

**SCREENING OF MEMBRANE-ACTIVE MECHANISM OF
ANTIBACTERIAL PEPTIDE, PAM-5, AGAINST *Pseudomonas*
*aeruginosa***

By

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ABSTRACT

SCREENING OF MEMBRANE-ACTIVE MECHANISM OF ANTIBACTERIAL PEPTIDE, PAM-5, AGAINST *Pseudomonas* *aeruginosa*

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Most of the current antibiotics could only act upon specific bacterial structure or enzymatic targets. This may not be beneficial in the long run as bacteria could easily develop resistance strategies against such mechanisms. Antibacterial peptides (ABPs) are among the suitable alternatives to the conventional treatments due to their distinguished mode of actions. The cationic and amphipathic nature of ABPs may contribute to their ability to exert membrane-active mechanisms which are relatively difficult for pathogens to evade. Previously, a novel synthetic ABP, PAM-5, was found to possess promising antibacterial effect on *Pseudomonas aeruginosa*. However, its membrane-active potency was yet to be elucidated. Therefore, in this study, PAM-5 was screened for its bactericidal effects on the bacterial membrane via scanning electron microscope (SEM) analysis and SYTOX[®] Green uptake assay. SEM analysis revealed striking morphological changes, such as blebbings and surface roughening, of the target bacteria upon peptide treatment. In addition, PAM-5 also exhibited membrane-permeabilizing activities as

treated bacteria showed an increment in the uptake of the green fluorescent nucleic acid stain. Collectively, the obtained results demonstrated that PAM-5 is able to induce bactericidal effects by disrupting the bacterial membrane, which is one of the essential elements for bacterial survival. Therefore, this peptide offers promising prospects in the search for novel antibacterial agents against antibiotic-resistant bacteria.

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I would also like to thank all my labmates, especially Li Hui, Li Jing and Michelle for their companions and moral support. I would also like to express my appreciation to my dearest family and friends for always being there when I needed support and motivation.

DECLARATION

I hereby declare that this project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

(EVEIYN PHOON WENG YAN)

APPROVAL SHEET

This project report entitled **“SCREENING OF MEMBRANE-ACTIVE MECHANISM OF ANTIBACTERIAL PEPTIDE, PAM-5, AGAINST *Pseudomonas aeruginosa*”** was prepared by EVEIYN PHOON WENG YAN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **EVEIYN PHOON WENG YAN** (ID No: **12ADB02904**) has completed this final year project entitled "**SCREENING OF MEMBRANE-ACTIVE MECHANISM OF ANTIBACTERIAL PEPTIDE, PAM-5, AGAINST *Pseudomonas aeruginosa***" under the supervision of Mr. Yuen Hawk Leong from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in PDF format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(EVEIYN PHOON WENG YAN)

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LIST OF ABBREVIATIONS

ABPs	Antibacterial peptides
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
CFU	Colony Forming Unit
CL	Cardiolipin
CLSI	Clinical and Laboratory Standard Institute
DiSC3(5)	3,3'-Dipropylthiadicarbocyanine Iodide
DNA	Deoxyribonucleic acid
HPLC	High performance liquid chromatography
K	Lysine
L	Leucine
LB	Luria-Bertani
M	Methionine
MBC	Minimum bactericidal concentration
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
NPN	N-phenyl-1-naphthylamine

P	Proline
PBS	Phosphate buffered saline
PG	Phosphatidylglycerol
PS	Phosphatidylserine
PuroB	Puroindoline B
R	Arginine
RNA	Ribonucleic acid
rpm	Revolution per minute
SEM	Scanning electron microscope
TEM	Transmission electron microscope
V	Valine
W	Tryptophan

CHAPTER 1

INTRODUCTION

The development of antibiotics was a great breakthrough in modern medicine as they can cure life-threatening infections. However, most antibiotics are typically directed at specific bacterial targets. This may limit their long-term usage in the clinical settings as such mechanisms could easily be circumvented through mutations. This could lead to the development of antibiotic resistance which has now become a major global health concern. Therefore, it is vital for researchers to search for alternative antibacterial therapies with minimal risk of bacterial resistance.

Antibacterial peptides (ABPs) have been gaining popularity as one of the potential substitutes of antibiotics. One of the main advantages of these peptides over the conventional treatments is the low risk of resistance development in bacteria. ABPs are believed to possess membrane-active mechanisms which are difficult for the pathogens to evade. This mode of action is usually lethal and hence, could result in rapid killing of the evading microbes.

Nevertheless, despite many researches on the antibacterial potency of novel ABPs, limited work was done to study their actions on the bacterial membrane. Knowledge of the membrane-active mechanisms may provide detailed insights

to researchers on the lethal mode of actions of these peptides, which may contribute to the design of more effective drugs in the battle against multidrug-resistant bacteria.

Previously, a hypothetical ABP, PAM-5, was designed and screened for its antibacterial potency against *Pseudomonas aeruginosa* ATCC 27853. Using microbroth dilution assay, it was shown that the peptide was bactericidal against the bacteria with the minimal bactericidal concentration (MBC) of 8 µg/mL (Lee, 2015). However, the mechanism of bacterial killing by this peptide is yet to be investigated.

Hence, the objective of this study is:

1. To screen for the membrane-active mechanisms of PAM-5:
 - i. By observing the morphological changes of *P. aeruginosa* upon treatment by PAM-5 through SEM analysis.
 - ii. By determining the changes in the bacterial membrane integrity via SYTOX[®] Green uptake assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibacterial peptides

2.1.1 Overview of antibacterial peptides

The emergence of multidrug-resistant bacteria is a matter of great concern to the healthcare community as it reduces the potency of the commonly used antibiotics (Wang, et al., 2015). Consequently, treatment of bacterial infections becomes difficult and this is usually associated with high morbidity and mortality among cases of nosocomial infections. As such, the search for novel classes of antibacterial compounds that possess better features as compared to the conventional antibiotics is urgently needed.

Antibacterial peptides (ABPs) are among the potential candidates to replace antibiotics (Seo, et al., 2012). They are normally 10-50 amino acid residues long and can be found in various organisms, such as mammals, arthropods, plants and bacteria (Guihelmelli, et al., 2013). They play vital roles in the innate immune system as one of the chemical barriers to counter the invasions by pathogenic microbes. Apart from that, ABPs can also be chemically synthesized or modified based on the templates of naturally occurring peptides. Many research groups are also turning to *de novo* synthesis of ABPs following high throughput screening of large peptide library in order to design and engineer novel ABPs (Diehnelt, 2013).

2.1.2 Advantages of antibacterial peptides in comparison to antibiotics

ABPs may serve as potential substitutes to antibiotics due to their better potency and low risk of resistance development in bacteria. The distinctive modes of actions of these peptides in damaging the bacterial membrane make it difficult for the bacteria to recover. As such, this could lead to rapid bacterial death (Wimley and Hristova, 2011). In addition, the wide range of utilizable antimicrobial mechanisms of ABPs may contribute to their broad spectrum activities in different types of bacteria (Lakshmaiah and Chen, 2015). Moreover, the peptides may act synergistically with the conventional antibiotics to boost the antibiotics' therapeutic efficacy (Park, Park and Hahm, 2011).

2.2 Mode of actions of antibacterial peptides

2.2.1 Membrane-active mechanisms

The findings of some noteworthy characteristics that are commonly present in many ABPs from diverse phyla suggested the significance of these features associated with the antibacterial activities of the peptides. Particularly, ABPs are mostly cationic at physiological pH as they commonly contain cationic amino acids such as lysine and arginine. Their cationicity range from +2 to +9 with an average net charge of +3.2 (Yeaman and Yount, 2003; Wang, et al., 2015). This is vital for the selective electrostatic interaction between the cationic peptides and the negatively-charged bacterial membranes that are composed of highly electronegative phospholipids such as phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylserine (PS). On

the other hand, the membrane of the eukaryotic cells are generally zwitterionic as they normally constituted of neutrally-charged molecules such as phosphatidylcholine and sphingomyelin, making them less desirable for the peptides to bind to (Malmsten, 2016). In addition, these peptides are usually amphipathic in which they consist of both hydrophilic and hydrophobic domains (Yeaman and Yount, 2003). Such structures allow the ABPs to have efficient binding to the hydrophobic lipid components and the hydrophilic phospholipid head groups of the bacterial membrane (Bahar and Ren, 2013). Thus, these structure-function relationships suggest a leading hypothesis that ABPs primarily act on the bacterial membrane (Wang, et al., 2015).

An example of ABP that targets bacterial membrane is Magainin 2. This peptide was found to be effective in permeabilizing both the outer and inner membranes of *Escherichia coli* (Matsuzaki et al., 1997). Membrane-active mechanisms were also documented for other ABPs such as polybia-MPI (Wang et al., 2013) and esculentin-1b (Marcellini et al., 2009) as well as trichoplaxin (Simunić et al., 2014).

However, many questions on the peptides' subsequent actions after their initial interaction on the bacterial surface as well as upon entry into the bacterial intracellular compartment still remain unanswered. This is due to the lack of verification assays that are able to define the post-membranous actions from the perspective of biophysical and chemi-physical aspects (Wimley and Hristova, 2011). Nevertheless, it is known that aggregated ABPs on the

bacterial membrane are able to destabilize the lipid head groups, followed by formation of pores on the membranes that lead to the efflux of intracellular contents (Wimley, 2010).

As the exact mechanism of ABPs on the membrane perturbation is still a matter of debate, few models depicting the alteration of the bacterial membranes' integrity induced by the ABPs were proposed. These include 'barrel-stave', 'toroidal pore' and 'carpet' models. It was suggested that these events could occur in the presence or absence of pore formation. Both 'barrel-stave' and the 'toroidal' pore models are the pore forming models while the carpet-like mechanism is a nonpore-forming method (Park, Park and Hahm, 2011). **Figure 2.1** illustrates the three main models of membrane disruption.

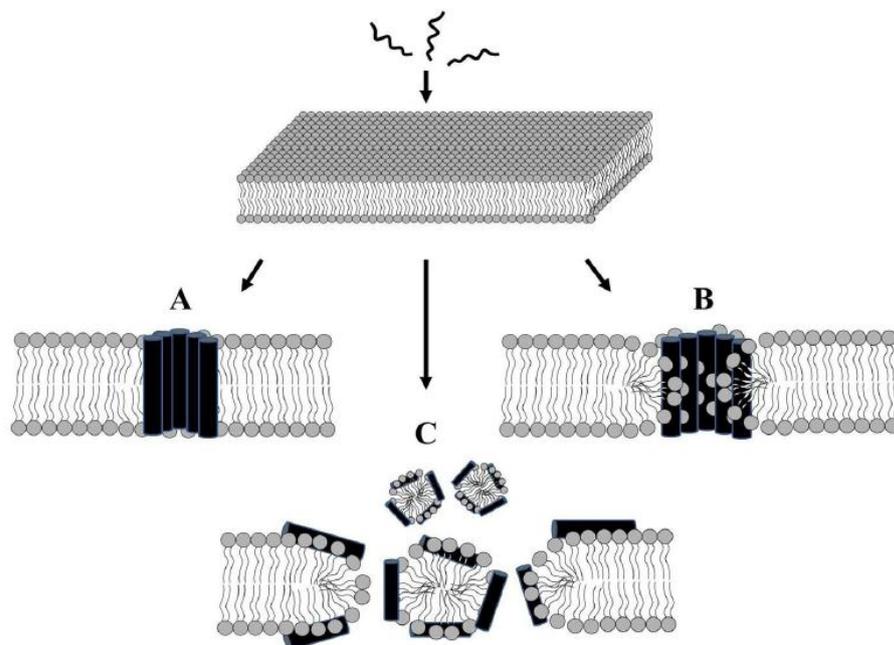


Figure 2.1: The three models of peptides mode of actions on membranes. (A) barrel-stave model; (B) toroidal pore model; (C) carpet model [Adapted from Park, Park and Hahm, 2011].

