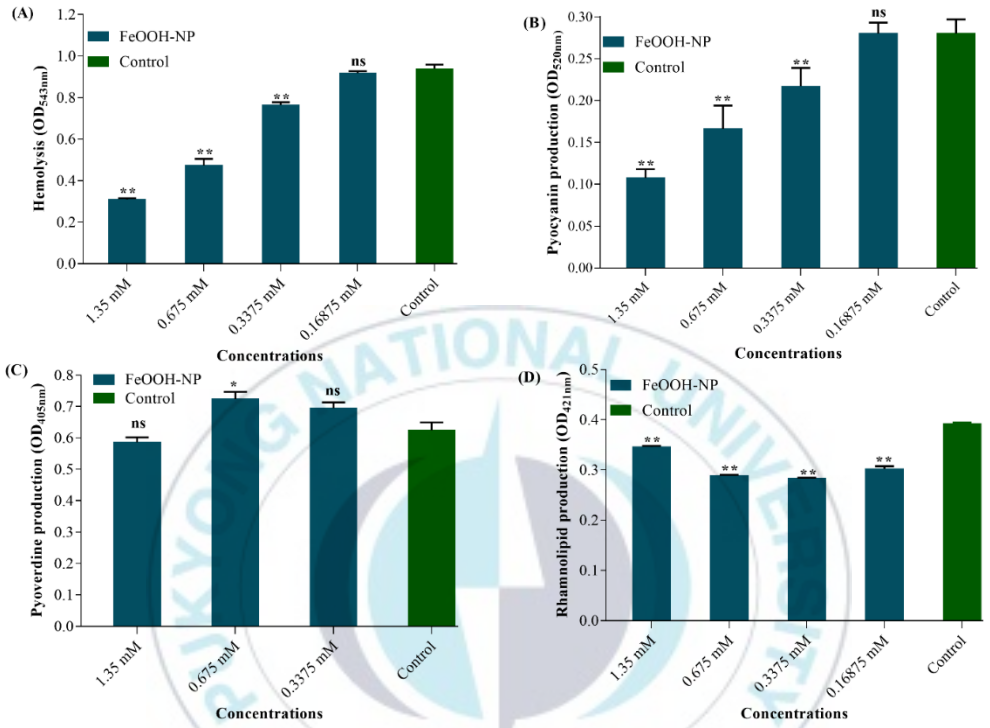

Fig. 6. Hemolytic activity of FeOOH-NP to *Pseudomonas aeruginosa* (A) and its anti-virulence effects at different concentrations on pyocyanin (B), pyoverdine (C) and rhamnolipid (D). Each assay was performed in three independent cultures.

6. Effects of FeOOH-NP on production of protease

Since protease enzymes were also found associated with acute *P. aeruginosa* infections, it is thus necessary to access its production level under FeOOH-NP treatments. Several FeOOH-NP concentrations ranged from 0.16875 to 1.35 mM were tested in skim milk agar plates and results indicated by clear zone surrounding the treatment-containing holes (cm) were reported in **Fig. 7A** and **B.** Upon the presence of FeOOH-NP, protease production was clearly in a concentration-dependent manner, becoming more limited as the treatment was increasingly concentrated.

7. Effect of FeOOH-NP on bacterial motility

As swimming and swarming to desired surfaces determine the initial attachment of bacterial biofilm formation, the effect of FeOOH-NP on these motions was also examined. As demonstrated in **Fig. 8**, result of swimming motility is opposite to swarming. In comparison to control, the presence of FeOOH-NP significantly enhanced the bacterial swimming (**Fig. 8A** and **B**) while slightly reduced its swarming movement (**Fig. 8C** and **D**).

