Bactericidal Capacity of a Heterogeneous TiO₂/ZnO Nanocomposite against Multidrug-Resistant and Non-Multidrug-Resistant Bacterial Strains Associated with Nosocomial Infections

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ABSTRACT: The surge of medical devices associated with nosocomial infection (NI) cases, especially by multidrug-resistant (MDR) bacterial strains, is one of the pressing issues of present health care systems. Metal oxide nanoparticles (MNP)s have become promising antibacterial agents against a wide range of bacterial strains. This work study is on the bactericidal capacity of heterogeneous TiO₂/ZnO nanocomposites with different weight percentages and concentrations against common MDR and non-MDR bacterial strains. The profiles on disk diffusion, minimum inhibitory concentration, minimum bactericidal concentration, tolerance determination, time-kill, and biofilm inhibition assay were determined after 24 h of direct contact with the nanocomposite samples. Findings from this work revealed that the heterogeneous TiO₂/ZnO nanocomposite with a 25T75Z weight ratio showed an optimal tolerance ratio against Gram-positive and -negative bacteria, indicating their bactericidal capacity. Further observation suggests that higher molar ratio of Zn²⁺ may possibly involve generation of active ion species that enhance bactericidal effect against Gram-positive bacterial strains, especially for the MDR strains. Nano-based technology using MNP.s may provide a promising solution for the prevention and control of NIs. Further work on biocompatibility and cytotoxicity profiles of this nanocomposite are needed.

INTRODUCTION

Nosocomial infections (NIs) contribute to current public health issues because of their potential to endanger the safety of patients, prolong hospital stays, and increase the treatment costs and result in high mortality and morbidity rates. This type of infection is acquired after 48 h of hospitalization or when a wound treatment lasts up to 30 days in a nursing home, long-term care facility, hospital, or haemodialysis clinic. About 90% of NIs are contributed by Gram-positive and -negative bacterial strains besides protozoans, fungi, viruses, and mycobacteria. Most common Gram-positive bacterial strains associated with NIs are Staphylococcus spp (Staphylococcus aureus), Enterococcus, and Streptococcus, whereas the Gram-negative pathogens are Escherichia coli (E. coli) and Klebsiella pneumoniae (K. pneumoniae).

Indwelling medical devices such as peripheral venous catheters, central venous catheters (CVCs), and urinary catheters pose a higher risk compared to other device-related NIs during a blood transfusion, nutrient, urine collection, medication transfer, and dialysis treatment. NIs become hard to tackle with antibiotics when the bacterial strain develops a biofilm, that is a group of structured bacterial community within the extracellular polymeric matrix permanently usually attached to inert medical appliance surfaces. Sahli et al. (2017) reported that K. pneumoniae (26.5%), S. aureus (23.5%), E. coli (2.9%), and others (46.8%) were among the causative microorganisms for NIs. Furthermore, catheter-associated S. aureus and E. coli infection is life-threatening and may result in septic thrombosis, meningitis, diarrhea, peripheral abscesses, endocarditis, urinary tract infections (UTIs), and death. A high percentage of NIs are also caused by highly multidrug-resistance (MDR) bacteria known as methicillin-resistance S. aureus (MRSA). It has been reported that among the S. aureus isolates, 36.3% were MRSA collected from biometric attendance devices at hospital setting. It can be spread among healthcare workers and...
transmitted to the patients through direct contact, open wounds, or contaminated hands.10 In China, MRSA accounts approximately 35–80% of total staphylococcal infection.13

Another opportunistic bacterial strain, \textit{K. pneumoniae}, is also frequently associated with UTIs, sepsis, or pneumonia, especially among immunocompromised individuals. Inadequate hygiene measures during the installation or maintenance of CVC promote cross-contamination of NI bacterial strains from medical devices to patients.14 Other factors, such as transmission through ward transfers and direct and indirect interaction among patients, visitors, and staff can also increase the risk of NI.14,15 In the last few decades, the top three CVC-NI bacterial strains have developed resistance to methicillin, vancomycin, linezolid, ampicillin, and carbencillin antibiotics.14–18 The inability to act against MDR bacterial strains is a worldwide health concern. Thus, alternative approaches, such as the application of metal oxide nanoparticles (MNPs) as new antibacterial agents against the bacterial strains associated with NIs have been studied.

