Methods Article

Progress in Microbes and Molecular Biology

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An Optimized Anti-adherence and Anti-biofilm Assay: Case Study of Zinc Oxide Nanoparticles versus MRSA Biofilm

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Abstract: Biofilms form protective layers over bacteria that are associated with a majority of the hospital infections contributing to antibiotic resistance development in susceptible strains. Nowadays, there is a pressing need for developing effective anti-biofilm agents to help address the growing problem of biofilm-producing bacteria associated with antibiotic resistance. In recent years, zinc oxide nanoparticles (ZnO-NPs) has emerged as a prospective candidate for new anti-biofilm agents. The present method paper described an optimized anti-adherence and anti-biofilm assay using ZnO-NPs. The antibiotic-resistant bacteria Methicillin-resistant *Staphylococcus aureus* (MRSA ATCC4330) and vancomycin were used as the growth control and positive control, respectively. The result showed concentration-dependent anti-adherence and anti-biofilm activity. The ZnO-NPs effectively prevented attachment of bacterial cells onto walls of wells with 51.69 ± 2.55% at the highest concentration tested (65.4 µg/mL). ZnO-NPs was also able to break-up 50% pre-formed MRSA biofilm at the lowest concentration of 13.5 µg/mL. Interestingly, ZnO-NPs at lower concentrations demonstrated significantly stronger anti-biofilm activity than that of the positive control vancomycin, demonstrating that ZnO-NPs is a promising anti-biofilm agent. This method could be used as a preliminary screening of transition metal oxide nanoparticles as potential anti-adherence and anti-biofilm agents followed by other specific anti-biofilm assays.

Keywords: Methicillin-resistant *Staphylococcus aureus*; MRSA; anti-biofilm; anti-adherence; Zinc oxide nanoparticles; ZnO-NPs

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Received: 3rd May 2020
Accepted: 3rd June 2020
Published Online: 12th June 2020

***Citation*:** Kemung HM, Tan LT-H, Khaw KY, *et al*. An Optimized Anti-adherence and Anti-biofilm Assay: Case Study of Zinc Oxide Nanoparticles versus MRSA Biofilm. Prog Microbes Mol Biol 2020; 3(1): a0000091. https://doi.org/10.3687/pmmb.a0000091.

Introduction

Over the course of history, nature through its subsidiary plants and microbes, has proven to be an essential player in driving development of future drugs by virtue of their potential in producing secondary metabolites with anti-bacterial, anti-cancer, anti-oxidant and neuroprotective activities[1–18]. Even to this day, nature continues to instill its significance in society as a prominent resource for future antibiotics in treating antibiotic-resistant infections[19]. Despite the use of current antibiotics, infectious diseases acquired either in hospitals or through consumption of foods contaminated by food-borne

pathogens[20–26] remain a major public health problem. Additionally, the unscrupulous practice of antibiotics for various ailments has encouraged antibiotic-susceptible infectious bacteria to form natural defenses against them. One such defense established by these bacteria is the biofilm[19].

Biofilm is a term that describes a community of microorganisms within a self-produced matrix of biopolymers attached on surfaces[27]. Microbes tend to produce biofilm on surfaces evading harmful effects of antibiotics as well as detergents and persists in hospitals causing many internalized hospital-related infections. It was estimated that biofilm contributes to approximately 60 to 80% of hospital infections[28–30]. Given that *Staphylococcus aureus* normal flora is the skin, suggests that it is among the most common causative agent in hospital-acquired infections associated with medical implants[31,32]. Moreover, it was shown that *S. aureus* was tolerant against higher doses of antibiotics and may thus contribute to development of antibiotic resistance in susceptible strains[32].