8. Effect of FeOOH-NP on *P. aeruginosa* growth kinetics under agitation

The impact of all tested concentrations of FeOOH-NP on *P. aeruginosa* growth was evaluated by measuring the OD at 600 nm. As shown in **Fig. 9**, the

bacteria reached maximum population at incubation period of 6 h under shaking condition (120 rpm). The growth rate was varied in response to the FeOOH-NP concentrations, by which at concentrations of 1.35, 0.675, and 0.3375 mM, the *P. aeruginosa* growth rate was 0.168, 0.174 and 0.174, respectively. Nevertheless, these results were approximately equal to the control (0.165).

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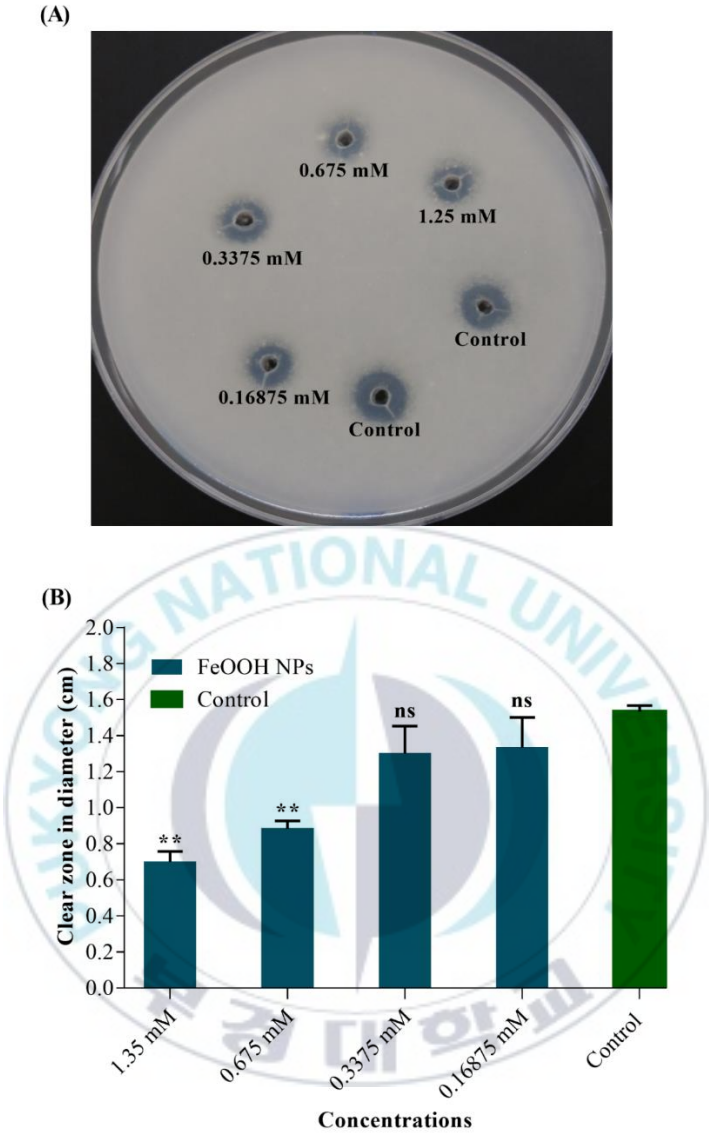

Fig. 7. Effects of FeOOH-NP on production of protease enzyme from *Pseudomonas aeruginosa*. The image of skim milk agar plate showing protease activity (A) and diameter (cm) of clear zones (B) surrounding the holes loaded with FeOOH-NP. Each concentration was performed in three independent cultures.