2.2.2 Non-membrane-active mechanisms

It remains debatable on whether membrane disruption by ABPs is always the lethal event to the target bacteria as non-membrane-active mechanisms have also been reported (Hale and Hancock, 2007). Some ABPs can penetrate and exert their actions on intracellular targets resulting in the inhibition of vital cellular functions. For example, an ABP named puromycin B (PuroB) was found to be bactericidal without disrupting the bacterial membranes. Surprisingly, further studies on the ABP revealed that it could bind to bacterial deoxyribonucleic acid (DNA) and inhibit the synthesis of the macromolecules (Haney, et al., 2013). The DNA-binding capability has also been described for Burforin II (Park, et al., 1998). It is proposed that cationicity of the ABPs may play a role in mediating their binding to the negatively-charged phosphate group in the nucleic acid backbone (Park, et al., 1998).

It is also possible that a single ABP can exert multiple killing mechanisms that simultaneously lead to the death of the microorganism. Skerlavaj and colleagues (1990) demonstrated that two ABPs, namely Bac5 and Bac7, possess membrane permeabilization activities and are able to disrupt the bacterial proton motive force. The inhibition of one of the oxidase systems in the electron transport chain could interfere with the efficiency of the bacterial aerobic respiration resulting in decreased quantity of intracellular ATP and disruption of energy-dependent cellular processes. This ultimately leads to the death of the bacteria.

2.3 Rationale of studying membrane-active mechanisms of antibacterial peptide

Despite the wide range of applicable killing mechanisms, the initial interaction between the ABPs and the bacterial membrane must be established first before subsequent actions could occur. Due to the cationic nature exhibited by majority of the ABPs, it is assumed that electrostatic interaction could mediate the binding between the peptides and the anionic bacterial membranes. Moreover, the amphipathicity of ABPs enables the interaction of both the hydrophobic and hydrophilic portions of the peptide with the lipid bilayer. This may drive the perturbation of the peptides into the membrane leading to its disruption (Ebenhan, et al., 2014). As such, most of the researches conducted on novel ABPs will include membrane-active mechanisms as their preliminary study.

2.4 Previous findings of the actions of antibacterial peptides on the bacterial membranes through scanning electron microscope analysis

Scanning electron microscopy (SEM) imaging is a powerful technique that allows the visualization of ultrastructural alterations to bacteria induced by an antibacterial compound. This is because it could provide high-resolution images depicting the general damage of the cell membranes.

Hartmann and colleagues (2010) had studied the effects of Gramicidin S and PGLa on *Escherichia coli* and *Staphylococcus aureus* through electron microscopy analysis. It was found that both peptides were able to hinder the maximum growth of *E. coli* as decrement in length of the bacteria upon the peptide-treatment was noted. In addition, both Gramicidin S and PGLa could also evidently induce multiple blisters on the surface of the Gram-negative bacteria. These features were absent in the untreated bacteria. Interestingly, both the ABPs could also cause distinct morphological changes to the Gram-positive bacterium, *S. aureus*. When compared to the smooth surface of the untreated bacteria, the treated specimens appeared to have multiple dents on its membranes. Besides, lysed cells could also be observed. Therefore, both Gramicidin S and PGLa are inferred to possess membrane-active mechanisms on both bacteria.

In another SEM study, Han, et al. (2013) also successfully demonstrated significant morphological changes to *E. coli* upon treatment by their synthetic peptides. Based on their SEM observation, the membrane surfaces of bacteria treated with LFP-20, LF2A, LF-2, LF-4 and LF-6 were found to have small protuberant structures or blebbings. These suggested that all the five antibacterial peptides could exert their antibacterial effects on the bacterial membranes.

Lv and colleagues (2014) conducted similar SEM analysis on their modified ABP named GI24, which was derived from cathelicidin PMAP-36. Both the variant and its parental peptide were able to cause surface roughening and corrugation to *E. coli* upon peptide-treatment. This implied that both ABPs are membrane-active peptides. **Figure 2.2** depicts the surface roughening of *E. coli* induced by PMAP-36 as compared to the smooth surface of the untreated sample. It is clearly seen that SEM could provide a clear picture on the morphological and structural changes to the surface of the ABP-treated bacteria, which strongly indicate the membrane-active mechanisms of the ABP.

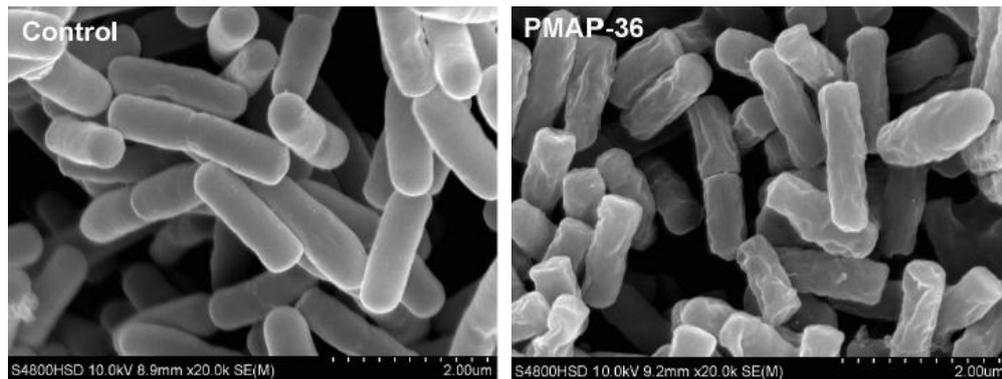


Figure 2.2: SEM micrographs of untreated *E.coli* and PMAP-36-treated bacteria [Adapted from Lv, et al., 2014].

2.5 Previous findings on the membrane-permeabilizing activities of antibacterial peptides via SYTOX[®] Green uptake assay

Loss of membrane integrity is considered as one of the criteria for determining cell viability. Hence, the usage of membrane-impermeable dyes such as SYTOX[®] Green can aid in cell-viability assessment. SYTOX[®] Green is impermeable to live cells due to their intact membranes. Hence, it can only enter membrane-disrupted cells (Roth, et al., 1997). Its subsequent association with nucleic acid upon penetrating the dead cells will result in fluorescence emission. Hence, fluorescence emission of this dye is indicative of the disrupted bacterial membrane.

After performing a high-throughput screening, Rathinakumar, Walkenhorst and Wimley (2009) successfully identified ten antibacterial peptides that possess membrane-active mechanisms. After treatment with the respective peptides, both *E.coli* and *S. aureus* revealed significant increment of fluorescence intensity as compared to the untreated samples. This indicated the influx of SYTOX[®] Green into the permeabilized cells of the ABP-treated *E. coli* and *S. aureus*, which implied that their peptides have direct impacts on the bacterial membranes.

The same assay was conducted by Hammer and colleagues (2010) to study the membrane-active action of their synthetic ABP, NK-2 on *E. coli*. The outcomes revealed that the influx of SYTOX[®] Green into NK-2-treated bacteria occurred via a dose-dependent pattern. They concluded that the degree of membrane

disruption on the bacteria is proportionate to the concentration of ABPs, and these observations were apparent under the SYTOX[®] Green uptake assay.

Based on the above-mentioned studies, it is clear that SYTOX[®] Green uptake assay is a useful method to assess the membrane disruption activity of an ABP. Thus, it was also included in this study to screen for the membrane-active mechanism of PAM-5 on bacterial membrane.

2.6 Linear synthetic peptide PAM-5

2.6.1 Previous studies on PAM-5

In 2015, Lee had designed a hypothetical antibacterial peptide, namely PAM-5 (KWKWRPLKRKLVLRM). With the incorporation of positively-charged amino acid, arginine (R) and lysine (K), this peptide became cationic with a net positive charge of +7. As mentioned earlier, cationicity of an ABP plays an essential role in the electrostatic binding of the peptide to the anionic bacterial membrane. Moreover, the introduction of valine (V), tryptophan (W), methionine (M) and leucine (L) into the peptide enhanced the peptide hydrophobicity to 46% in consideration that hydrophobicity allows ABPs to have higher binding affinity to the hydrophobic region of the bacterial membranes. With these modifications, it was found that PAM-5 was able to exert bactericidal effect on *Pseudomonas aeruginosa* ATCC 27853 via microbroth dilution assay. The minimum bactericidal concentration (MBC) of the peptide against the bacteria was 8 µg/mL (Lee, 2015).

However, the mode of actions detailing the peptide's antibacterial activities is yet to be studied. It is notable to study the type of mechanisms by PAM-5 to kill the target bacteria as this could give better insight on how this peptide works to defend infections. Being cationic and amphipathic, PAM-5 is hypothesized to have the capability to exert membrane-active mechanisms on the target bacteria.

CHAPTER 3

MATERIALS AND METHODS

3.1 General experimental design

The 15-mer linear synthetic peptide, PAM-5, which had been previously studied for its antibacterial activities as mentioned in **Section 2.6.1**, was screened for its membrane-active effect in this study. Firstly, its *in vitro* antibacterial effect against *P. aeruginosa* was verified again via microbroth dilution assay. Next, the peptide-treated bacteria was processed and observed under scanning electron microscope to screen for any morphological changes on the membrane surface of the bacteria as compared to the untreated sample. In addition, SYTOX[®] Green uptake assay was carried out as a measurement of membrane-permeabilizing activities of the bacteria by the studied peptide. All the assays were conducted in triplicates to ensure data reproducibility.

3.2 Materials

3.2.1 Bacterial strain

The reference strain of the Gram-negative bacteria *Pseudomonas aeruginosa* ATCC 27853 which was used throughout this study was kindly provided by Dr. Sit Nam Weng from the Department of Biomedical Science, Universiti Tunku Abdul Rahman (UTAR). The bacteria samples were cultured on Mueller-Hinton (MH) agar and grown in MH broth. For long term storage, the bacteria

were preserved as glycerol stock in Luria-Bertani (LB) broth with the addition of 20% (v/v) glycerol and stored at -80 °C. Prior to the assays, the bacterial glycerol stock was thawed and revived by inoculating it on MH agar as master culture. The latter was kept at 4 °C for a maximum of seven days.