MNPs exhibit effective action against a wide range of Gram-positive and -negative bacterial strains due to high photocatalytic activity, good biocompatibility, and non-toxicity.18–20 MNPs such as zinc oxide (ZnO/Z) and titanium dioxide (TiO\textsubscript{2}/T) present as excellent antibacterial agents because of their ability to over-accumulate reactive oxygen species (ROS) and metal ions to disrupt normal bacterial cell homeostasis.21–25 However, individual performance of MNPs are limited. Therefore, present technology on heterogeneous MNPs may improve their performance especially on band gap of TiO\textsubscript{2} and visible light absorption.24,25 In this study, the bactericidal and bacteriostatic effects of heterogeneous TiO\textsubscript{2}/ZnO nanocomposites were tested against \textit{S. aureus} ATCC 25923, MRSA ATCC 38591, \textit{E. coli} ATCC 25922, and \textit{K. pneumoniae} ATCC 700603. The antibacterial activities of heterogeneous TiO\textsubscript{2}/ZnO nanocomposites at various molar ratios were assessed quantitatively by the broth disk micro-dilution technique and time-kill assay in 96-well microplates and test tubes. Biofilm inhibition activity was analyzed using the crystal violet assay. Among the collection of selected strains, MDR bacterial strains (MRSA and \textit{K. pneumoniae}) received the greatest concern because they are intrinsically resistant to multiple drugs.

\section{MATERIALS AND METHODS}

\textbf{Synthesis and Characterization.} Heterogeneous TiO\textsubscript{2}/ZnO nanocomposite was synthesized and characterized as the protocol described previously.26 The heterogeneous TiO\textsubscript{2}/ZnO nanocomposites with different molar ratios; P2S (commercial TiO\textsubscript{2}), 100T, 100Z, 25T75Z, 50T50Z, and 75T25Z were tested.

\textbf{Bacterial Culture.} Four strains of bacteria were obtained from American Type Culture Collection (ATCC); \textit{S. aureus} ATCC 25923, MRSA ATCC 38591, \textit{E. coli} ATCC 25922, and \textit{K. pneumoniae} ATCC 700603. The bacteria were inoculated in 10 mL of LB broth overnight at 37 °C in a 150–200 rpm in a shaker-incubator. The strains were adjusted to 0.5 McFarland, with OD\textsubscript{625} nm (0.08–0.13) which is equivalent to 10\textsuperscript{8} CFU/mL.

\textbf{Kirby–Bauer Disk Diffusion Assay.} The disk-diffusion susceptibility test was performed according to the CLSI MO2-A11 (Clinical and Laboratory Standard Institute MO2-A11) guideline.27 In brief, bacterial culture with McFarland turbidity standard (0.5) was swabbed on the Luria–Bertani agar plates (Merck, Germany) using sterile cotton swabs. Sterile black disks were gently placed on the agar surface and each antibacterial agent with different concentration (100, 200, 500, 800, and 1000 μg/μL) were pipetted into the disks. Positive control and negative control were represented by the standard antibiotic and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich), respectively. Dishes were incubated at 37 °C for 24 h, and the inhibition zones was measured as the zone of inhibition (ZOI).

\textbf{Minimum Inhibitory Concentration and Minimum Bactericidal Concentration Determination.} The antibacterial activity of the heterogeneous TiO\textsubscript{2}/ZnO nanocomposite was defined by the broth dilution method on 96-well plates.28,29 Four bacterial suspensions; \textit{S. aureus}, MRSA, \textit{E. coli}, and \textit{K. pneumoniae} were collected after being cultured in LB broth. The suspension was adjusted to a 0.5 McFarland standard turbidity (1.5 × 10\textsuperscript{8} CFU/mL) of a prior experiment. Samples were diluted in two-fold concentration at the following concentration; 10; S; 2.5; 1.25; 0.625; 0.31; 0.16; 0.08; 0.04; and 0.02 mg/mL. Each well of plate was aliquoted with 50 μL of LB, 12th well (blank control) was added with 100 μL of LB, and 11th well (negative control) was added with LB and 10% DMSO. The dissolved samples (50 μL) with different concentrations were added into first wells till the 10th well. Later, the plates were incubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) absorbance reading at 530 nm wavelength were recorded before and after incubation. The MIC value was defined as the lowest concentrations of samples visually inhibited bacterial growth with clear suspension and no increment of absorbance readings after 24 h incubation. The minimum bactericidal concentration (MBC) of the heterogeneous TiO\textsubscript{2}/ZnO nanocomposites was measured after reading the MIC by aliquoted 100 μL of selected wells onto an agar plate.