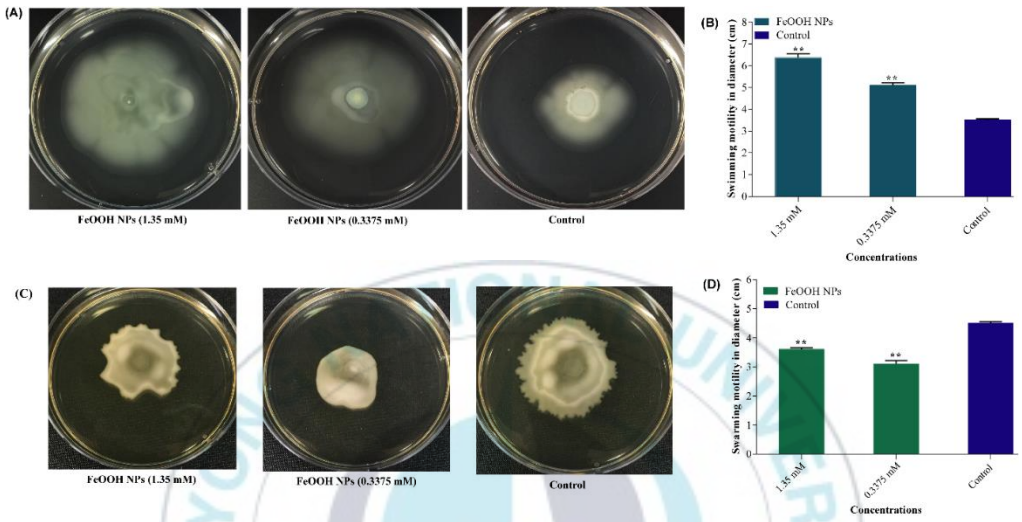

Fig. 8. Effects of FeOOH-NP on *Pseudomonas aeruginosa* motilities. Swimming photos (A), swimming statistics (B), swarming photos (C), and swarming statistics (D). The visible motility was shown by diameter of expansion starting from the center of the plate where *P. aeruginosa* culture was loaded. Each experiment was repeated two times using two independent cultures.

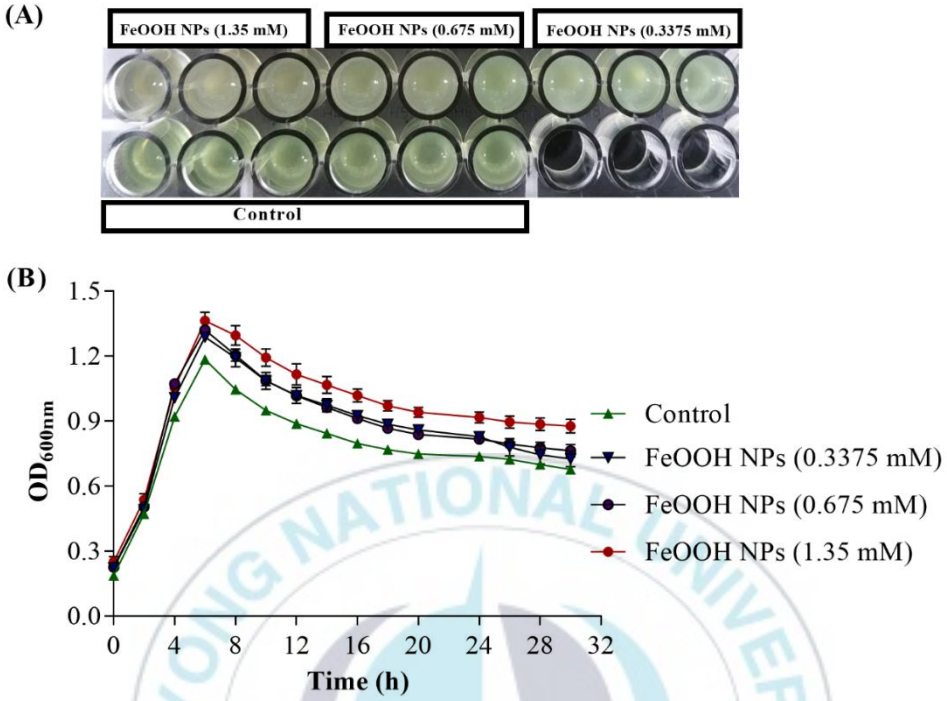

Fig. 9. Effects of FeOOH-NP on *Pseudomonas aeruginosa* cell growth kinetics under agitation condition. Image of change in color of *P. aeruginosa* culture in the presence of FeOOH-NP (A) and growth kinetics of *P. aeruginosa* in the presence of FeOOH-NP (B). Each experiment was repeated two times using three independent cultures.

