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Study to elucidate effect of titanium dioxide nanoparticles on bacterial membrane potential and membrane permeability

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Abstract
The interaction of metal oxide nanoparticles (NPs) with cells and lipid bilayers is precarious in various fields such as antibacterial and drug or gene delivery. These require a strong control over NPs–cell interactions, an understanding of how the NPs surface impact their interaction with lipid bilayers and cells. Therefore, to elucidate Titanium dioxide (TiO2) NPs of size 8–10 nm and 90–100 nm and their interaction with lipid bilayer of Escherichia coli and Staphylococcus aureus, we studied membrane potential, membrane permeability. Results of the traditional method of checking antibacterial activity - minimum inhibitory concentration (MIC) was co-related with change in membrane potential and membrane permeability. TiO2 NPs 8–10 nm have profound action on depolarization of membrane potential of E. coli cells, while of S. aureus were not affected. TiO2 NPs 90–100 nm have very less effect on membrane potential and permeability of both organisms. It is observed that there exists a strong co-relation between antibacterial activity of the TiO2 NPs and change in the membrane potential and membrane permeability. These observations are also supported by membrane leakage test by estimation of protein, deoxyribonucleic acid (DNA) and potassium ion (K+) ion content.

1. Introduction
With the development of nanotechnology, there has been a tremendous growth in the application of NPs for drug delivery systems, antibacterial materials, cosmetics, sunscreens, and electronics [1, 2]. The interaction of metal oxide nanomaterial with cells and lipid bilayers is critical in many applications such as antibacterial and drug/gene delivery. These require a strong control over NPs–cell interactions. Most metal oxide NPs including TiO2 NPs are known to possess strong antibacterial properties [3]. Antibacterial activity of NPs is studied by traditional method- MIC determination. The bactericidal effect of TiO2 generally has been attributed to the decomposition of bacterial outer membranes by reactive oxygen species (ROS), primarily hydroxyl radicals (OH), which leads to phospholipid peroxidation and ultimately cell death [3]. This traditional method (MIC), though effective, is time consuming. The aim of this study was to assess the co-relation between antibacterial properties TiO2 NPs against E. coli and S. aureus and corresponding changes in membrane potential and membrane permeability. Bacteria normally maintain an electrical potential gradient (membrane potential, Δψ) of over 100 mV across the cytoplasmic membrane, with the interior side negative [4]. Membrane potential (MP) plays a key role in cell physiological processes and could therefore serve as a better parameter for physiological activity since it is part of the generation of proton motive force and is closely involved in adenosine triphosphate (ATP) generation, substance transport, bacterial chemotaxis and survival at low pH [5]. However bacteria have no mitochondria, MP also reflects membrane integrity and energy status as well as viability of the cells [6]. Bacterial membrane also acting as a permeability barrier for most molecules and serving as the location for the transport of molecules into the cell. While impermeability of the membrane to certain classes of fluorescent
dyes, on the other hand, have been used as the main indicators of the physiologic state of the cytoplasm membrane and the viability of the organism. Hence, membrane potential and membrane permeability are indicator of viability of bacterial cells and for this reason we decided to explore the possibility of using measurement of membrane potential and membrane permeability as indicator of antibacterial activity of TiO₂ NPs.

2. Material and methods

2.1. TiO₂ NPs

TiO₂ NPs with size 8–10 nm synthesized by hydrothermal method (at Fergusson college, Pune, India) These NPs were characterized by x-ray diffraction (XRD) analysis, ultraviolet (UV)-visible spectroscopy, field emission scanning electron microscopy (FE-SEM) and Transmission Electron Microscope (TEM) & High resolution transmission electron microscope (HRTEM). Nanoparticle size was calculated by Debye–Scherrer’s equation

\[ D = \frac{0.9\lambda}{\beta \cos \theta}, \]

where, \( D \) is the average nanoparticle size obtained in nm, \( \lambda \) is the wavelength of the x-ray radiation (\( \lambda = 0.15406 \) nm), \( k \) is Scherrer’s constant (\( k = 0.89 \)), and \( \beta \) is the line width at the half maximum height. The average nanoparticle size obtained is 8–10 nm [7].

While 90–100 nm size TiO₂ NPs were procured from Sigma Aldrich (Product Code: 677469). Both NPs were suspended in sterile saline and were sonicated for uniform mixing. These were used for the following experiments.