Recent years has seen a growing interest in the study of biofilm inhibitors acting as adjuvant agents in reducing biofilm layer of pathogenic bacteria[33]. This has led to the use of anti-biofilm assays to identify alternative sources as potential inhibitors of microbial biofilm. Previous studies have highlighted the antibacterial potential of transition metal oxides for crop protection[34] and disease eradication[35–37]. Nanoparticles especially those of metallic nature are one of the newest emerging systems which have great potential in inhibiting the formation of biofilms accredited to their high anti-microbial and anti-bacterial properties. The use of nanoparticles as anti-biofilm agents have found its way in many different sectors such as in healthcare (drug delivery, therapeutics and dentistry) or even in the food industry with a plethora of tailored applications[38,39].

The potency of these metallic nanoparticles in resisting the production of biofilm is high due to its nanoscale size and active participation in most of the stages in biofilm production. If the nanoparticles can successfully prevent adherence of microbes, then cycle of biofilm production is halted from the start. Sometimes, these nanoparticles disrupt the biofilm at the proliferation or even maturation stages, generally through the formation of radicals and reactive oxygen species (ROS) which affects gene expressions and breaks DNA strands[40]. In this context, the ZnO-NPs were chemically synthesized using a zinc nitrate precursor and subsequently characterized to confirm its identity. This includes conducting elemental analysis, Fourier-transform infrared spectroscopy and morphological analysis using electron microscopy. The ZnO-NPs synthesized as an anti-adherence and anti-biofilm agent have nanorice morphologies and have an average size of 250 nm.

The aim of this methodology article is to present step-by-step and optimized anti-adherence and anti-biofilm assays to evaluate the efficacy of ZnO-NPs as anti-adherence and anti-biofilm agents[41] (Figure 1). To validate the test method, Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 and vancomycin hydrochloride were used as the control bacteria and positive control, respectively. The experiment set-up consisted of a 96-well plate with a flat bottom, crystal violet as a staining agent and a 96-well microplate reader for quantification of both the anti-adherence and anti-biofilm activities. The result obtained indicate ZnO-NPs has anti-adherence and anti-biofilm properties against MRSA ATCC 4330. Given that crystal violet anti-adherence and anti-biofilm assay is an indirect measure of biofilm biomass, this study could be used as a preliminary screening to investigate the anti-biofilm properties of transition metal oxide nanoparticles prior to studying the mechanism of action of anti-adherence and anti-biofilm properties.

Method Details

Synthesis of ZnO-NPs

A weighted measurement of 1.90 g of zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) is first dissolved in 100 mL of ultrapure water under constant stirring. Subsequently, the pH of the mixture was adjusted to pH 10 using 1M of sodium hydroxide (NaOH) solution. Next, the solution is heated for 1 hour at 85°C under continuous stirring. The white suspension was then centrifuged for 5 minutes at 7000 rpm. Upon removing the supernatant, the residue is washed with distilled water and then subjected to another cycle of centrifugation before removing the supernatant again. The residue was then dried in an aerated oven at 60°C overnight, yielding a white powder.

Anti-adherence and Anti-biofilm Assay

Materials

- Biosafety level 2 cabinet, functional incubator, 96-wells microplate reader
- Sterile disposable consumables: 96-wells microtiter, 15 mL centrifuge tubes
- Bacterial cells (American type culture collection strain- ATCC)
- Bacteria nutrient-rich media. The use of Tryptic soy broth (TSB) which contains glucose and stimulates biofilm formation especially with Methicillin-resistant *Staphylococcus aureus*.
- Aqueous crystal violet (0.1% w/v)
- Glacial acetic acid (30% v/v)
- 1×Phosphate buffer saline
- Multichannel pipette (preferable)
- Vancomycin hydrochloride drug as the positive control
- Zinc oxide nanoparticles (ZnO-NPs) prepared in different test concentrations

Procedure

Bacterial culture preparation

Inoculate 3 to 5 pure colonies of MRSA ATCC43300 from

the culture plate into 15 mL TSB. Revive the bacteria in the shaker incubator at 200 rpm and at 37°C for 18 to 24 hours prior to the experiment so that they are preferably in their log phase of growth. Ensure sterile TSB is used by autoclaving TSB at 121°C for 15 minutes.