Discussion

The formation of biofilm as a structural component and production of functional virulence factors play the central role in *P. aeruginosa* pathogenesis. Enclosed within an extracellular polymeric matrix (EPS), the bacterial biofilm is safely protected from human immune and environmental pressures and enabled to adhere to various surfaces. In addition, biofilm also enhances the *P. aeruginosa* resistance to traditional antibiotics by promoting intercellular metabolism and communication (Neethirajan et al. 2014; Wang et al. 2017). In such situation, metal-based NP is introduced as an alternative approach to inhibit the formation of biofilm by *P. aeruginosa*. The antimicrobial and anti-biofilm activities of NPs are known to derive from release of (1) metal ions or (2) reactive oxygen species (ROS) that would impair several bacterial cellular components (cell membrane, DNA, proteins) and functions (transcription, electron transport chain) (Dizaj et al. 2014; Lemire et al. 2013; Pelgrift and Friedman 2013). With this knowledge, the present study therefore synthesizes a new iron-based nanoparticle, FeOOH-NP and applies to biofilm inhibition in *P. aeruginosa*.

The characterization study showed that synthesized FeOOH-NP had the size of 40 nm and a rod-shaped morphology. The size and morphology of NP are extensively known to primarily affect the loading capacity, permeability, toxicity, control release and bioactivity of the NPs (Peulen and Wilkinson 2011;

Wang et al. 2017). Here FeOOH-NP had the size that can be considered small in the range of NPs size (1-100 nm in one side) and rod shape (Khan et al. 2017). Both properties are highly advantageous for enhanced bactericidal effect and penetration through bacterial membrane and biofilm structure (Preedia Babu et al. 2017; Slomberg et al. 2013; Wang et al. 2017). In contrast to size and morphology, the effect of FeOOH-NP concentration and its interaction with other parameters were less discussed, especially in the case of iron-based NPs. Nevertheless, concentration is considered crucial for NP application as it is related directly to toxicity, according to previous studies (Joo and Aggarwal 2018). For example, the present study has shown that responses of biofilm formation and several virulence factors production of *P. aeruginosa* were highly dependent on FeOOH-NP concentrations. Thus, concentration of the NPs, particularly, iron NPs, is much noteworthy that it indeed requires more research.

This study demonstrated that FeOOH-NP inhibited *P. aeruginosa* biofilm at the initial stage of biofilm formation and development. According to the results obtained from crystal violet assay and bacterial colony count, the concentration-dependent inhibitory activity of FeOOH-NP was observed from initial stage of biofilm establishment. Similar assays were also carried out in pre-existing matured biofilm, revealing that the synthesized FeOOH-NP also exhibited eradication to biofilm at the maturation stage. The present report is in closely agreement with previously studies in anti-biofilm property by metallic NPs such as the silver NP against the *P. aeruginosa* biofilm

throughout the biofilm development (Kalishwaralal et al. 2010; Martinez-Gutierrez et al. 2013). Compared to the zinc oxide NP or gentamycin-loaded Au-NP, which mostly inhibited the functional biofilm either at initial stage or at maturation stage, respectively, FeOOH-NP could be considered more preferred (Hassani Sangani et al. 2015; Mu et al. 2016). Visualization results from SEM and fluorescence microscopy analyses showed that biofilm structure formed on the nylon membrane and glass surfaces upon exposure to 1.35 mM of FeOOH-NP was more disrupted than the control.