3.2.2 Synthesis and preparation of peptides

PAM-5 (KWKWRPLKRKLVLRM) was synthesized and purchased from Bio Basic Inc. (Canada) and it was received in lyophilized form. The purity of the peptide was 79.48% as determined by reverse-phase HPLC by the manufacturer while through mass spectrometry analysis, its molecular mass was determined as 2038.84 Dalton. The lyophilized peptide was stored at -20 °C before use. Prior to dissolving the peptide, it was allowed to equilibrate to room temperature in the dark for one hour. As the peptide contains methionine residues in which their side-chains are prone to oxidation, the peptide was dissolved in sterile, degassed distilled water. A peptide stock solution was prepared at the concentration of 1, 024 µg/mL. It was then two-fold serially diluted by consecutively transferring 500 µL of the dissolved peptides into 500 µL sterile degassed phosphate buffered saline (PBS) to yield a range of peptide concentrations from 1, 024 µg/mL to 4 µg/mL as shown in **Figure 3.1**. The peptide solutions were then kept in 4 °C and to be used for a maximum of seven days.

Polymyxin B which was used as the positive control throughout this study was purchased from EMD Chemicals, Inc. (Canada). Its preparation methods were similar as described for PAM-5.

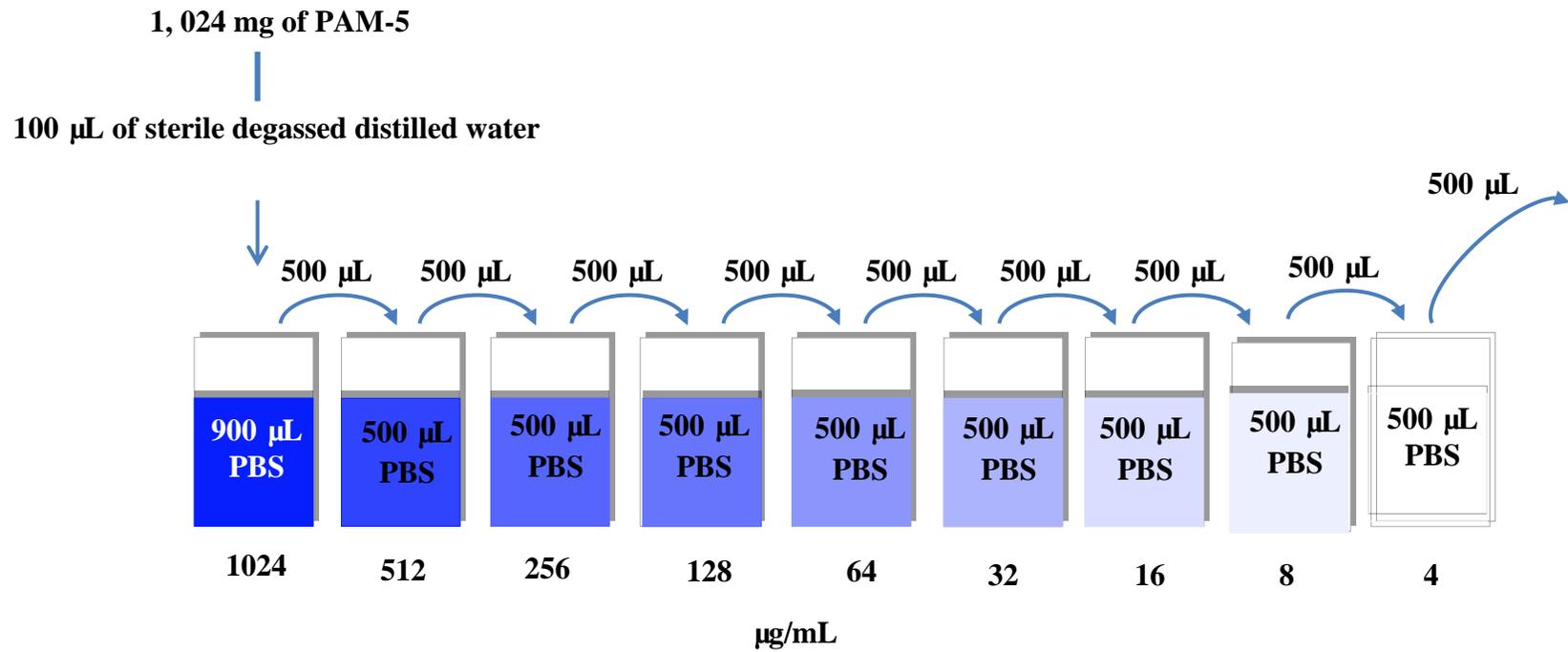


Figure 3.1: Illustration of the method to dissolve and dilute PAM-5 into different concentrations.

3.2.3 Preparation of buffers, media and reagents

Refer to Appendix A

3.3 Lab wares and equipment

Refer to Appendix B

3.4 Methodology

3.4.1 Antibacterial assay

PAM-5 was verified for its bactericidal or bacteriostatic potency against *P. aeruginosa* ATCC 27853 via microbroth dilution assay which is the gold standard assay. It was done according to the guidelines as recommended by the Clinical and Laboratory Standards Institute (CLSI) with modest modifications to the procedures.

Firstly, a bacterial suspension was prepared by inoculating three to four bacterial colonies from the master culture plate into 10 mL of Mueller-Hinton (MH) broth. It was then incubated in the orbital shaker with agitation of 200 rpm for 16 to 18 hours at 37 °C. On the subsequent day, a 100-fold dilution of the overnight bacterial culture was performed by transferring 200 µL of the culture into a conical flask filled with 20 mL of fresh MH broth. The diluted culture was then subjected to incubation at 37 °C with agitation of 200 rpm until the bacteria reached its mid-exponential growth phase ($OD_{600} \sim 0.5-0.6$).

The growth medium was removed by centrifugating the bacterial culture at 6000 x g for 6 minutes. The pelleted bacteria were then washed twice using phosphate buffered saline (PBS, pH 7.4) and recentrifuged. After the second wash, the bacteria pellet was again re-suspended with PBS and serially diluted by 10-folds in order to obtain the titer of 10^3 CFU/mL.

A volume of 100 μ L of the bacteria suspension with the initial inoculation titer of 10^3 CFU/mL was loaded into the wells of a transparent 96-well microplate, followed by the addition of equal amounts of two-fold serially diluted PAM-5 with final concentrations that ranged from 2 μ g/mL to 256 μ g/mL. Bacterial suspensions that was added with equal volume of two-fold serially diluted Polymyxin B with final concentrations similar to PAM-5 was used as positive controls while the bacterial suspension in PBS was regarded as the negative control. **Table 3.1** summarizes the volume and the contents that were loaded into each well.

Table 3.1: Contents and volume of the test wells and controls (positive and negative) wells for microbroth dilution assay.

Contents	Test wells	Positive control wells	Negative control wells
Bacterial suspension	100 μ L	100 μ L	100 μ L
PAM-5	100 μ L	-	-
Polymyxin B	-	100 μ L	-
PBS (pH 7.4)	-	-	100 μ L
MH broth	50 μ L	50 μ L	50 μ L

* The hyphen in the table represents the absence of the particular variable.

After pre-incubating the microtiter plate with the contents at 37 °C for 1 hour, 50 μ L of MH broth was added into each well. The microplate was then subjected to another 16 to 18 hours of incubation at 37 °C. On the next day, the contents in the wells of the microplate were inspected for turbidity that indicated bacterial growth. Next, 10 μ L of the culture suspension from each well was inoculated onto MH agar plates for bacterial viability assessment and determination of the MIC and MBC of PAM-5. The contents in the wells which had developed turbidity were serially-diluted and plated out in order to determine the bacterial titer. The inoculated media were incubated overnight at 37 °C before enumerating the bacterial colonies in order to compare the titer between peptide-treated bacteria and untreated bacteria. The assay was carried out in triplicates to ensure reproducibility; hence the MBC or MIC obtained was an average value of the three assays.

Since high bacterial titer were required for subsequent analyses by scanning electron microscopy analysis and SYTOX[®] Green uptake assay, the MBCs of PAM-5 for the bacterial titer of 10^7 CFU/mL and 10^6 CFU/mL were determined as well.

3.4.2 Determination of MIC and MBC via microbroth dilution assay

The minimum inhibitory concentration (MIC) for PAM-5 was defined as the lowest concentration of the peptide which resulted in no visible growth of bacteria in the well after 18 hours of incubation. On the contrary, the minimum bactericidal concentration (MBC) was assigned to the lowest concentration of PAM-5 that successfully prevents any growth of bacterial colonies on the inoculating media. **Figure 3.2** portrays the manner in which how the MIC and MBC were determined through the microbroth dilution assay.

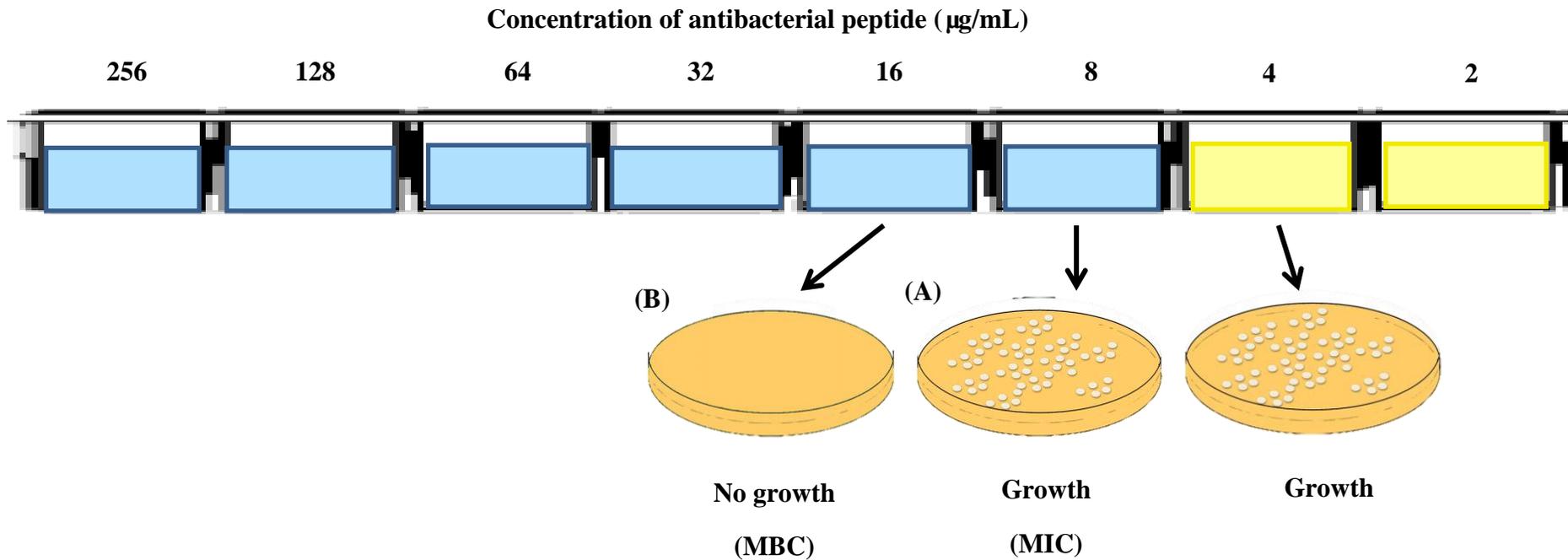


Figure 3.2: Microbroth dilution assay performed using 96-well microplate. (A) The MIC is determined as the lowest concentration of antibacterial agents that disallows the visible growth of bacteria in the well. The concentration of antibacterial peptide (ABP) that yielded the MIC according to this figure is 8 µg/mL; (B) The lowest concentration of antibacterial agent that resulted in no bacterial colonies after inoculation onto antibiotics-free media is determined as the MBC. In this illustration, the concentration of ABP that was assigned as the MBC is 16 µg/mL.