2.2. Bacterial culture conditions

Escherichia coli NCIM 2065 type strain, and Staphylococcus aureus ATCC 6538 were used in all experiments. Cultures were maintained on nutrient agar (NA) slants. From this, a single pure colony was inoculated into 10 ml of nutrient broth (NB) (Hi-media, Mumbai, India) and incubated overnight at 37 °C in shaker incubator. Cells had reached an optical density (OD) at 600 nm of approximately 0.1 with a cell density of 10⁶ CFU/ml.

2.3. Antibacterial test

Different concentrations TiO₂ NPs of both 8–10 nm and 90–100 nm size NPs (10, 20, 30, 40, 50, 60, 70, 80, 90, 100 μg ml⁻¹) were prepared in 10 ml of NB. A loopfull culture of 0.1 OD of E. coli and S. aureus were inoculated into each concentration and incubated at 37 °C in shaker incubator for 24 h. Control was without NPs. Absorbance was measured at 600 nm. The OD of the tests and control was used to calculate the percentage of cell viability as follows: percentage of cell viability = \( \frac{A_{\text{treatment}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\% \) (where, A = absorbance).

2.4. Cytoplasm leakage

Cytoplasma leakage analysis was conducted for protein, DNA and K⁺ ions leakage. 0.1 OD culture of E. coli and S. aureus were added to 50 ml of sterile NB and incubated at 37 °C for 24 h. The broth was then centrifuged at 5000 rpm for 10 min Supernatant was discarded and cell pellet was collected then cells were resuspended into 50 ml of saline solution with 100 μg ml⁻¹ of TiO₂ NPs (of both 8–10 nm and 90–100 nm size) as test sample and cells without NPs in 50 ml saline were kept as control and both incubated overnight at 37 °C. It was then centrifuged at 5000 rpm for 10 min. The supernatant was collected for performing Bradford’s assay for protein content [8], DNA estimation by diphenyl amine (DPA) method [9] and K⁺ content by flame photometry [10].

2.5. Measurement of membrane potential by flowcytometry and confocal microscopy

Bacterial growth was grown in NB. Healthy bacteria were obtained from log-phase cultures. Bacterial culture was diluted to approximately 1 × 10⁶ cells per ml in filtered phosphate buffered saline (PBS). Similar dilution of bacterial cells was done for NP treatment, where both the bacteria were treated with 100 two different size of TiO₂ NPs separately and incubated at 37 °C overnight. 1 ml of cell suspension was taken into 3 tubes for a test, depolarized control and an unstained control each. 10 μl of 500 μM CCCP (Component B) was added to the depolarized control sample. 10 μl of 3 mM DiOC₂(3) (Component A) was added to each flow cytometric tube and mixed well. Samples were incubated at room temperature for 15–30 min. Stained samples were analyzed after incubation by flowcytometry equipped with a laser emitting at 488 nm. Samples were analyzed on a flow cytometer with 488 nm excitation using emission filters appropriate for DiOC₂(3) dye. Gate on the cells, excluding debris. Using the CCCP-treated sample, perform standard compensation. Forward scatter (FS) and side scatter (SS) was measured to identify single cells. Pulse processing is used to exclude cell doublets from the analysis.

For confocal microscopy analysis a smear was prepared on the grease free slide of above mentioned stained samples including controls, then cover slip was placed on the slide and the samples were examined with a
Confocal microscope (NIKON A1 R) using a 100X oil objective. 3D Confocal stacks were acquired. 488 laser line was selected.

2.6. Measurement of membrane permeability by flowcytometry

Bacterial culture was grown in NB. Healthy bacteria were obtained from log-phase cultures. Bacterial culture was diluted to approximately $1 \times 10^6$ cells per ml in filtered PBS. 1 ml of cell suspension was taken into two tubes out of these, one tube was kept as potassium iodide (PI) stained control and anther an unstained control. Similar dilution of bacterial cells was done for TiO2NPs treatment, where both the bacteria were treated with two different size NPs separately and incubated at 37°C overnight. For staining with PI in all the tubes 5 mM solution of PI were added and incubated for 10 min at room temperature in dark before flow cytometry measurements. Samples were analyzed on a flow cytometer with 488 nm excitation using emission filters appropriate for PI dye. Unstained samples were used to set voltage in flow cytometer. Then, Stained samples were analyzed after incubation by flowcytometry equipped with a laser emitting at 488 nm. Gate were set on the cells, excluding debris. Forward scatter (FS) and side scatter (SS) were measured to identify single cells. Pulse processing is used to exclude cell doublets from the analysis. Data obtained from acquisition software of equipment was analyzed by DeNovo FCS express analyzer.