The growth property of *P. aeruginosa* in the presence of FeOOH-NP under static as well as shaking conditions reflected no toxicity effect. The FeOOH-NP did not disrupt the bacterial growth, regardless of different concentrations and incubation conditions. Although agitation was hypothesized to minimize NPs aggregation, thus potentially enhancing NPs bioactivities, no growth disruption was recorded. Similar result was obtained in gold NP, in which the NP did not exhibit toxicity to *P. aeruginosa* yet only inhibited its biofilm formation (Yu et al. 2016). It was proposed that NPs treatment might have enhanced nutrients and energy which in turns promoted cellular metabolism and growth (Auffan et al. 2008; Ehrlich 1997; Shan et al. 2005). This finding on the antibacterial properties of FeOOH-NP adds to the variation in anti-bacterial effect of metal NPs. Previously, copper oxide NPs was also found non-lethal to *Pseudomonas chlororaphis* O6 as the bacteria population continued growing despite of increasing NP concentration (Dimkpa et al. 2012b). In contrast, zinc oxide (ZnO), Fe and iron-silica NPs exhibited

concentration-dependent inhibition against the growth of *P. chlororaphis* O6, *Serratia* sp. and *P. aeruginosa*, respectively; as the more concentrated the NPs were, the more bacterial cells were destroyed (Dimkpa et al. 2012a). Overall, combining the results from biofilm formation and growth rate assays under various concentrations and incubation conditions, it can be concluded that FeOOH-NP is effective in *P. aeruginosa* biofilm inhibition and is genuinely non-toxic to the bacteria. This finding is helpful for future studies on antimicrobial and anti-biofilm applications of FeOOH-NP.

P. aeruginosa hemolysis refers to the bacterial ability to damage erythrocytes structure (Golubeva et al. 2010; Van Delden and Iglewski 1998). As *P. aeruginosa* causes hemolysis to RBCs, the sheep RBCs was selected for the experiment. Hemolysis was found significantly reduced by FeOOH-NP treatment. The effect was more profound at high concentrations of FeOOH, indicating that high concentration is recommended for FeOOH-NP to prevent the bacterial hemolysis.

P. aeruginosa produces several functional virulence factors to increase bacterial pathogenesis and nutrients uptake, which in turn causes infections within host tissues (Olejnickova et al. 2014; Van Delden and Iglewski 1998). Among *P. aeruginosa* virulence factors, pyocyanin, pyoverdine, rhamnolipid, protease enzyme and hemolysis were accessed in this study. The present study found that FeOOH-NP at the highest concentration (1.35 mM) was found the most effective in reducing production of pyocyanin but not pyoverdine and rhamnolipid. In fact, each factor has shown different responses to FeOOH-NP

treatment. Such variations in pyocyanin, pyoverdine and rhamnolipid responses to FeOOH-NP treatment is probably due to their diverse and complex genetic regulatory systems (Lau et al. 2004; Maier and Soberon-Chavez 2000; Ringel and Bruser 2018). Pyoverdine and rhamnolipid are exceptional cases to consider, as in contrast to pyocyanin and hemolytic activity, their responses to FeOOH-NP were insignificant when FeOOH-NP was applied. Pyoverdine is known as a major iron-recruiting agent (siderophore) produced when the environmental iron level is insufficient for the bacterial needs for metabolism and survival (Maura et al. 2016). Iron in the form of ferric ion (Fe^{3+}) is chelated with pyoverdine to form a ferripyoverdine (Fpv) complex, which is recognized by Fpv receptor located in bacterial outer membrane. After the complex is imported into cellular cytoplasm, Fe^{3+} is reduced to Fe^{2+} by FpvG, released from pyoverdine and utilized by FpvDE (Brillet et al. 2012; Dimkpa et al. 2012b; Ganne et al. 2017). Rhamnolipid is a virulence factor which is involved in the bacterial biofilm formation and swarming motion (Davey et al. 2003; Soberon-Chavez et al. 2005). In this study, treatment with FeOOH-NP slightly affected the pyoverdine production, in which high concentration slightly reduced and lower concentration slightly enhanced the production. For the case of rhamnolipid, production of this factor was slightly reduced regardless of FeOOH-NP concentrations.