\textbf{Tolerance Level.} The tolerance levels against the heterogeneous TiO\textsubscript{2}/ZnO nanocomposites were determined by using the stated formula

\[
\text{Tolerance} = \frac{\text{MBC}}{\text{MIC}}
\]

The bacterial and bacteriostatic capacity of the samples was determined by the tolerance level. It was categorized as a bacteriostatic agent if the ratio is ≥16 and as bactericidal agent, if the ratio ≤4.30

\textbf{Time-Kill Assay.} The antibacterial activity of the heterogeneous TiO\textsubscript{2}/ZnO nanocomposite against time was carried out using the time-kill assay.31,52 The adjusted bacterial suspension to 0.5 McFarland standard turbidity was used and diluted with sample solution with the final concentration of 10 mg/mL. The tubes were then incubated in a rotary shaker at 37 °C with 150–200 rpm agitation. A 100 μL aliquot of the treated bacterial suspension was pipetted from each tube at different incubation times (0.5, 3, 6, and 12 h) and swabbed on LB agar. Each plate was incubated at 37 °C for 24 h. The number of viable bacterial colonies were counted as log(CFU/mL). All tests were done in triplicate.

\textbf{Assessment of Biofilm Formation.} The biofilm mass of bacterial strains treated with the heterogeneous TiO\textsubscript{2}/ZnO nanocomposite was evaluated by the crystal violet staining assay as described by Mathur et al. (2006) with slight modifications.33 The bacterial strains were adjusted to a 0.5 McFarland standard turbidity prior experiment. Each well of a 96-well plate was aliquoted with 100 μL bacterial suspension and 100 μL of (1% glucose) and incubated at 37 °C for 24 h to
allow biofilm formation. The plate was washed several times with sterile distilled water and existing biofilms were incubated at 37 °C with samples (10 mg/mL) for additional 24 h. Later, the plate was washed gently with sterile distilled water and stained with 0.1% solution of crystal violet for 30 min. Then, the plate was washed again with distilled water and incubated at 37 °C for 15 min. Biofilm-bound CV was eluted with 95% ethanol for 15 min. 95% ethanol was used as the negative control (blank). Results were presented based on the OD reading value at 570 nm. To compensate for background absorbance, the OD reading value (570 nm) of the blank was deducted from the sample values. Biofilm production strength was classified based on the optical density of 0.120 for non-biofilm producer, 0.120–0.240 for moderate biofilm producer, and 0.240 for strong biofilm producers. To compensate for background absorbance, the OD reading value (570 nm) of the blank was deducted from the sample values.

Statistical Analysis. All data are expressed as the mean ± standard deviation (SD). Under the assumption of normal distributions and equal variances, two-way ANOVA and post-hoc analyses were applied to perform statistical comparisons between groups, and P values of less than 0.05 and less than 0.001 were considered statistically significant.
RESULTS AND DISCUSSION