3.4.3 Scanning electron microscope (SEM) analysis

The morphological changes to the membranes of *P. aeruginosa* ATCC 27853 after treatment with PAM-5 were studied using scanning electron microscope (SEM). The preparation of overnight culture, growth of the bacteria to its mid-logarithmic phase and the washing of the bacterial pellet were similar to the steps as described earlier for the microbroth dilution assay. In this assay, the bacterial suspension was diluted with PBS (pH 7.4) to 10^7 CFU/mL before being treated with PAM-5 at 128 $\mu\text{g/mL}$. Bacteria treated with Polymyxin B of the same concentration served as the positive control while bacteria in PBS suspension were regarded as the negative control.

The peptide-treated bacteria together with the positive and negative controls were then incubated for 1 hour at 37 °C before centrifugation for 5 minutes at 6000 x g in order to obtain the bacterial pellets. The latters were then washed twice with PBS (pH 7.4) before fixing with 500 μL of 3% (v/v) glutaraldehyde in 0.1 M PBS for 18 hours at 4 °C. On the subsequent day, the glutaraldehyde was removed by centrifugation at 4000 x g for 5 minutes followed by two-times washing of the bacterial samples with PBS (pH 7.4). The specimens were then dehydrated sequentially using a series of ascending concentrations of ethanol as follows:

- i. 25% (v/v) ethanol, 5 minutes
- ii. 50% (v/v) ethanol, 10 minutes
- iii. 75% (v/v) ethanol, 10 minutes

iv. 95% (v/v) ethanol, 10 minutes

v. 100% (v/v) absolute ethanol, 10 minutes (three changes)

The samples were then further dehydrated by subjecting them to freeze drying for 18 hours. Thereafter, sufficient amount of the specimens were transferred to a carbon tape adhered to a copper stub. Following the coating of the samples with platinum for one minute, the copper stubs were placed onto the specimen holder and were observed under 18,000X and 30,000X magnification by SEM (JSM-7610F).

3.4.4 SYTOX[®] Green uptake assay

The integrity of the membrane of *P. aeruginosa* ATCC 27853 after treatment with PAM-5 was also assessed via the usage of the membrane impermeant SYTOX[®] Green dye. Similar steps were performed for the preparation of the bacteria as described for the microbroth dilution assay and SEM analysis. A volume of 100 μL of the bacteria at the titer of 10^6 CFU/mL was loaded into the 96-well microplate, followed by the addition of equal volume of PAM-5 with the final concentrations ranging from 2 $\mu\text{g/mL}$ to 256 $\mu\text{g/mL}$. For positive control, the bacteria were treated with two-fold serially diluted Polymyxin B with similar final concentrations as PAM-5 while bacteria suspended in filtered PBS (pH 7.4) served as the negative control.

The microplate was then incubated for one hour at 37 °C. Subsequently, 100 µL of the suspension was transferred to a flat, white opaque 96-well plate using a multichannel pipette. Next, the samples were added with 50 µL of 1 µM of SYTOX[®] Green dye and incubated in the dark for 15 minutes before measuring the fluorescence signals by Tecan Infinite 200 microplate reader. The excitation wavelength was set at 485 nm while the emission wavelength was at 520 nm. The assay was carried out thrice in order to ensure reproducibility.

CHAPTER 4

RESULTS

4.1 Antibacterial properties of PAM-5

The antibacterial potency of PAM-5 against *P. aeruginosa* ATCC 27853 was verified using microbroth dilution assay. Its potency can be indicated by the minimum bactericidal concentration (MBC) which is the lowest concentration of the peptide that exhibits total killing of the bacteria. This is reflected by the inability of the PAM-5-treated bacteria to grow on the antibiotic-free media. In addition, the efficacy of PAM-5 can also be determined through minimum inhibitory concentration (MIC) which is the lowest concentration of the peptide that impede the growth of the bacteria as manifested by a reduction in the bacterial titer as compared to the negative control.

4.1.1 Determination of MBC and MIC of PAM-5 through microbroth dilution assay

After the peptide treatment and overnight incubation, the visual turbidity of the bacterial culture in the wells was examined and the gross view of the visual inspection is presented in **Figure 4.1**. Based on the figure, the first column of wells (A1 to A8) in the 96-well microplate were filled with cultures of *P. aeruginosa* ATCC 27853 treated with two-fold serially diluted PAM-5, while the second column of the wells (B1 to B8) served as the positive controls in which the bacteria were treated with Polymyxin B. Negative control in wells

C1 and C2 was represented by the untreated bacteria in PBS. Turbidity in the wells of the negative control (C1 and C2) indicated bacterial growth. On the other hand, wells B1 to B8 containing bacteria treated with Polymyxin B showed no signs of turbidity, implying complete killing of the bacteria by the strong ABP. As for the samples, wells A7 and A8 that were filled with PAM-5-treated bacteria at the concentrations of 2 and 4 $\mu\text{g}/\text{mL}$ exhibited signs of bacterial growth as indicated by the turbidity similar to the negative control. On the contrary, wells A1 to A6 containing bacterial culture treated with higher concentrations of PAM-5 from 8 to 256 $\mu\text{g}/\text{mL}$ appeared non-turbid, implying absence of the bacteria growth.

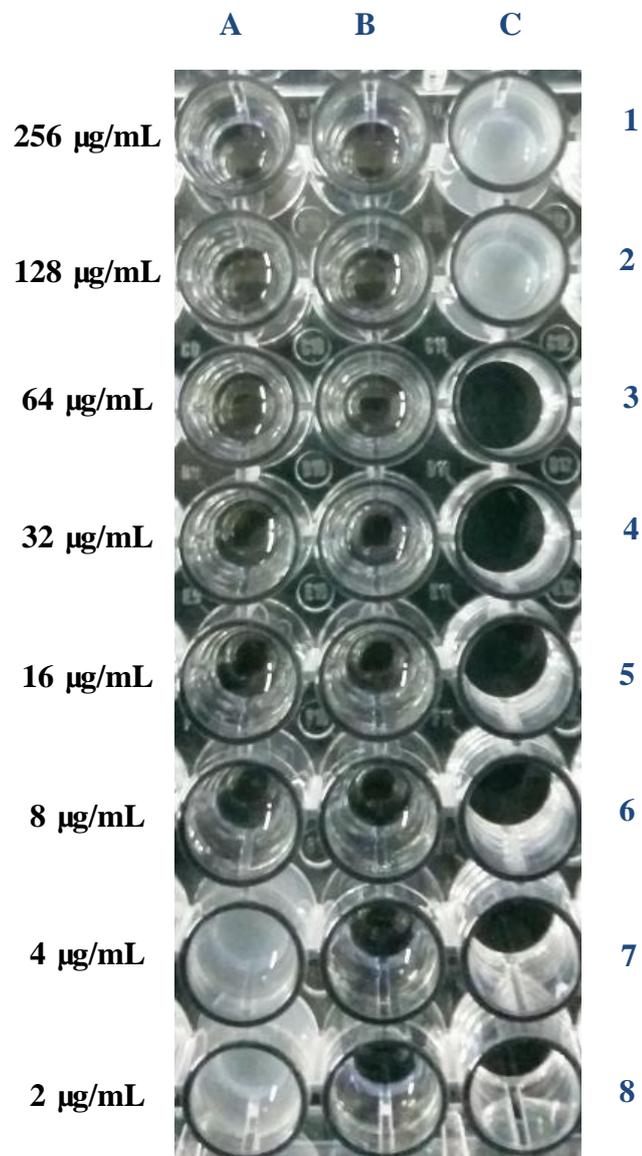


Figure 4.1: Visual inspection of the microbroth dilution assay of *P. aeruginosa* ATCC 27853 after overnight treatment with PAM-5 (A1 to A8) and Polymyxin B (B1 to B8) from 2 to 256 $\mu\text{g/mL}$ in the 96 well microplate. Bacteria suspended in PBS (C1 and C2) were used as the negative control.

In order to verify the results of the visual inspection as mentioned above, inoculation of the cultures from the wells onto culture media was performed to determine the presence of any growing colony. The outcome of this inoculation is presented in **Figure 4.2**, which depicts the inoculation *P. aeruginosa* ATCC 27853 that had been treated with: (A) Polymyxin B at the final concentrations ranging from 2 to 256 $\mu\text{g/mL}$, and (B) PAM-5 of the same concentrations. (C) represents untreated bacteria in PBS which served as the negative control.

As shown in the figure, Polymyxin B (A1 to A8) which served as the positive control killed the bacteria completely at all concentrations ranging from 2 to 256 $\mu\text{g/mL}$ as no bacterial colonies were observed. Notably, the concentrations of PAM-5 which were bactericidal to *P. aeruginosa* ATCC 27853 ranged from 8 to 256 $\mu\text{g/mL}$ (B1 to B6), as implied by the absence of bacterial colony on the agar plates. However, at the lower concentrations of 2 $\mu\text{g/mL}$ (B8) and 4 $\mu\text{g/mL}$ (B7), PAM-5 was not bactericidal towards the target bacteria as indicated by heavy bacterial growth on the inoculating media similar to the negative control. In addition, PAM-5 did not exert any MIC on the target bacteria as no distinct reduction of bacterial colonies was noted on the media plates as compared to the negative control.