One of the mechanisms of biofilm inhibition of metal-based nanoparticles is known as the release of the positively charged metal ions which interfere with the negatively charged constituents present in biofilm such as EPS, adhesins or

extracellular-DNA (eDNA). However, in the case of iron nanoparticles, the increase in free iron availability when iron ions from the nanoparticle are released into the surrounding media, would adversely stimulate biofilm formation (Cai et al. 2010). To investigate this hypothetical scenario, the level of pyoverdine which is the siderophore produced in iron shortage condition was evaluated in the presence of FeOOH-NP. Results showed that compare to the control, pyoverdine level of *P. aeruginosa* was enhanced when treated with FeOOH-NP, which possibly indicated that the environmental iron availability remained unchanged when the FeOOH-NP was applied. Combining the results from pyoverdine assay and biofilm assay, it can be concluded that FeOOH-NP inhibited *P. aeruginosa* biofilm formation not via the release of metal ions mechanism.

Such high variability in production of pyoverdine, rhamnolipid and pyocyanin virulence factors in response to different concentrations of FeOOH-NP is also observed in treatment with ZnO NPs, and was proposed to result from their specific biosynthesis, signal sensing and regulation mechanisms (Garcia-Lara et al. 2015). Therefore, in general, FeOOH-NP can be considered as a modulator for *P. aeruginosa* virulence factors production.

P. aeruginosa protease is also targeted as its secretion is crucial to combat the host cell proteins, aiding infection and bacterial population growth (Das et al. 2016; Hoge et al. 2010). The present study found that FeOOH-NP at 0.675 mM – 1.35 mM was the most effective for protease inhibition.

Proper attachment to biotic or abiotic surfaces and effective colonization of

P. aeruginosa are initiated by its translocation movements, which involve swimming, swarming and twitching (Harmsen et al. 2010; Mulcahy et al. 2014; Neethirajan et al. 2014). Here *P. aeruginosa* swimming and swarming motions in the presence of FeOOH-NP at high (1.35 mM) and low (0.3375 mM) concentrations were evaluated. Results showed that FeOOH-NP stimulated the bacterial swimming, while exhibiting slight inhibition effect on bacterial swarming. These findings suggest that beside biofilm inhibitory activity, FeOOH-NP also modulate the bacterial production of virulence factors and motility, which can be considered helpful in controlling *P. aeruginosa* infections.

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Conclusion

The formation of biofilm structure significantly enhances *P. aeruginosa* resistance against antibiotics treatments. With recent development of nanotechnology, NP is a promising anti-biofilm agent against *P. aeruginosa* due to the high volume-to-surface ratio, low toxicity and control release. In the present study, FeOOH-NP was characterized and evaluated for its potential in inhibiting formation of the bacterial biofilm, along with secretion of essential virulence factors and surface movements. Results from biofilm assays and microscopic observations showed that FeOOH-NP exhibited inhibitory activity throughout the biofilm development stages. However, FeOOH-NP was found non-toxic to the bacteria. Subsequent analyses on bacterial hemolytic activity and infection-associated virulence factors such as pyocyanin also showed the noticeable effectiveness of the nanoparticles in inhibiting their production. However, the effect of FeOOH-NP treatment in production of rhamnolipid and pyoverdine was not significant. Taking all results into consideration, FeOOH-NP can be regarded as an effective and non-toxic anti-biofilm agent and virulence modulator against *P. aeruginosa*. Findings from the present study could be considered as a platform which provides insights for the anti-biofilm potential of an iron-based NPs. However, future studies are required to understand the mechanism of FeOOH-NP inhibition against *P. aeruginosa* biofilm formation. Furthermore, the interactions between the

FeOOH-NP and the bacterial cellular components, as well as the underlying genetic regulations of the bacterial virulent responses and motility properties towards FeOOH-NP are some of the questions that demand further research in the future. Biochemical analysis of different virulence factors production from the respective mutant strain of *P. aeruginosa* in the presence and absence of FeOOH-NP is also an important field that demands more understandings.

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