3. Results

3.1. Antibacterial activity of TiO2 NPs E. coli and S. aureus

Antibacterial assay of TiO2 NPs was studied by using broth dilution method. (figure 1). When the cell of E. coli and S. aureus were treated separately with increasing concentrations of each size of TiO2 NPs, the viable number of cells decreased linearly. The percent decrease in viability of E. coli and S. aureus in presence of TiO2 NPs of 8–10 nm was greater than TiO2 NPs of 90–100 nm. As can be seen from figure 1, more than 50% reduction in viabilities of E. coli and S. aureus were observed at 10 μg ml$^{-1}$ of TiO2 NPs of 8–10 nm, whereas more than 50 μg ml$^{-1}$ of TiO2 NPs of 90–100 nm were required to bring similar effects on E. coli and S. aureus. Interestingly, TiO2 NPs were more effective on cells of E. coli than on the cells of S. aureus.

3.2. Cytoplasm leakage test

To confirm lysis of E. coli and S. aureus cells due to NPs, cytoplasm leakage was checked by measuring protein, DNA and K$^+$ content (figure 2) of supernatant of treated cells. In presence of TiO2 NPs of 8–10 nm, the protein leakage, from E. coli and S. aureus was 0.065 and 0.05 mg ml$^{-1}$ respectively, whereas in presence of TiO2 NPs of 90–100 nm, the protein leakage was 0.05 and 0.03 mg ml$^{-1}$ respectively. Interestingly, the protein leakage from E. coli and S. aureus was more in presence of TiO2 NPs of 8–10 nm. The cytoplasmic leakage for DNA was studied by measuring the content of DNA by spectrophotometer. In presence of either type of TiO2 NPs, there was a leakage of cellular DNA (figure 2). The DNA leakage from the cells of E. coli was more prominent than from the cells of S. aureus. A more conclusive study on the cytoplasmic leakage was performed by measuring leakage of K$^+$. When cells of E. coli and S. aureus were treated with TiO2 NPs, leakage of K$^+$ were observed.
3. The leakage of K$^+$ was prominent in *E. coli*, which was, respectively, 0.04 and 0.01 mM, in presence of TiO$_2$ NPs of 8–10 nm and TiO$_2$ NPs of 90–100 nm.

3.3. Measurement of membrane potential

Membrane potential is measured by two different methods. In confocal microscopy, *E. coli* and *S. aureus* cells were treated with different sizes of TiO$_2$ NPs. Normal *E. coli* cells and *S. aureus* cells (stained with DiOC$_2$(3) dye used as positive control) appear red because of higher membrane potential (figures 3(A) and (E)). CCCP treated *E. coli* cells and *S. aureus* cells (used as negative control) appered green stained with DiOC$_2$(3) dye (figures 3(B) and (F)) because of less membrane potential. There was different effect of different size TiO$_2$ NPs on membrane potential of *E. coli*. Cells treated with 8–10 nm size TiO$_2$ NPs appered green compared to control (figure 3(C)) and cells treated with 90–100 nm size TiO$_2$ NPs appered red compared to control (figure 3(D)). While in *S. aureus* cells treated with different size TiO$_2$ NPs appeared red as same as positive control (figures 3(G) and (H)). The presence of membrane potential in *E. coli* and *S. aureus* cells treated with different sizes of TiO$_2$ NPs stained using DiOC$_2$(3) was also analyzed by scatter plots of green versus red fluorescence. CCCP-treated cells were used to generate ‘depolarized’ control populations. Subsequently, gates were drawn based on these controls with subpopulations present in ‘polarized’ or ‘depolarized’ regions and expressed as a percentage of the total profile shown in figures 4 and 5. The results were similar to Confocal microscopy analysis and red: green ratio has been shown to vary with the intensity of the proton gradient of *E. coli* and *S. aureus* cells treated with different sizes of
TiO$_2$ NPs. Depolarization of membrane was observed in presence of TiO$_2$ NPs of size 8–10 nm and no such depolarization is observed in *S. aureus* in either sized NPs.