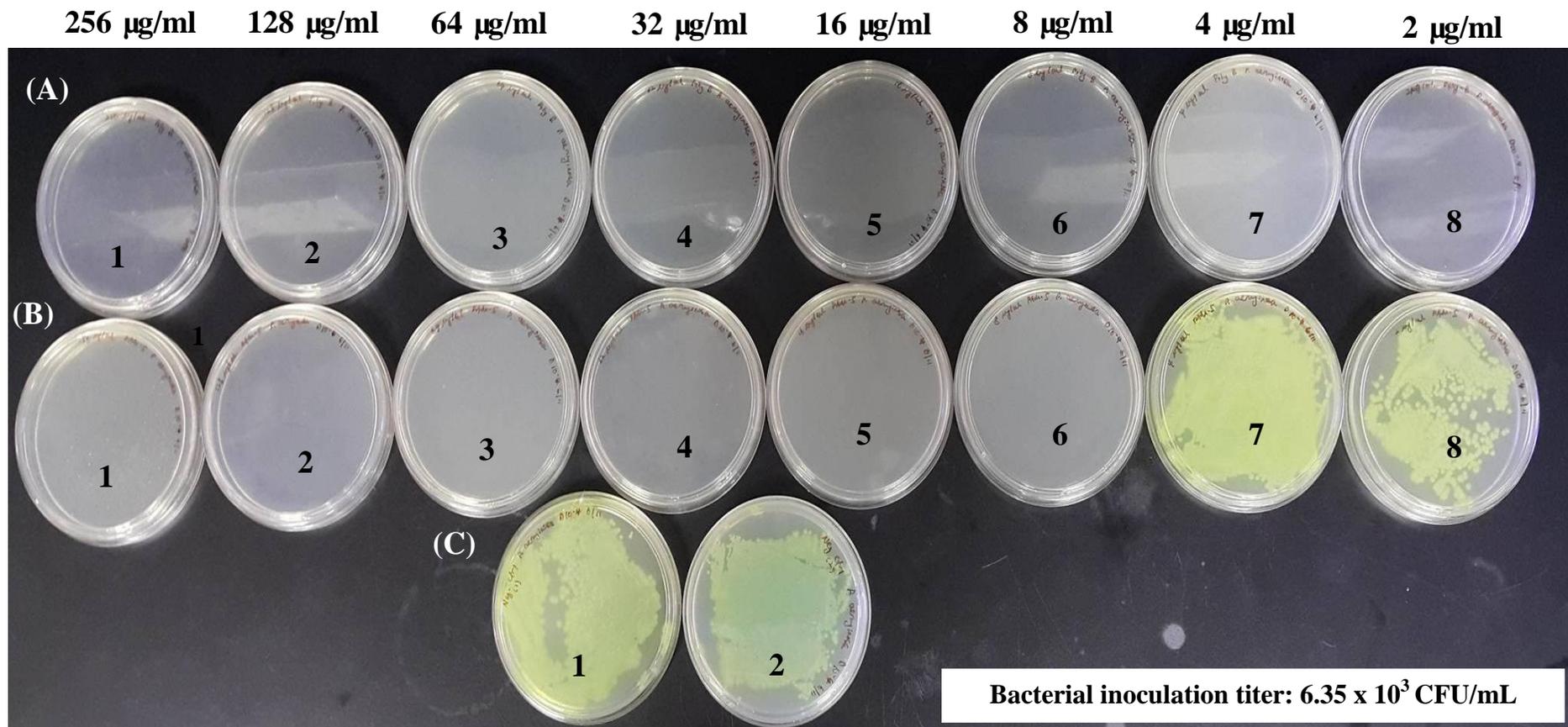


Figure 4.2: Inoculation of cultures of *P. aeruginosa* ATCC 27853 treated with antibacterial peptides. (A) Polymyxin B-treated bacteria from 2 µg/mL to 256 µg/mL as the positive controls; (B) Bacterial cultures that had been treated with PAM-5 with similar range of concentrations as Polymyxin B; (C) Bacteria suspension in PBS, which served as the negative controls.

Microbroth dilution assay was performed in order to determine the titer from those bacterial cultures that survived the peptide treatment. As this assay was carried out in triplicates with similar initial inoculation bacterial titer of 10^3 CFU/mL, an average of the data was obtained and presented in **Figure 4.3**. As demonstrated by the figure, *P. aeruginosa* treated with 2 $\mu\text{g/mL}$ shows a reduction of 0.5 LOG_{10} CFU/mL when compared to the negative control; while at 4 $\mu\text{g/mL}$, the bacterial culture did not show any decrement in bacterial titer as compared to the negative control. These data suggested that PAM-5 is unable to inhibit the target bacteria effectively at these lower concentrations. However, complete bactericidal effects similar to the positive control, Polymyxin B, were apparent at higher concentrations of PAM-5 (8 to 256 $\mu\text{g/mL}$). Thus, the MBC of PAM-5 against *P. aeruginosa* ATCC 27853 at the titer of 10^3 CFU/mL was determined at 8 $\mu\text{g/mL}$ as it was the lowest concentration of PAM-5, which prevented any bacterial growth.

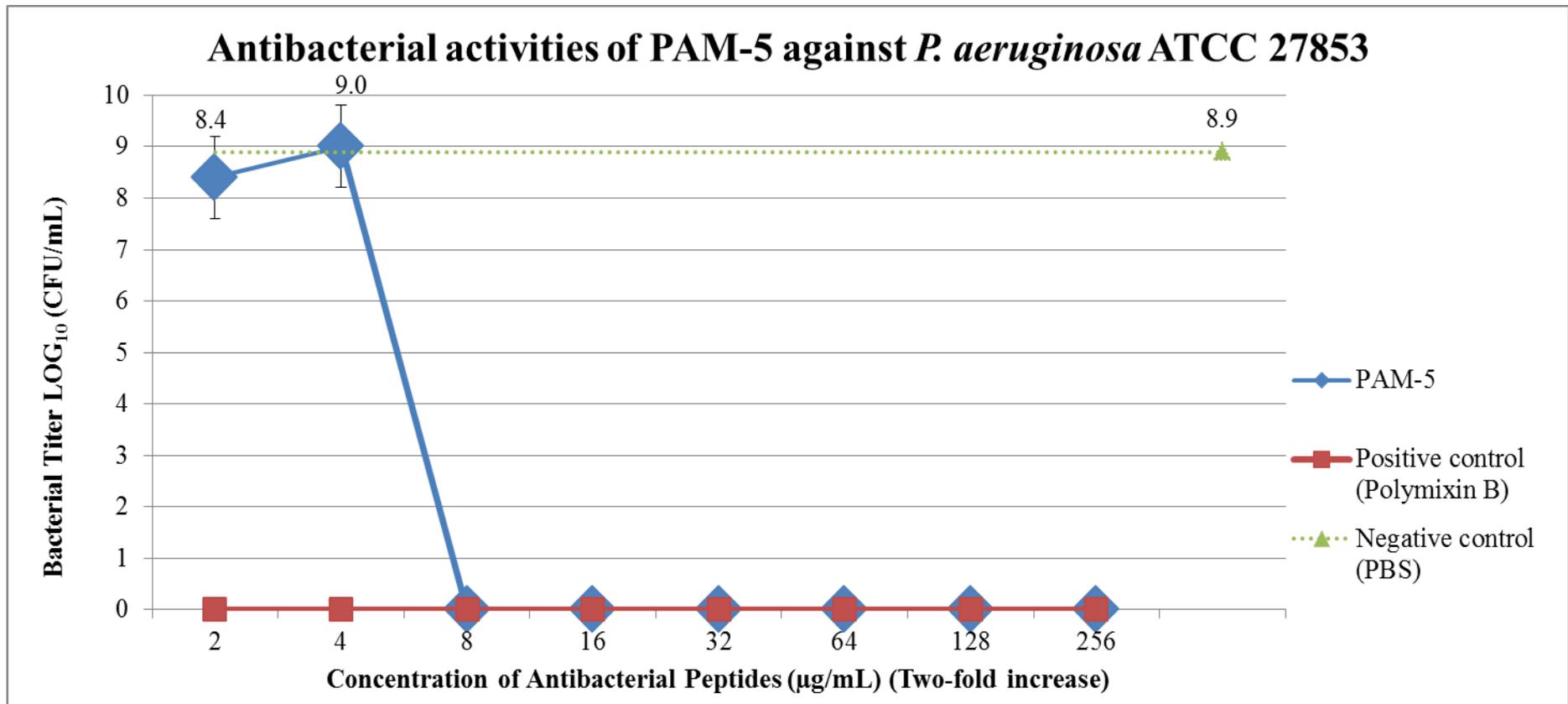


Figure 4.3: The antibacterial properties of PAM-5 against *P. aeruginosa* ATCC 27853. The target bacteria with the initial inoculation titer of 10^3 CFU/mL was treated with two-fold serially diluted PAM-5 and Polymyxin B with final concentrations ranging from 2 µg/mL to 256 µg/mL. Untreated bacteria in PBS suspension were regarded as the negative control.

4.2 Membrane-active mechanisms of PAM-5

As PAM-5 was ascertained to have bactericidal effects against *P. aeruginosa* ATCC 27853 via microbroth dilution assay, screening for the type of mechanisms in which PAM-5 employed to kill the bacteria is noteworthy. As PAM-5 is a cationic linear synthetic peptide, it is anticipated to have the classic mode of actions of an antibacterial peptide on the anionic bacterial membranes. The utilization of scanning electron microscope (SEM) can aid in the visualization of any structural or morphological changes of the bacterial membranes while the usage of SYTOX[®] Green dye allows the determination of membrane integrity of the target bacteria after the peptide treatment.

4.2.1 Scanning electron microscopy (SEM) analysis

As shown by the micrograph in **Figure 4.4 (A)**, untreated bacteria which served as the negative control possessed intact and smooth surfaces when they were viewed under low magnification of 18,000X. The bacterial length ranged from 1.6 μm to 2.6 μm with an average of 2.0 μm . In contrast, as seen in **Figure 4.4 (B)**, which represents the positive control, SEM observation on the Polymyxin B-treated bacteria revealed severe corrugation to their membranes and the length of the bacteria was shortened to a range of 1.1 to 2.0 μm . The average length calculated was 1.5 μm . Interestingly, PAM-5 could also induce surface roughening or corrugation to the membranes of *P. aeruginosa* similar to the positive control after one hour of peptide treatment as shown in **Figure 4.4 (C)**. In addition, decrement in length of the PAM-5-treated-bacteria was also apparent as they were only 1.2 to 1.7 μm long with an average of 1.4 μm ,

indicating that the target bacteria may not be able to grow to its maximum length.

