Assessment on the Filtration Ability and Antimicrobial Activity of Nanomembranes with *Mycobacterium tuberculosis*

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ABSTRACT

Nowadays, tuberculosis (TB) is still an important public health problem worldwide especially in low and middle income countries. Because *Mycobacterium tuberculosis,* the causative agent of TB mostly spreads and infects people via aerosol droplets or droplet nuclei, the elimination of tubercle bacilli from the air is very essential to reduce risk of infection. Here, we evaluated three candidates of silver-containing nanomembranes namely P1, P2 and P3, fabricated from polyvinyl alcohol (PVA) and additives by electrospinning technique, using *M. tuberculosis* H37Ra as the model organism. For filtration test, 3×10^5 of mycobacterial cells were filtered through each nanomembrane. There was no significant difference ($P<0.01$) of CFU count from filtrates passed through all nanomembranes compared to the standard commercial filter. The P1 nanomembrane showed the highest filtration efficiency at 99.9974% . For antimycobacterial test, dead cells trapped on the nanomembranes were observed using LIVE/DEAD fluorescent staining assay. The P3 showed the highest antimycobacterial activity more than 98% after 24 hours of exposure and sterilization appeared within 48 hours.

Keywords: *Mycobacterium tuberculosis*, Silver-containing nanomembrane

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Introduction

Tuberculosis (TB) is one of the infectious diseases that, has succeeded to spread worldwide. In 2015, WHO reported 10.4 million active TB cases. It is one of the top 10 diseases which causes 1.4 million deaths worldwide and more than those caused by HIV. About 84% of them are the people living in African and South-East Asian region. Nowadays, global prevalence of TB is very high about one-third of human populations (more than 2 billion people) got asymptomatic infection and 5-15% of them will develop into the active disease (World Health Organization [WHO], 2016). The etiologic agent of TB is mostly *Mycobacterium tuberculosis* (*M. tb*) which can spread easily through the air by coughing, sneezing and shouting or even speaking of the TB patient. The infectious route is mostly through respiratory tract by inhalation of the airborne particles containing tubercle bacilli (1-5 microns in diameter). These particles can suspend in the air and retain infectious activity for several hours. Moreover, infectious dose of the agent is absolutely low, less than 10 bacilli that can cause the disease in human (Dean *et al.*, 2005; Centers for Control Disease and Prevention [CDC], 2008). Hence, the eradication of tubercle bacilli floating in the air is very important to reduce the risk of infection and to achieve global TB ending program.

Air filtration using HEPA filter is a good way to get rid of *M. tb* from the air. However, it is sometimes too expensive for some low-income countries to afford. Thus, the filter made up from cheaper materials and assemblies become desirable. One of candidate materials is polyvinyl alcohol (PVA). PVA is the largest volume of water-soluble synthetic polymer produced in the world which has no color and odor. It is very stable and chemically inert and nontoxic. Moreover, another advantage of PVA is thought to be biodegradable. It has been applied to many fields including industrial, medical, commercial and food sections and used to produce many products such as lacquer, surgical thread, wound dressing and hydrogel (Hassan, Peppas, 2000; Muppalaneni *et al.*, 2013; Gaaz *et al.*, 2015). Therefore, researchers from National Nanotechnology Center, Ministry of Science and Technology, Thailand are interested in developing multifunctional filter membrane using PVA as the base material. The first beneficial function of the PVA membrane proposed is filtration ability. Second one is antimicrobial activity and the third one is reusability. PVA was fabricated into nanofibrous membrane using electrospinning technique. Three candidates (represented as a code) of PVA membrane are different in component and post-fabrication process. P1 has crosslinks between nanofibers. P2 has crosslinks and the addition of water repellant substance and P3 has no crosslinks but has the addition of water repellant substance. Silver was added to all membranes to confer their antimicrobial activity.

Silver has been reported as one of the most powerful antimicrobial metals because it has ability to break disulfide bonds and to bind strongly with sulfhydryl residue of proteins causing denaturation of any proteins both inside and on surfaces of the bacterial cells including DNA aggregation. Many reports have already approved the antimicrobial activity of silver in both gram positive and negative bacteria (Feng *et al.*, 2000; Jung *et al.*, 2008; Ramirez *et al.*, 2012). It was also reported about the antiviral activity (Park *et al.*, 2014). As nanoparticles, silver has ability to reduce 98.7% of *M.*

smegmatis at the concentration less than 5 ppm (Islam *et al.*, 2013) and inhibit growth of *M. tuberculosis* at 8 ppm (Seth *et al.*, 2010)

In this study, we aimed at testing two important functions of the filters: filtration ability and antimycobacterial activity. The two tests were done using a conventional method and fluorescent staining technique respectively. *M. tuberculosis* H37Ra served as the model organism. Mycobacterial cells were prepared in liquid suspension instead of aerosolized form which is more impractical and dangerous.