Disk Diffusion and MIC/MBC Determination. The heterogeneous TiO$_2$/ZnO nanocomposite showed antibacterial activities against Gram-positive bacteria (S. aureus and MRSA) with molar ratios of 100Z, 25T75Z, and 50T50Z (Table 1, Figures 1 and 2). However, pure TiO$_2$ (100T) and 75T25Z did not show any antibacterial activities against Gram-positive and -negative bacterial strains. Results indicate that higher ZnO content played an essential role as an antibacterial agent. Furthermore, from MIC and MBC findings, MRSA has MIC values ranging from 0.08 to 2.5 mg/mL, indicating that it is more sensitive to each MNP (Table 2). Observation on MIC values for MRSA were lower than those of S. aureus possibly due to the smaller colony size of the former. In line with the present results, previous works revealed that the lowest MIC and MBC were 1.0 and 2.0 mg/mL for S. aureus and MRSA, whereas the highest MIC and MBC with ZnO treatment were 8–16 mg/mL. This result showed that the heterogeneous TiO$_2$/ZnO nanocomposites inhibit and kill bacterial strains at a low concentration (0.15–10.0 mg/mL).

Tolerance Determination. Findings on the tolerance level test are based on the MBC/MIC ratio which showed bacterial susceptibility or resistance of the bacteria to the heterogeneous TiO$_2$/ZnO nanocomposite. The tolerance level of a non-MDR strain against the heterogeneous TiO$_2$/ZnO nanocomposite was calculated and was less than 4. It was considered as a bactericidal agent which can kill the bacteria. Whereas, the tolerance level of MDR strain against the heterogeneous TiO$_2$/ZnO nanocomposite was much lower but still less than 4. In our study, the heterogeneous TiO$_2$/ZnO nanocomposite (25T75Z) exerted a good antibacterial agent against both non-MDR and MDR strains.

Time-Kill Assay with Heterogeneous TiO$_2$/ZnO Nanocomposites. This assay is chosen to evaluate bacterial growth and death and to observe the antibacterial effects with time. The highest concentration of each nanocomposite (100Z, 25T75Z, and 50T50Z) was chosen based on the MBC results to evaluate the effects of heterogeneous TiO$_2$/ZnO nanocomposites in different treatment periods. It is considered as a bactericidal agent if the antibacterial activity is $\leq 3$ log$_{10}$ in the CFU/mL. As shown in Figure 3, the bacterial count data revealed that bactericidal activity for both 100Z and 25T75Z cause a significant reduction with $\leq 3$ log$_{10}$ for Gram-positive strain. The nanocomposites showed a reduction in the viable count from 4.3 log$_{10}$ to 3 log$_{10}$ after 12 h of incubation for S. aureus and 6 h for MRSA. In addition, bactericidal endpoints for MRSA treated with nanocomposites was reached after 12 h of incubation as compared to S. aureus, which needed longer...
time to be killed completely. Whereas, the 100Z and 25T75Z samples potentially inhibit and reduce E. coli and K. pneumoniae colony count at the range of less than 4.0 log_{10} after being treated for 12 h. The untreated bacterial strains showed no reduction in colony counts even after 12 h incubation periods.

From the obtained results, the heterogeneous TiO_{2}/ZnO nanocomposites enhance the antibacterial action against non-
MDR and MDR Gram-positive bacterial strain. Both samples of 100Z and 25T75Z had much similar bactericidal effect against Gram-positive bacterial growth in a shorter time compared to that for Gram-negative strains. The difference in antibacterial activity between both strains may attribute to the structure of their different cell walls and electrostatic attraction between negatively charged bacterial cells and positively charged Zn$^{2+}$.

Inhibition of Biofilm Formation. According to the results of MIC, MBC, tolerance level, and time-kill assay, the three selected samples effectively killed NI strains after 24 h of treatment. Biofilm biomass grown for 24 h before being treated with samples was subsequently evaluated using the standard crystal violet assay to determine biofilm inhibition activities. Ghasemian et al. (2016) proved that S. aureus and MRSA are potentially effective biofilm producers based on the microtiter tissue plate assay.\textsuperscript{42} The results shown in Figure 4 indicated that the selected samples were more effective in eradicating preformed Gram-positive biofilm than Gram-negative biofilm due to the differences in cell membrane organization, peptidoglycan, and the absence of an outer membrane. In general, Gram-positive bacterial strains have a thicker, negatively charged peptidoglycan layer (30 mm thickness) compared to that in Gram-negative bacterial strains (approximately 3–4 mm).\textsuperscript{39} However, they naturally lacked the outer membrane layer, known as lipopolysaccharide, that serves as a permeability barrier against deleterious molecules.\textsuperscript{40} This structure also allows Gram-negative strains to induce endotoxins associated with septicemia; hence, they require a longer time to be killed compared to Gram-positive strains.\textsuperscript{41}