Anti-adherence Assay

1. Inoculate 50 µL of ZnO-NPs and vancomycin into designated wells at a series of concentration. (vehicle control e.g. DMSO is also needed to be aliquoted into appropriate wells if used as the diluent for the test substance)
2. Prepare a bacterial suspension (~1 x 10^8 CFU/mL equivalent to UV absorbance reading of 0.08 to 0.1 with wavelength at 600 nm) in 15 mL from a 24-hour bacterial culture. Alternatively, a 0.5 McFarland standard can be used to determine the optimal bacterial suspension. Make a 1:100 dilution in a separate centrifuge tube to obtain a 10^6 CFU/mL bacterial suspension.
3. Add 50 µL diluted bacterial concentration in respective wells using an appropriate multichannel pipette. Using a multichannel pipette is a faster and more efficient mean of adding the bacterial suspension into the wells.
4. Add sterile distilled water to the 4 corners of the microplate to prevent evaporation of water from the test wells. Evaporation of water in test wells can interfere with the results. Alternatively, the microplate can be kept in a container placed with moist filter paper during the incubation.
5. Cover the plate with the lid and place the plate in an incubator at 37°C for 18 to 24 hours.
6. Take the 96-well plate out from incubator and slowly remove the TSB either by decanting or pipetting. Rinse the plate thrice with sterile double distilled water and allow the plate to air dry under the biosafety cabinet. Turn the plates upside down to hasten the process of drying. Ensure it is dry before moving on to the next step.
7. Dispense 100 µL of aqueous crystal violet (1% w/v) into the test wells and let it stain the bacterial cell walls for 10 to 15 minutes. Decant the crystal violet either into a sink or onto clean disposable tissues.
8. Rinse the test wells three times with sterile double distilled water and allow the wells to dry under the biosafety cabinet. Alternatively, the plate can be bathed subsequently with 3 dishes of water.
9. Add 30% (v/v) glacial acid in water to solubilize crystal violet and leave it standing for 15 minutes. Ensure there is clear blue/violet solution with no visible residue in each of the test wells.
10. Read the UV absorbance of all the wells at 570 nm (suggested range would be between 570 to 600 nm)
11. Calculate the anti-adherence activity of test substance and vancomycin using the following formula:

$$\text{Anti-adherence activity\%} = \frac{\text{Absorbance of control-Absorbance of test sample}}{\text{Absorbance of control}} \times 100\%$$

Anti-biofilm Assay

1. Follow the steps stated in bacterial culture preparation and step 2 in anti-adherence assay to prepare 10^6 CFU/mL bacterial suspension. Inoculate 100 µL of the diluted bacterial suspension in TSB into respective well of a new 96 well microplate.
2. Incubate the plate at 37°C in an incubator for 24 hours.
3. Decant the TSB broth completely from the microplate, wash the well gently without disrupting the biomass formed attaching on the bottom and wall of the wells with sterile phosphate buffer saline (PBS) 3 times.
4. Add in 100 µL of freshly prepared sterile TSB broth (control well), ZnO-NPs suspended in TSB with test concentrations and TSB containing the vancomycin in test concentration.
5. Repeat the steps 5 to 10 from the above anti-adherence assay protocol.
6. Calculate the anti-biofilm activity of the test substance and vancomycin using the following formula:

$$\text{Antibiofilm activity\%} = \frac{\text{Absorbance of control-Absorbance of test sample}}{\text{Absorbance of control}} \times 100\%$$

Method Validation

Determination of Biofilm Formation

Based on the protocol, biofilm formation was indicated by the violet stains. This show that TSB media was adequate for biofilm formation whilst 0.1% (w/v) concentration of crystal violet was sufficient for visible observation with the naked eye and quantification by the spectrophotometer.