Objective of the study

To compare filtration ability and antimycobacterial activity of three different silver-containing nanomembranes with *M. tuberculosis* H37Ra.

Materials and Methods

Culture and preparation of single cell suspension

M. tuberculosis H37Ra employed in this study was cultured on Löwenstein-Jensen (LJ) medium, incubated at 37°C for 3-4 weeks and used for preparation of the bacterial cell suspensions as McFarland No.1.

Firstly, 3-5 drops of M7H9 medium with OADC (Becton-Dickinson company, New Jersey, USA) were added into sterile glass-bead containing tube by disposable Pasteur pipette, then mycobacterial colonies from LJ medium were scraped and stabbed into the tube. Clump colonies in the tube were broken with vortex mixer (VORTEX-GENIE-2, Scientific Industries, Inc., New York, USA). Six milliliters of M7H9 were added into the tube and mixed well. Then the tube was left for 20 minutes to let the large cell clump settle. Four milliliters of supernatant suspension were transferred into a sterile container.

Because single cell suspension is necessary for observation in both filtration and antimicrobial tests while cord factor production of *M. tb* cause the clumping of cells. Thus, the homogenization technique was exploited to disperse the clump cells into single cells. The suspension was drawn up and down (homogenization) using syringe (TERUMO-®SYRINGE, Tokyo, Japan) for 35-40 times through needle (No.26G, Becton-Dickinson company, New Jersey, USA) then 1 mL of homogenized suspension was transferred into a new sterile bottle and diluted with 2 mL of normal saline. Two milliliters of the diluted suspension were drawn using syringe and filtered through 5 µm membrane filter (Sigma-Aldrich, St. Louis, Missouri, USA) to separate fine clump of cells into single cells. The filtrate was adjusted the turbidity

to McFarland No.1 (1.75×10^7 CFU/mL, concentration already verified as reference in our laboratory) with normal saline and measured by densitometer (DEN-1B, biosan, Riga, Latvia).

Filtration Test of nanomembranes with mycobacterial cells

All membrane candidates were cut into circular shape (2.5 cm in diameter), packed into filter holder (Sartorius, Göttingen, Germany) and autoclaved at 121°C for 15 minutes. The sterile nanomembranes were then dried in hot-air oven at 60°C overnight. After that, the mycobacterial cell suspension (McFarland No.1) was serially diluted (10-fold dilution) into 1.75×10^5 cell/mL using normal saline. Then, 2 mL of the diluted suspension was filtered through a 0.45 µm membrane filter (Sartorius, Göttingen, Germany) as the control and 3 membrane candidates. Finally, 100 µL of each filtrate was spread on M7H10 plates (triplicates), incubated at 37°C for 3-4 weeks. The colony forming units (cfu) of mycobacterium are counted as CFU/mL. The filtration ability of nanomembranes was determined by the percentages of initial cell concentration minus passed cells per initial cell concentration (1.75×10^4 cells/mL).

Assessment of viability using Live/ Dead staining technique and examination with confocal laser scanning microscopy (CLSM)

The filtered membranes were cut into small pieces (pizza liked) and placed on M7H10 plates. The plates were incubated at 37°C with each piece of membrane picked up for Live/Dead staining after 24, 48, 72, 96 and 168 hours of the incubation.