In this study, influence of heterogeneous TiO$_2$/ZnO nanocomposites on biofilms formed on well plates was investigated after 24 h. Biofilm-forming potential of bacterial strains increases the bacterial resistance in NIs and makes them hard to eliminate.\textsuperscript{42} From the results obtained, biofilm formation was reduced in the presence of TiO$_2$ and ZnO MNPs against Gram-positive and -negative bacterial strains. Numerous studies have documented the ability of ZnO and TiO$_2$ alone or in combination with other MNPs as antibacterial agents against selected strains (S. aureus, E. coli, and K. pneumonia).\textsuperscript{25,43,44} Jesline et al. (2015) evaluated the antibacterial activity of ZnO and TiO$_2$ against biofilm-producing MRSA and showed the potential of both MNPs without any combination in inhibiting bacterial growth through the well-diffusion method.\textsuperscript{45}

Furthermore, there are two possible mechanisms that may explain this observation on the bacteriostatic/bactericidal effect: (1) the collision between the cell membrane and Zn$^{2+}$ and (2) the overaccumulation of ROS, such as hydroxyl radicals, superoxide, and hydrogen ions, which led to homeostasis imbalance and cellular membrane destruction.\textsuperscript{26} Dilution of nanocomposites with LB medium cause oxidative stress and the generation of superoxide radicals and hydrogen peroxide.\textsuperscript{25} Low crystallinity and voids may influence the water uptake and impede the release of ROS.

Present findings suggest that the 25T75Z nanocomposite had a better molar ratio combination as it inhibits and kills both Gram-positive and -negative bacteria. The efficient photocatalysis and charge separation between ZnO and c-Zn$_2$Ti$_3$O$_8$ enhanced the transportation of electron from the conduction band and h$^+$ from the valence band, leading to the production of active ROS species, such as ·OH, HO$_2^-$, H$_2$O$_2$, and O$_2^-$.\textsuperscript{45} Additionally, the negatively-charged elements found on bacterial cell wall components, such as teichoic acids and lipopolysaccharides, attract the H$_2$O$_2$ released from the heterogeneous TiO$_2$/ZnO nanocomposite by the concept of electrostatic interaction. It slowly diffuses into the inner cell membrane and caused common changes such as blister formation, clumping of the membrane, and blockage of electron-transport chain, eventually leading to cell death due to leakage of minerals and proteins.\textsuperscript{16,44} Further observation supports that 100T and 75T25Z displayed no antibacterial effects, thus confirming the previous findings that Zn$^{2+}$ is the other element that induces antibacterial activity.

**CONCLUSIONS**

Finding from this study explored the potential of the heterogeneous TiO$_2$/ZnO nanocomposites as bactericidal agents against non-MDR and MDR agents. The antibacterial activity of these nanocomposites was quantified based on several assays including disc diffusion, MIC, MBC, tolerance determination, time-kill, and inhibition of biofilm formation. The results highlight better bactericidal activity of 25T75Z compared to that of 50T50Z. In addition, it also demonstrates the role of Zn$^{2+}$ as an active species and generation of ROS to enhance bactericidal effect against Gram-positive bacterial strains. However, further work is needed to understand the detailed biocompatibility and cytotoxicity effects of heterogeneous TiO$_2$/ZnO nanocomposites embedded in synthetic polymers prior to their application in medical and healthcare industries.

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Author Contributions
S.S., K.A.S., and N.B. carried out the sample preparation and characterization. F.A. provided bacterial samples for this experiment. N.H.H. carried out the antibacterial assays, including bacterial preparation, disc diffusion, MIC, MBC, time kill-assay, and biofilm inhibition tests. W.N.W.M.Z. assisted in the experimental procedure. A.S. funded this experimental work. R.B.S.M.N.M. contributed in the experimental design, writing process, and gave final approval of this paper for publication. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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