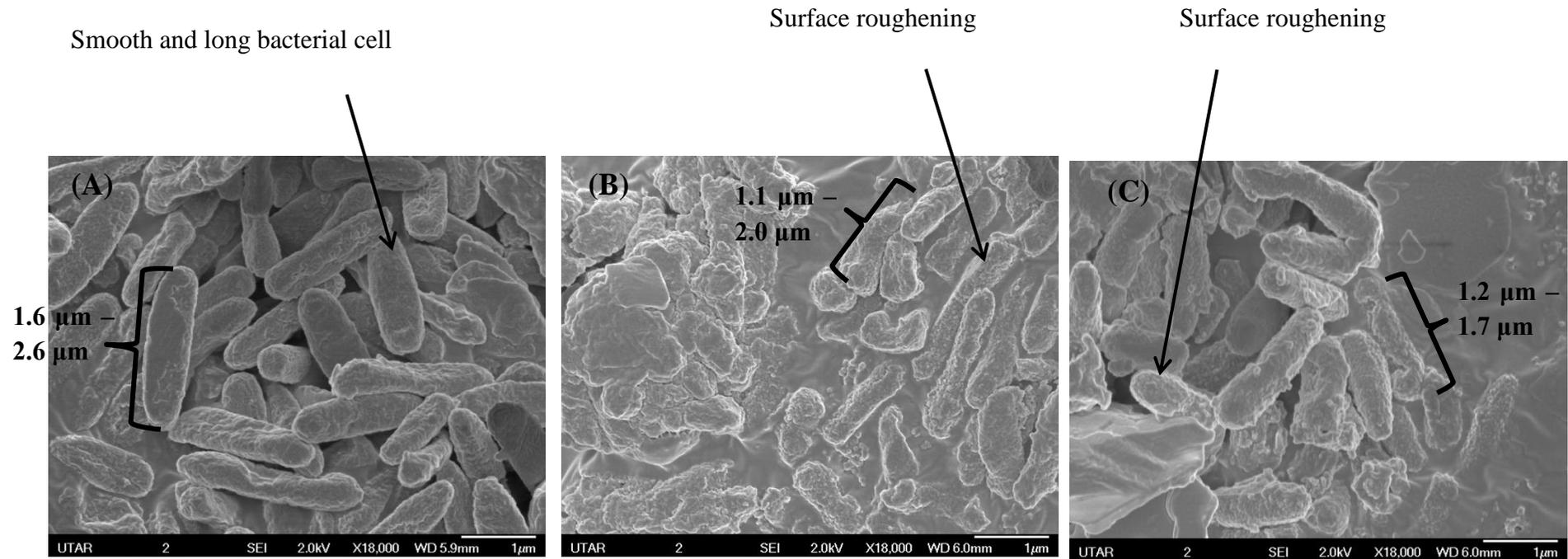


Figure 4.4: SEM micrographs of *P. aeruginosa* ATCC 27853 viewed using 18,000X magnification. (A) Untreated bacteria in PBS suspension that served as the negative control; (B) Bacterial cells treated with 128 $\mu\text{g}/\text{mL}$ of Polymyxin B were regarded as the positive control; (C) Bacteria treated with PAM-5 that was of the same concentration as the positive control.

When the specimens of the positive control were observed under higher magnification (30, 000X), they displayed small protuberant structures or blebbings on their surfaces as illustrated in **Figure 4.5 (B)**. Similar to the positive control, PAM-5-treated bacteria as depicted in **Figure 4.5 (C)** were characterized by the occurrence of blebbings. However, these features were absent in the untreated bacteria as shown in **Figure 4.5 (A)**.

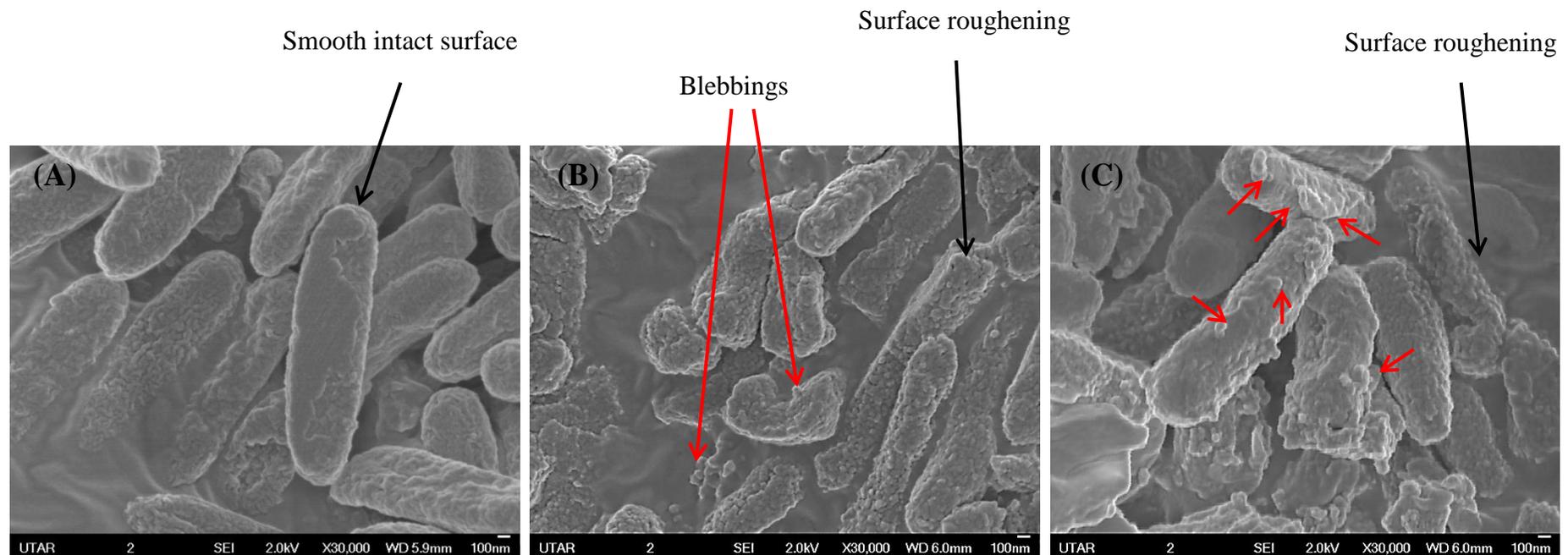


Figure 4.5: SEM micrographs of *P. aeruginosa* ATCC 27853 observed using a higher magnification of 30, 000X. (A) Negative control in which the untreated bacteria were suspended in PBS; (B) Polymyxin B-treated bacteria as the positive control; (C) PAM-5-treated bacteria. Red arrows indicate blebbings.

4.2.2 Bacterial membrane permeabilization as determined by SYTOX[®]

Green uptake assay

After discovering that PAM-5 could cause distortion to the membranes of *P. aeruginosa* ATCC 27853, SYTOX[®] Green uptake assay was then employed to determine the membrane-permeabilizing ability of the peptide. It is generally accepted that the loss of membrane integrity is one of the criteria in determining bacterial death. As mentioned earlier, SYTOX[®] Green is membrane-impermeable and can only enter cells with disrupted membranes, allowing its subsequent association with the intracellular nucleic acids to emit green fluorescence. Thus, the degree of fluorescence intensity emitted by the peptide-treated bacteria is corresponding to the amount of killed/dead bacteria.

Upon one hour of treating the bacteria with the peptides, the fluorescence intensity generated by the bacteria was measured using the Tecan Infinite 200 microplate reader. **Figure 4.6** presents the graph of the average green fluorescence intensity obtained from three independent assays. Based on the data, low fluorescence intensity was detected in the negative control, in which the untreated bacteria were suspended in PBS. In contrast, all the positive controls (except bacteria treated with 2 µg/mL of Polymyxin B) produced 5- to 6- times higher fluorescence intensity as compared to the negative control.

On the other hand, fluorescence intensity generated by the bacteria treated with PAM-5 at the concentrations of 2 µg/mL and 4 µg/mL were lower as compared to the other concentrations except for 256 µg/mL. The low fluorescence

intensity was comparable to that of the negative control. Interestingly, the fluorescence intensity was noted to increase gradually from 2 $\mu\text{g/mL}$, peaking at 64 $\mu\text{g/mL}$. After that, the fluorescence intensity was seen declining from bacterial culture treated with 128 $\mu\text{g/mL}$ to 256 $\mu\text{g/mL}$ of PAM-5 by 2-times reduction.

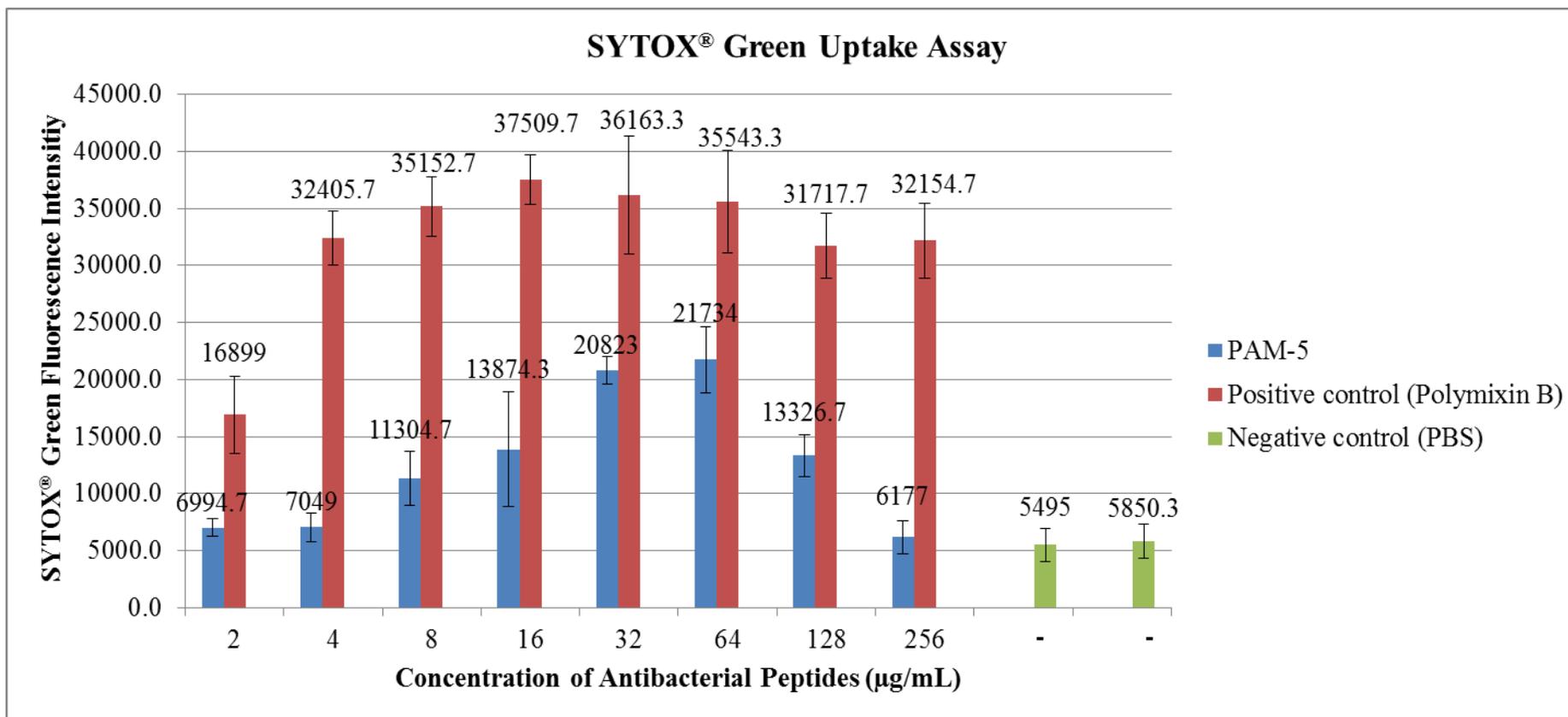


Figure 4.6: Bacterial membrane permeabilization activities of PAM-5 as determined by the detected fluorescence intensity of SYTOX® Green. The target bacteria with the titer of 10^6 CFU/mL was treated with two-fold serially diluted PAM-5 with final concentrations ranging from 2 µg/mL to 256 µg/mL.