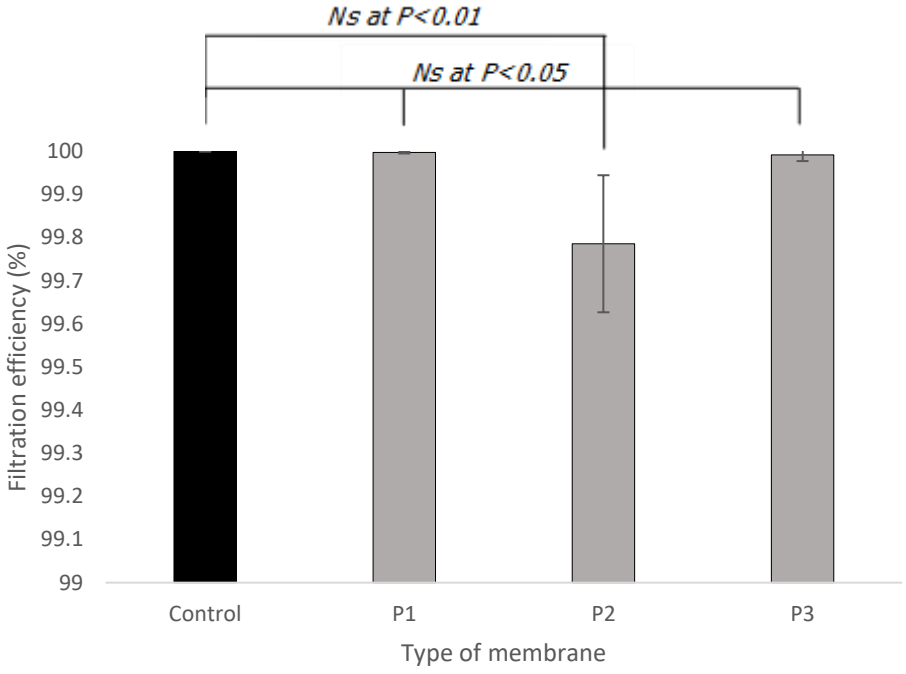
The live/dead staining was done to differentiate between live and dead cells trapped on nanomembranes and to count their numbers. In this study the steps of staining were modified from the manufacturer instruction. Briefly, 1.5 µL of propidium iodide (PI) and SYTO9 (1:1) (L7012, Molecular probes, Thermo Fisher Scientific, Massachusetts, USA) was mixed with 500 µL of normal saline in sterile container. A piece of each membrane was placed on glass slide. After that, 80 µL of the dye mixture was dispensed onto the membrane. Then, the slide was covered with a coverslip and sealed by fixing the edge with sealing agent. The slide was incubated for 15 minutes in the darkness.

After that, the viability of cells was observed under the confocal laser scanning microscope (CLSM) (ECLIPSE Ti-Clsi4 Laser Unit, Nikon, Tokyo, Japan). The fluorescence is triggered using laser beam from the microscope. At 488 nm in wavelength stimulates SYTO9 to emit green fluorescence and 543 nm in wavelength stimulates PI to emit red fluorescence. The fluorescent-emitting cells were designated and counted as red (dead) and green (live) cells, and the antimycobacterial activity of each nanomembrane was reported as the percentages of killed cells per total cells.

Results

Filtration efficiency of nanomembranes compared to control filter

The filtration efficiency of each membrane candidate was reported as the percentages of mycobacterial CFU filtered by the membranes compared to the initial cell concentration. P1, P3 and P2 showed very high filtration efficiency at 99.9974%, 99.9917% and 99.7856% respectively. P1 and P3 had no significant difference at $P<0.05$ (t-test) compared to standard membrane filter (100% filtration efficiency), while P2 showed no significant difference at $P<0.01$. Moreover, they also showed no significant difference in filtration ability among one another at $P<0.05$ (ANOVA) as shown in Figure 1.



Ns = Non-significant difference

Figure 1. The filtration ability of nanomembrane candidates compared to standard control

Antimycobacterial ability of nanomembranes

Fluorescent-emitting cells counted under confocal laser scanning microscope were reported as the percentages of viability reduction after *M. tb* cells were exposed with the membranes. The baseline control, silver-free PVA nanomembrane was used as a reference in this part. The trends of bacterial viability after exposure with each nanomembrane in different time intervals were showed in Figures 2, 3 and 4.