Assessment of Anti-adherence Assay

This protocol allows the determination of anti-adherence property of ZnO-NPs versus vancomycin using the 96-well plate. The biomass of the bacterial cell was quantitatively analyzed on the microplate reader at absorbance of 570 nm showing a decreasing trend of biomass attachment with increasing concentrations of ZnO-NPs tested. The result show that ZnO-NPs at 65 µg/mL achieved a significant anti-adherence activity of 51.69 ± 2.55%. However, vancomycin at 0.5 µg/mL did not exhibit significant anti-adherence activity when compared to negative control (TSB only) (Figure 2).

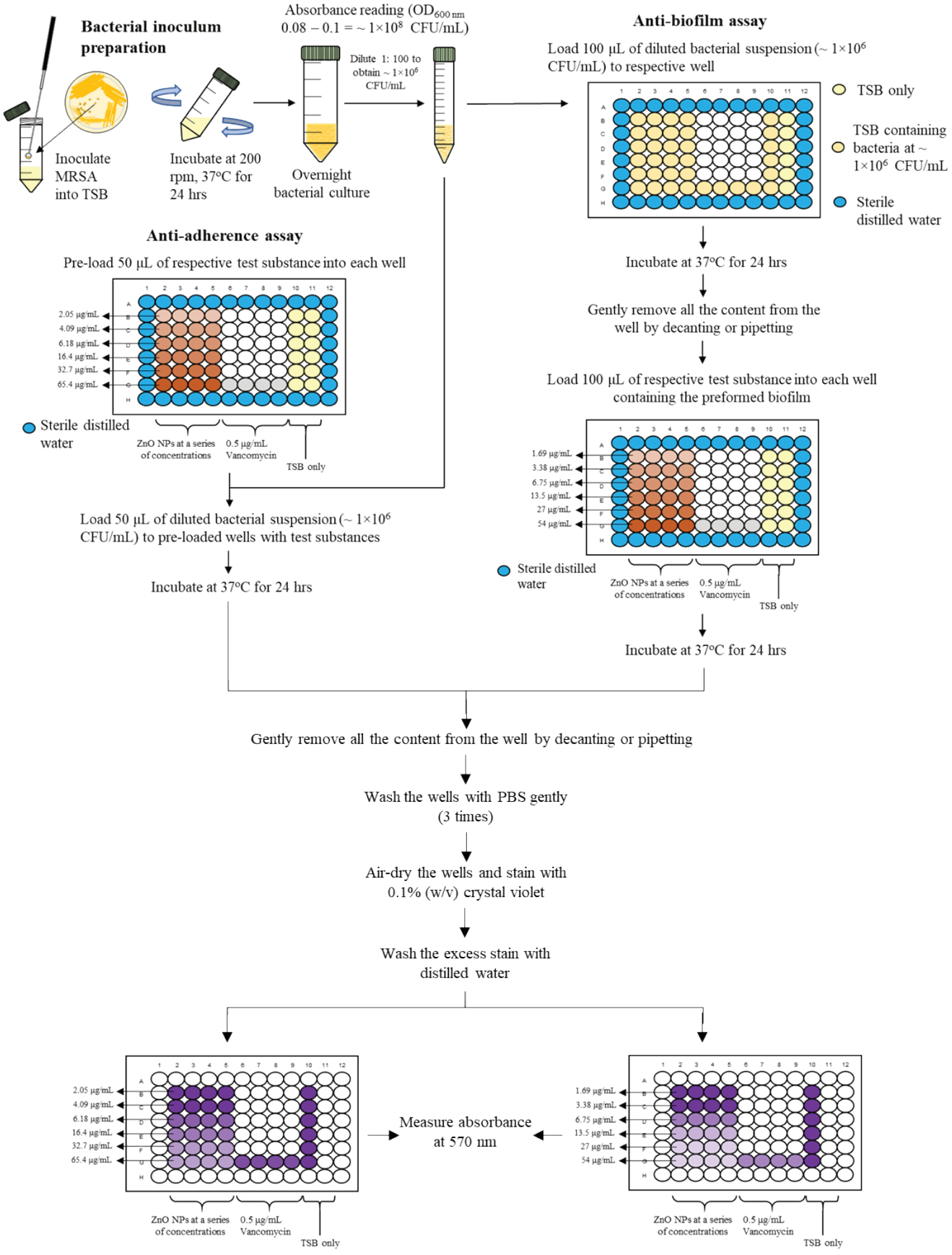

Figure 1. Schematic diagram shows the step-by-step protocol of the optimized ant-adherence and anti-biofilm assays. More detailed protocol should refer to the text.