CHAPTER 5

DISCUSSION

Antibacterial peptides (ABPs) have been regarded as one of the potential therapeutic agents against multi-drug resistant bacteria due to their advantages over the conventional antibiotics. ABPs have been shown to interact with bacterial membrane and are associated with the subsequent membrane disruption. However, there are also instances where these peptides could target intracellular targets such as DNA replication, RNA transcription as well as protein synthesis (Brogden, 2005). Studies regarding their modes of actions should be as vital as their antibacterial effects as many details on how they exert their antibacterial effects still remain unclear. The understandings on their mechanisms of action could provide essential clues that help in the design and synthesis of effective antibacterial agents that are able to overcome the limitations of the conventional antibiotics, such as bacterial resistance.

In this study, the novel antibacterial peptide, PAM-5 (KWKWRPLKRKLVLRM) was screened for its ability to disrupt the membrane integrity of *P. aeruginosa* ATCC 27853. This peptide was shown to be bactericidal against the bacteria at the MBC of 8 µg/mL (Lee, 2015). This finding was again verified by the current study as shown in **Section 4.1.2**. According to Hancock and Chapple (1999), an ideal ABP is able to kill bacteria *in vitro* with minimal inhibitory concentrations (MICs) ranging from 1 to 8 µg/mL. Thus, PAM-5 is considered to have fulfilled this criterion and

hence, can be regarded as a potent bactericidal ABP. Since PAM-5 is bactericidal, it is assumed that this peptide must be targeting at one of the bacterial targets which are essential for bacterial survival, such as its membrane. Thus, in this study, we focused on the effects of PAM-5 on the membrane of *P. aeruginosa* ATCC 27853.

5.1 Membrane-active mechanisms of PAM-5

As mentioned earlier, ABPs may inhibit or kill the bacteria via several mechanisms that target either the bacterial membrane or intracellular targets. Regardless of the mechanisms, it is essential for the ABPs to establish the initial interaction to the bacterial membrane before any further action can take place. As most of the natural and synthetic ABPs are cationic in nature, it is assumed that these peptides can bind to the anionic bacterial membrane through electrostatic interaction. Therefore, most of the researches on novel ABPs will include the study of their membrane-active mechanisms as the preliminary study. It was hypothesized that PAM-5 could exert its antibacterial effects via membrane disruption. As such, scanning electron microscope analysis and SYTOX[®] Green uptake assay were used to screen for the membrane-active mechanisms of PAM-5.

Both of these assays required higher bacterial titer than the one used in microbroth dilution assay. In order to generate sufficient amount of fluorescence to be detected by the microplate reader, the bacterial titer that was used for SYTOX[®] Green uptake assay was 10^6 CFU/mL and the corresponding

MBC of PAM-5 was 64 µg/mL. As for SEM analysis, the bacterial titer that was sufficient to be viewed under electron microscopy was 10⁷ CFU/mL and the corresponding MBC was 128 µg/mL. The increment in the MBCs due to the difference in the titer used could be explained by the inoculum effect of the bacteria, which had been studied by Lee in 2015. It is described as the decreasing efficacy of an antibacterial agent due to the increased in the amount of initial inoculated bacterial titer (Tan, et al. 2012).

5.1.1 Visualization of PAM-5-induced membrane disruption of *P. aeruginosa* ATCC 27853 via scanning electron microscope (SEM)

PAM-5, like many other antibacterial peptides, exhibits cationicity and moderate hydrophobicity. A number of ABPs, for instance, Magainin 2, have been previously shown to exert their antibacterial effects on the bacterial membranes by disrupting them and eventually lead to cell lysis (Bahar and Ren, 2013; Matsuzaki et al., 1997). As PAM-5 is a cationic peptide with a net positive charge of +7, it is believed that this peptide could bind strongly to the bacterial membranes that are commonly anionic (Melo, et al., 2009). In addition, amphipathicity of the ABPs might play an important role in the subsequent events after the initial binding to the bacteria. This feature allows the penetration of the peptide deeper into the bacterial membrane (Ebenhan, et al., 2014). Hence, this may eventually lead to membrane damage and disintegration (Bechinger and Lohner, 2006). In view of PAM-5, it also carries some amphipathicity which might contribute to the damaging effects of the bacterial membrane.

As reported in Chapter 4, distinct morphological changes such as surface roughening, blebbings or micelles and corrugation of the membrane surfaces were noted in the PAM-5- treated *P. aeruginosa* but not in the untreated bacteria, in which their intact membranes appeared smoother. The similar appearance of membrane disruptions can also be seen in the positive control when the bacteria was treated with Polymyxin B. These observations suggest that PAM-5 could cause similar morphological changes to the bacteria as compared to Polymyxin B, which is a strong membrane-disrupting antibacterial peptide.

The findings on the PAM-5-induced bacterial membrane disruption were corresponding to a few other studies by different research groups. For example, Saiman, et al. (2001) had demonstrated similar morphological changes on the membrane surface of *P. aeruginosa* PAO1 when the bacteria were treated with a potent antibacterial peptide named cathelicidin SMAP29. These changes included surface roughening, blebbings of the membrane and shortening of the bacteria. On the other hand, similar results were observed in *Escherichia coli* in another two studies by Song, et al. (2012) and Yenugu, et al., (2004) that treated the bacteria with HAHp2-3-1 and HE2 (α and β), respectively. The surfaces of these treated bacteria were found to have occurrence of numerous blebbings and wrinkling that were absence on untreated bacteria. The comparable results obtained for PAM-5 on *P. aeruginosa* ATCC 27853 in this study highly suggested that this peptide is a bactericidal antimicrobial peptide which kills bacteria via membrane disruption.

Several models have been proposed to describe how ABPs disrupt the bacterial membrane. The most well reported models are the ‘barrel-stave’, ‘toroidal pore’ and the ‘carpet’ models. In ‘barrel-stave’ model, ABPs aggregate on the bacterial membrane surface until certain threshold before spanning the lipid bilayer perpendicularly. This is followed by pore formations that lead to leakage of cellular contents and cell death. On the other hand, the ‘toroidal-pore’ model works similarly like the ‘barrel-stave’ model, but apart from inserting themselves parallel to the phospholipid bilayer, the ABPs also induce curvature to the lipid bilayer resulting in the lumen being lined by both the inserted ABPs and the lipid head groups (Melo, et al., 2009; Brogden, 2005). However, these two models require the ABPs to have a minimum length of 20 amino acids in order for them to be long enough to span the lipid bilayer (Song, et al., 2012; Shahmiri, Enciso and Mechler, 2015). As such, being a 15-mer ABP, PAM-5 may not be able to induce membrane damage through ‘barrel-stave’ and ‘toroidal-pore’ models.

In the ‘carpet’ model, ABPs orient themselves to lie at the interface that is parallel to the surface of the bacterial membrane. The peptides will then form a layer of ‘carpet’ that is able to induce weakness to the membrane by disrupting the bilayer curvature upon reaching their critical point. This will eventually lead to the collapse of the membrane into micellar configuration by a detergent-like action (Hale and Hancock, 2007; Shai, 2002). **Figure 5.1 (A)** illustrates the resemblance of a micelle structure from a disrupted membrane adapted from Brogden (2005), which lead to the appearance of blebbings as observed in the SEM micrograph of PAM-5-treated *P. aeruginosa* ATCC

27853 [Figure 5.1 (B)]. Thus, the blebbings found on the surface of the bacteria were probably micelles as the result of PAM-5-induced membrane collapse. The membrane disruption is also accompanied by membrane wrinkling which often suggests the loss of membrane integrity (Saiman, et al., 2001; Yenugu, et al, 2004).

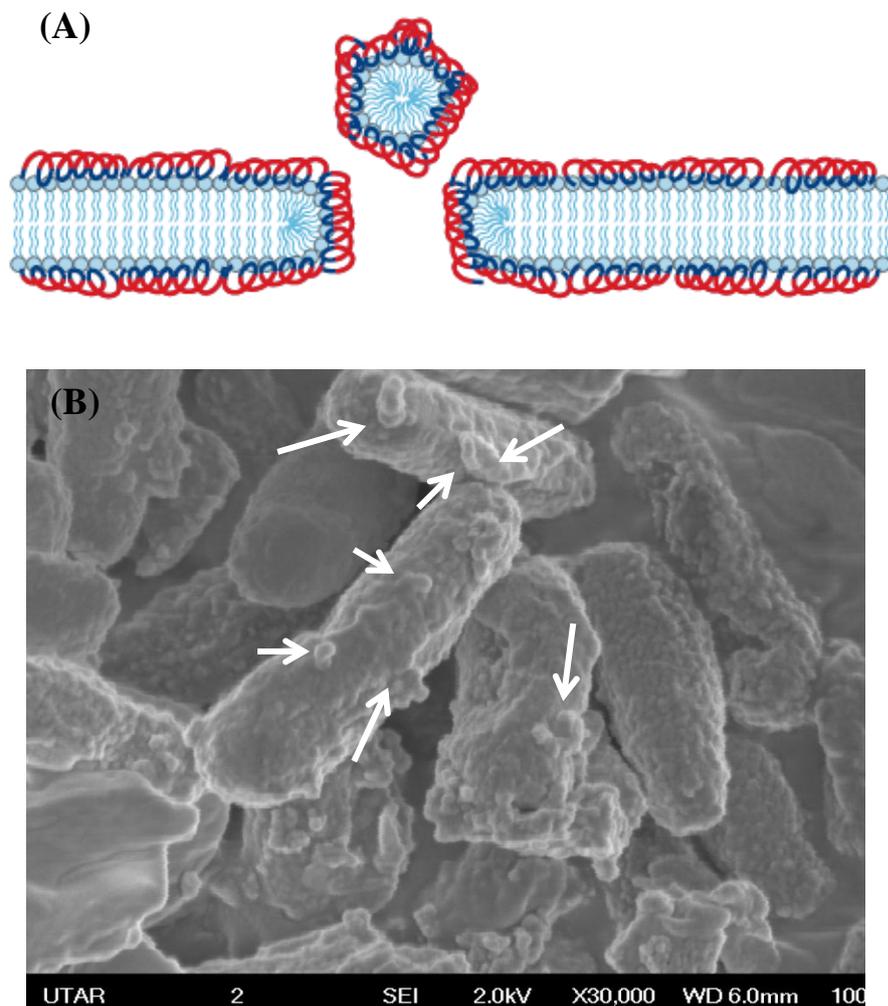


Figure 5.1: Resemblance of a micelle to the blebbings observed on the surface of PAM-5 treated bacteria. (A) Illustration of a micelle structure adapted from Brogden (2005); (B) SEM micrograph of PAM-5 treated *P. aeruginosa*. White arrows indicate the blebbings (micelles).

As the peptides proposed in this model of action do not insert themselves into the membrane or adopt any particular orientation during their course of action on the membrane, they do not need a minimum peptide length as required by the former two models to achieve their action. In addition, PAM-5 also fulfills an important criterion of the 'carpet' model. This peptide possesses positively-charged amino acids residues, arginine and lysine, that spread along the peptide chain. According to Shai and Oren (2001), the extended arrangement of these cationic amino acids allows them to be in continuous contact with the lipid head group during membrane permeation. As the SEM images in this study clearly show the presence of micelle-like structures on the PAM-5-treated bacteria, it could be assumed that PAM-5 exerted its antibacterial effects via the 'carpet' model.