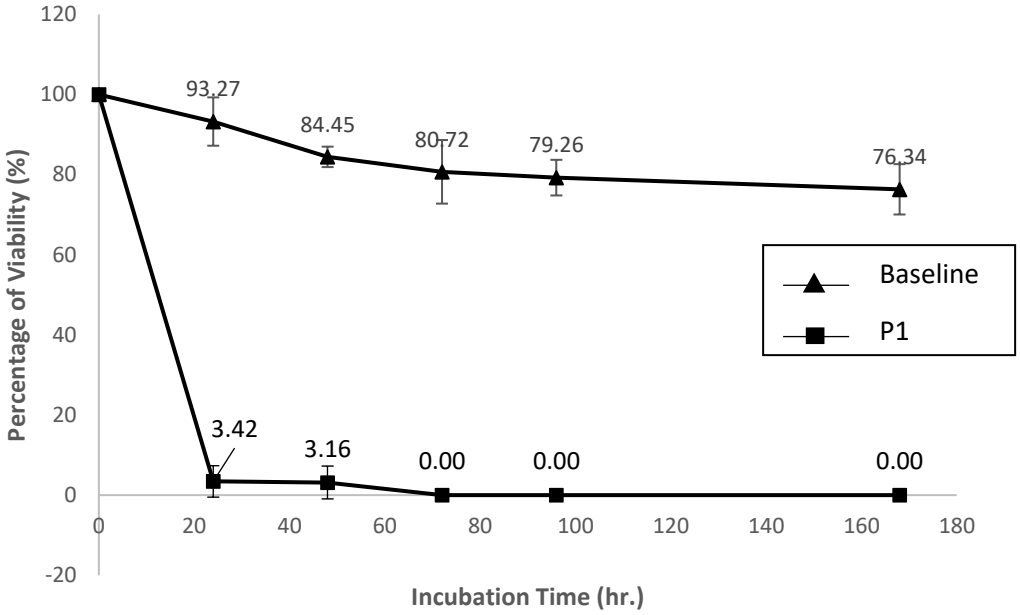


Figure 2. Trend of the cell viability of *M. tuberculosis* after exposure to the P1 nanomembrane compared with the control

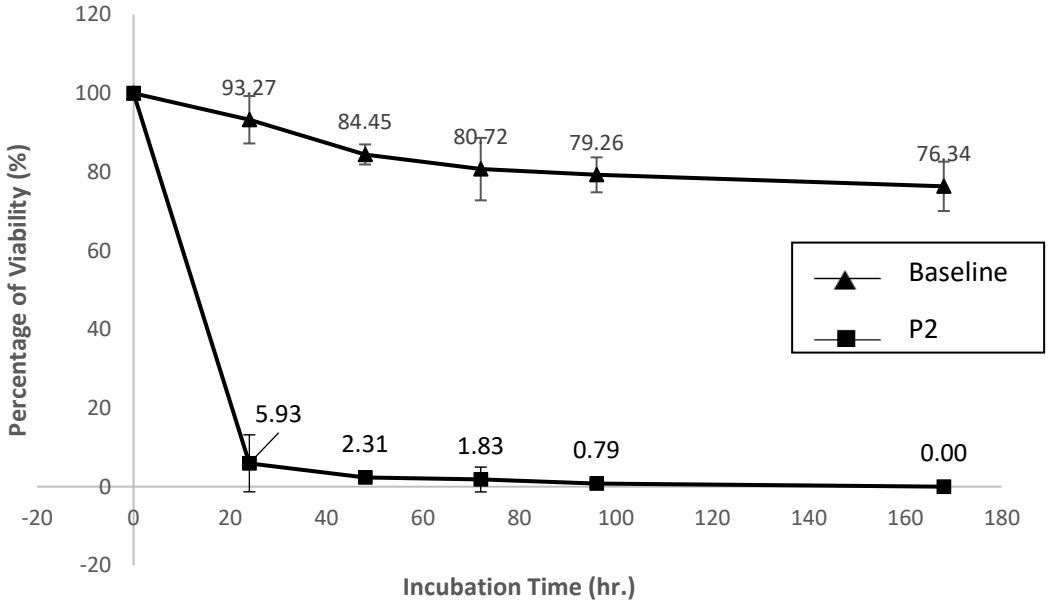


Figure 3. Trend of the cell viability of *M. tuberculosis* after exposure to the P2 nanomembrane compared with the control