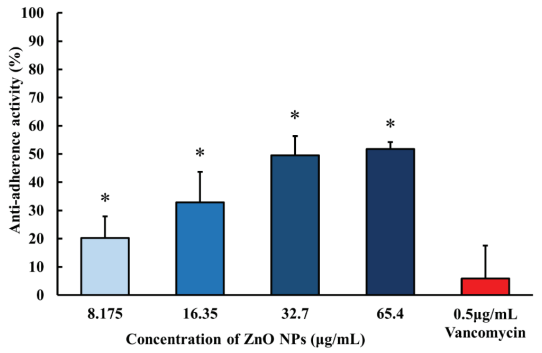

Figure 2. Anti-adherence assay using vancomycin as positive control and MRSA ATCC 43300 as growth control. Experiment evaluated based on quadruplicate results with standard deviation. (n = 4, $p < 0.05$). *indicates significant difference when compared to negative control (TSB only).

Assessment of Anti-biofilm Assay

This method allowed the determination of anti-biofilm property of ZnO-NPs versus vancomycin using the 96-well plate. The biomass of bacterial cell was quantitatively analyzed on the microplate reader at absorbance of 570 nm showing significant reduction of biofilm when increasing concentration of ZnO-NPs used when compared to the control (TSB only). The result shows that ZnO-NPs at 54 µg/mL exhibited significant anti-biofilm activity of 77.35 ± 2.67%. Meanwhile, the anti-biofilm activity of the positive control vancomycin was measured at 40 ± 8.39% at higher concentration of 100 µg/mL tested (Figure 3).

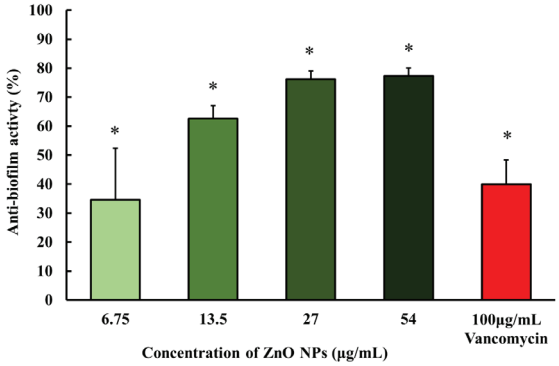

Figure 3. Anti-biofilm assay using vancomycin as positive control and MRSA ATCC 43300 as growth control. Experiment evaluated based on quadruplicate results with standard deviation. (n = 4, $p < 0.05$). * indicates significant difference when compared to negative control (TSB only).

Conclusion

Collectively, the present study shows step-by-step optimized protocol of anti-adherence and anti-biofilm assays which incorporate the crystal violet biofilm staining method of visualization and quantification of biofilm biomass in a 96-well microplate reader. The use of 96-well plates has allowed more samples that can be tested at any one time and is preferable to be carried out post-MIC assessment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

This work was inspired by Monash PhD Research Training Module which entitled "Bioprospective of microbes with biopharmaceutical potential with bioinformatics and drug discovery platforms" and financially by External Industry Grants from Biotek Abadi Sdn Bhd (vote no. GBA-81811A) and Monash Global Asia in the 21st Century (GA21) research grant (GA-HW-19-L-01 and GA-HW-19-S02) and Fundamental Research Grant Scheme (FRGS/1/2019/WAB09/MUSM/02/1 and FRGS/1/2019/SKK08/MUSM/02/7).

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