The shortening of the bacterial length from an average length of 2.0 μm in the untreated bacteria to averagely 1.4 μm as seen in the PAM-5-treated bacteria indicates the inability of the bacteria to grow to its maximum length. Leakage of cytoplasmic contents such as potassium ions, adenosine triphosphate (ATP) or other soluble products such as protein and nucleic acid may follow after the disruption of the bacterial membrane (Johnston, et al., 2003). These molecules, especially ATPs, play important roles in the metabolic and anabolic events of the bacteria. Thus, the loss of these substances from the cells could results in the cessation of bacterial growth as indicated by the overall shortening of the peptide-treated bacteria.

5.1.2 Membrane permeabilization activities of PAM-5 as studied by SYTOX[®] Green uptake assay

To further elucidate the capability of PAM-5 in permeabilizing the membranes of *P. aeruginosa*, SYTOX[®] Green uptake assay was employed. SYTOX[®] Green is a membrane-impermeable green fluorescent nucleic acid dye. As intact *P. aeruginosa* possess functional resistance-nodulation cell division (RND)-type multidrug efflux pump, it could easily eliminate any foreign substance such as antibiotics or dyes from the interior of the bacteria. Permeabilized *P. aeruginosa* no longer possess such functional efflux pumps, hence the dye could enter and bind to the bacterial nucleic acid easily before emitting fluorescence (Dreier and Ruggerone, 2015). As such, SYTOX[®] Green could be used as an indicator of dead cells.

P. aeruginosa treated with PAM-5 at concentrations from 2 µg/mL to 64 µg/mL showed increment in the influx of SYTOX[®] Green as compared to the untreated bacteria. As such, it could be inferred that PAM-5 indeed has membrane-permeabilizing activities that could lead to the entry of SYTOX[®] Green into the cell.

Theoretically, if a higher concentration of ABPs is used to treat the bacteria, one could simply predict higher fluorescence signals would be emitted by the untreated bacteria. This is because there are more peptides available to permeabilize the bacterial membrane rendering more dye to bind to the bacterial nucleic acid, which leads to increased fluorescence emission.

Nevertheless, it was noted that there was a decrement in the fluorescence intensity of the samples that were treated with PAM-5 at 128 $\mu\text{g}/\text{mL}$ and 256 $\mu\text{g}/\text{mL}$. These findings could not support the notion that higher peptide concentrations are associated with greater membrane permeabilization as one would predict.

There are two possible explanations for the decrement in the fluorescence intensities from the bacteria treated with 128 $\mu\text{g}/\text{mL}$ and 256 $\mu\text{g}/\text{mL}$ of PAM-5. DNA degradation or changes in the DNA topology may follow after membrane damage and these are dependent on the environment and species of the bacteria. Such occurrence was observed in the experiment performed by Lebaron, Catala and Parthuisot in 1998. By using flow cytometry, they recorded an initial great difference in the SYTOX[®] Green fluorescence intensities between live (intact) and heat-treated (permeabilized) bacteria at the onset of bacterial starvation. However, the difference in fluorescence intensities between the two cohorts of bacteria decreased as the starvation period prolonged. They discovered that this was attributed to the decline in the fluorescence emission by the permeabilized bacteria as the result of decrement in the DNA content of the dead cells. Thus, this reduce the amount of SYTOX[®] Green probe that were able to bind to the nucleic acid and subsequently followed by reduction of fluorescence emission.

Secondly, PAM-5 may possess other killing mechanisms apart from membrane permeabilization only. Studies had found that ABPs can associate themselves with DNA and may compete with SYTOX[®] Green for the same binding site and hence, lead to quenching of fluorescence from the probes. Taute, et al. (2015) obtained a similar SYTOX[®] Green uptake assay result where the bacteria treated with lower concentration of an ABP, named Os, demonstrated higher fluorescence intensities than those treated with higher concentrations of the peptide. They speculated that their ABP could have DNA-binding affinity. This speculation was eventually confirmed by gel retardation assay performed by the research group. It was found that ABPs which possess DNA-binding affinity could compete with nucleic acid probes such as SYTOX[®] Green to bind to the bacterial nucleic acid. Thus, high ABP concentrations may reduce the amount of SYTOX[®] Green that is able to bind to the bacterial DNA, followed by reduction of fluorescence emission.

5.2 Implications of study

Most of the conventional antibiotics only act upon specific bacterial target. In contrast, majority of the ABPs, including PAM-5, could carry out their antibacterial effects via membrane destruction. This mechanism poses several advantages over antibiotics as there is a low risk for the development of bacterial resistance against ABPs. This is due to the fact that a change in the entire lipid composition of the bacterial membrane will be required in order for them to evade the membrane-damaging bactericidal effects of ABPs. Synthesis of the essential macromolecules of the anionic membranes, for instance,

phospholipids and lipid A, is rather complex and requires high energy expenditure of the bacteria. As such, it is unlikely that bacteria will be able to replace these cell-membrane structures with novel molecules that could hinder the binding of the ABPs (Peschel and Sahl, 2006). On top of that, the membrane-active PAM-5 may allow the peptide to kill bacteria rapidly. As a result of this, the target bacteria are deprived of sufficient time to acquire any resistance mechanism via mutation. Therefore, all these features make ABPs one of the potential therapeutic agents to replace antibiotics, which are slowly losing their efficacies due to bacterial resistance.

5.3 Limitation of current study and proposed future studies

Although this study confirms the membrane-active activities of PAM-5 on *P. aeruginosa*, further additional killing mechanism of PAM-5 such as DNA-binding or inhibition of protein synthesis are yet to be elucidated. In particular to the latter, it is strongly believed that PAM-5 might bind to bacterial nucleic acids with high affinity, as indicated by the possible displacement of SYTOX[®] Green from the bacterial DNA as discussed previously. Hence, DNA-binding properties of this peptide should be evaluated via gel retardation assay in future studies.

Additional membrane-active mechanisms should be analyzed in order to gain better insight on how PAM-5 disrupts the bacterial membrane. Outer membrane permeabilization activities of PAM-5 can be studied using the N-phenyl-1-naphthylamine (NPN) uptake assay. If the outer membrane of the

bacteria could be disrupted by PAM-5, the hydrophobic dye, NPN, will enter the periplasmic space and emits fluorescence. In order to determine the inner membrane depolarization properties of PAM-5, dipropylthiadicarbocyanine iodide [diSC₃(5)] assay could be performed. This cationic dye could self-quench once it enters the cytosol of the bacteria. If PAM-5 is able to disrupt the inner membrane, the dye will be released out from the cytoplasm and fluoresce. Moreover, transmission electron microscope (TEM) analysis is worth conducting as it allows the observation of the internal ultrastructural damage of the bacteria. By carrying out these studies, it is hope that the membrane-active mechanisms of PAM-5 can be further elucidated.

CHAPTER 6

CONCLUSION

In a nutshell, the novel synthetic antibacterial peptide, PAM-5, could exert its bactericidal effects via membrane-active mechanisms. Through SEM analysis, PAM-5 was able to induce morphological changes to *P. aeruginosa* ATCC 27853 upon one hour treatment as blebbings and corrugation of its surface could be observed. In addition, PAM-5 was also found to possess membrane-permeabilizing activities as it allowed the uptake of SYTOX[®] Green into peptide-treated bacteria resulting in fluorescence emissions. These significant findings highlight the potentials of PAM-5 as an alternative to conventional antibiotics which could act upon one of the essential bacterial targets, the membrane.

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APPENDIX A

PREPARATION OF BUFFERS, MEDIA AND REAGENTS

Preparation of Bacterial Glycerol Stock

Few colonies of *Pseudomonas aeruginosa* ATCC 27853 was inoculated into a conical flask containing 20 mL of LB broth and it was then incubated in the orbital shaker with 200 rpm at 37 °C for 4.5 hours. A volume of 5 mL of 80% glycerol was then added into the bacterial suspension and this made up a final concentration of 20% (v/v) glycerol solution. After the bacterial suspension was adequately mixed, 500 µL was aliquoted into the 1.5 mL microcentrifuge tubes. They were then stored overnight at -20 °C before perpetually keeping them at -80 °C. In order to revive the bacteria, the bacterial glycerol stock was thawed on ice for at least half an hour before inoculating it onto MH agar as the master culture plate. The latter was incubated overnight at 37 °C and was eventually stored at 4 °C for a maximum period of seven days.

Luria-Bertani (LB) broth

Eight grams of LB broth (Merck Millipore Corporation) was dissolved in 400 mL of distilled water. The medium was then subjected to autoclave at 121 °C and 15 psi for 20 minutes.

Mueller-Hinton (MH) agar

The Difco™ MH agar powder was weighed at 26.6 g and dissolved in 700 mL of distilled water. The medium was then autoclaved at 121 °C and 15 psi for 20 minutes. It was then poured into sterile petri dishes. Once the agar has solidified, the media plate will be left to dry in the laminar flow for 45 minutes before storing them in 4 °C.

Mueller-Hinton (MH) broth

The medium was prepared by suspending 8.4 g of Difco™ MH broth powder in 400 mL of distilled water. It was then autoclaved at 121 °C and 15 psi for 20 minutes.

Phosphate buffered saline (PBS) solution (pH 7.4)

Four PBS tablets (free from magnesium and calcium) manufactured by MP Medicals, LLC. were dissolved in 400 mL of distilled water before autoclaving the solution at 121 °C and 15 psi for 20 minutes.

Glutaraldehyde (3%) in PBS

The solution was prepared by mixing 12.5 mL of 0.1 M PBS with 6 mL of 25% Glutaraldehyde manufactured by Sigma-Aldrich Co., LLC. The solution was then topped up with distilled water to 50 mL.

APPENDIX B

LIST OF LAB WARES AND EQUIPMENT USED AND THEIR REPECTIVE MANUFACTURERS

Lab wares/ Equipments	Manufacturers
2nd level Biosafety Cabinet	TELSTAR, Philippines
15 mL centrifuge tube	Greiner, Germany
50 mL centrifuge tube	Axygen [®] Scientific, USA
96-well clear microplate, flat-bottomed	Greiner CELLSTAR [®] , Germany
96-well white opaque, flat-bottomed	Greiner CELLSTAR [®] , Germany
Auto Fine Coater	JEOL (JFC-1600), USA
Beaker	GQ, Malaysia
Bunsen burner	Campingaz, France
Centrifuge	Eppendorf (5430 R), Germany
Freeze dryer	Scanvac COOLSAFE [™] , Denmark
Incubator	Memmert, Germany
Measuring cylinder	GQ, Malaysia
Media bottle	Schott DURAN [®] , Germany
Microcentrifuge	Thermo Scientific, USA
Microcentrifuge tube	Axygen [®] Scientific, USA
Microplate reader	Tecan Infinite 200, Switzerland

Micropipette set	Gilson, France
Micropipette tip	Axygen® Scientific, USA
Petri dish	BIOAN, Malaysia
Scanning electron microscope	JEOL (JSM-6701F), USA
Spectrophotometer	Thermo Scientific Genesys 20, Malaysia
Vortex mixer	VELP® Scientific, Europe