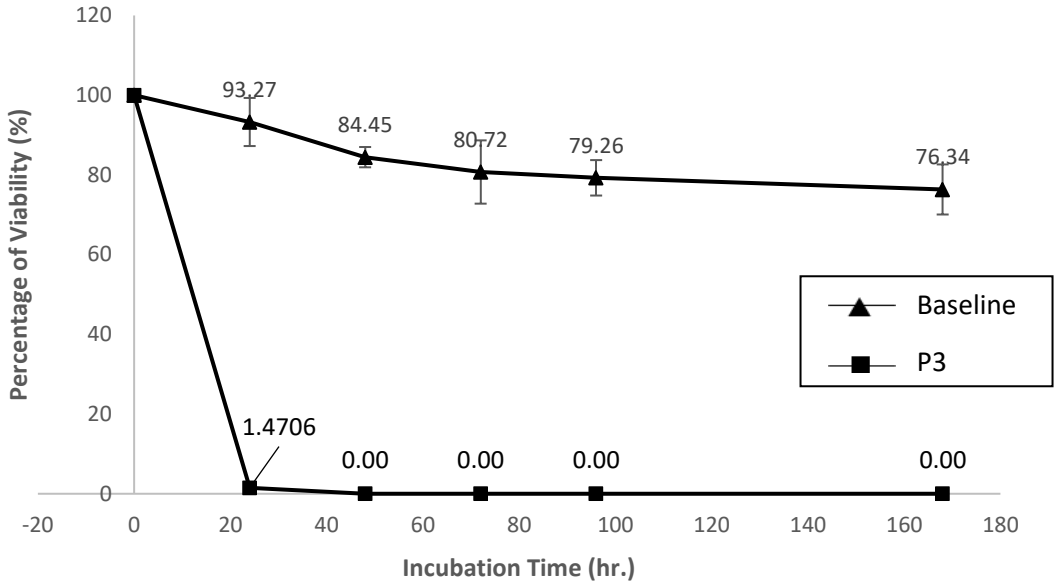


Figure 4. Trend of the cell viability of *M. tuberculosis* after exposure to the P3 nanomembrane compared with the control

P3 showed the highest antimycobacterial activity which revealed more than 98% dead cells within 24 hours and sterilization appeared within 48 hours. However, all candidates showed no significant difference in antimycobacterial activity at $P<0.05$ (ANOVA) . When compared to the control, they also exhibited significant difference in antimycobacterial activity at $P<0.01$. However, P2 showed the least sterilization effect which appeared after 100 hours and before 168 hours of exposure.

Discussion

According to WHO report, TB incidence cases in 2015 are 10.4 million and increasing when compared to those of 2014 with 9.6 million (WHO, 2016 and 2015). This is prompting us to be aware and work hard to find out the effective way which can eradicate tubercle bacilli, the etiologic agent of tuberculosis. Here, we evaluated three different silver-containing nanomembranes in the ability to filter and to kill *M. tuberculosis* in solution form and the results were very satisfactory in both filtration and antimycobacterial activity. However, tuberculosis is grouped as air-borne transmitted disease, the infection is mostly caused by tubercle bacilli floating in the air as aerosol droplets. This might not be actually representative because in reality, the droplet nuclei contain only trace amount of water which releases silver ion (Ag^+) from the silver nanoparticles to kill the cells (Franci *et al.*, 2015). In contrast, this study, we prepared *M. tb* cells as the suspended solution which contains a lot of water. At real condition, antimycobacterial activity might be lower than our results (more than 94% within 24 hr. in all candidates) because the lower amount of Ag^+ are released from nanoparticles.

Another point is that the results of baseline control (Figure 2, 3 and 4) showed some antimicrobial activity possibly due to aldehyde compound employed as a crosslinking agent which has been firmly reported to have antimycobacterial activity (Rutala, Weber, 2008)

For the filtration ability, P2 and P3, contained water repellant substance to impart self-cleaning function of the nanomembranes. Their hydrophobic property obstructed the filtration test. This water repellence sometimes impeded water transmission, built up pressure and caused leakage of mycobacterial cells from the nanomembranes during the filtration process. On the other hands, P1 that has no water repellant function did not show this effect. This suggested that the aerosolized simulation might be more suitable to test their activities. However, the aerosolization of *M. tb* cells is considered to be dangerous process and must be performed under restricted facility at least in biosafety level 3 or P-3 room. As a consequence, the assessment of filtration efficiency and antimycobacterial activity using tubercle bacilli in fluid could be quite safer.

Conclusions

This study aimed at filtration efficiency and antimycobacterial activity evaluation of 3 different silver-containing PVA nanomembranes with *M. tuberculosis*, causative agent of tuberculosis. The results exhibited very high potential of the nanomembrane as antimycobacterial filter to eliminate *M. tuberculosis* in the environment.

Acknowledgements

PVA nanomembranes used in this study were supported by Dr. Varol Intasanta and Mr. Nakarin Subjalearndee, National Nanotechnology Center, Ministry of Science and Technology, Thailand. We would like to thank for their kind support. AC and CT are supported by Chalermprakiat Grant, Faculty of Medicine Siriraj Hospital, Mahidol University.

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