3.4. Measurement of membrane permeability

Membrane permeability study of *E. coli* and *S. aureus* cells treated with 8–10 nm and 90–100 nm sizes of TiO$_2$ NPs shows that there are intermediate population of cells in both organisms, intermediate population of cells is the population of the cells which is dead but not reacted to PI dye. In figure 6, untreated and unstained cells of *E. coli* and *S. aureus* are represented by A and D respectively. Figures 6(B) and (C) represent *E. coli* cells treated with 8–10 nm and 90–100 nm size TiO$_2$ NPs respectively. Here population of intermediate cells is more in 90–100 nm size TiO$_2$ treated *E. coli* cells as compared to 8–10 nm size TiO$_2$ treated *E. coli* cells. In case of *S. aureus* population intermediate cells is similar in both sizes of NPs (figures 6(E) and (F)).
4. Discussion

Antibacterial properties of NPs and its co-relation with lipid bilayer membrane interaction with respect to changes in membrane potential and membrane permeability have not yet reported. Sara Akhtar et al recently reported that TiO$_2$-nano-colloids of size 8 nm showed antibacterial and antiviral activity at very low concentration and the possible mechanism of action include damage to the lipids found in viral envelop [11]. Therefore, we started to answer the question by investigating the antibacterial property to TiO$_2$ NPs viz., TiO$_2$ NPs 8–10 nm and TiO$_2$ NPs 90–100 nm on $E$. coli and $S$. aureus. We started with traditional broth dilution method and observed a reduction in the viability of $E$. coli and $S$. aureus in presence of TiO$_2$ NPs of both size. Two interestingly features were observed in present study. TiO$_2$ NPs 8–10 nm had superior antibacterial activity than TiO$_2$ NPs 90–100 nm and TiO$_2$ NPs 8–10 nm had superior antibacterial activity on $E$. coli over $S$. aureus. The size-dependent antibacterial activity of NPs is an interesting phenomenon wherein antibacterial potential of NPs increases with decrease in the size [3, 12]. Recently, palladium NPs of three different sizes, viz., 2.0, 2.5 and 3.1 nm were tested on cells of $E$. coli and $S$. aureus and it was observed that NPs of size 2.0 nm showed far better antibacterial activity over NPs of sizes, 2.5 and 3.1 [13]. Apart from size dependent antibacterial activity, TiO$_2$ NPs also showed organism specific antibacterial property. This is interesting because it implies that the interaction of NPs with cells depends on the physiological features of cell walls, particularly, $E$. coli possess a thin layer of peptidoglycan, the cell wall’s backbone, offering strength to the cell wall and surrounded by a layer of lipopolysaccharide. Unlike $E$. coli, $S$. aureus possesses a thick layer of peptidoglycan. In comparison, therefore, it appears that the thick layer of peptidoglycan offers a resistance to NPs and thus, $S$. aureus being less susceptible to NPs action [3, 12]. Recently, copper oxide and silver NPs were tested on $E$. coli & $S$. aureus, and the minimum inhibitory and microbicidal cells concentration for $E$. coli was found to be less than $S$. aureus [3, 12].

After establishing antibacterial properties of TiO$_2$, we started to establish co-relation between changes in membrane potential and membrane permeability. Membrane damage in $E$. coli and $S$. aureus cells in presence of TiO$_2$ NPs may occur due to two reasons (i) depolarization of active membrane potentials (ii) change in the membrane permeability. There has been a close correlation between membrane permeability and active membrane potentials of a cells. It has been documented that the membrane permeability in cells occur due to depolarization of active membrane polarization [14, 15]. Therefore, we decided to study the active membrane potential of $E$. coli and $S$. aureus in presence of TiO$_2$ NPs. Charged molecules such as that are sufficiently lipophilic to pass readily through the lipid bilayer portion of the membrane partition across the membrane in response to the potential gradient. Positively charged lipophilic dyes, such as DiOC$_2$(3), are concentrated inside

Figure 6. Membrane permeability study by Flow-cytometer. Populations of $E$. coli and $S$. aureus after subjecting to TiO$_2$ NPs and stained with propidium iodide (PI). A and D are untreated and unstained cells of $E$. coli and $S$. aureus respectively. B and C represent intermediate population of $E$. coli cells treated with 8–10 nm and 90–100 nm sizes of NPs respectively. These represent dead cells, but PI dye is not reacted. As seen in figure B and C, percent of intermediate cells is more in 90–100 nm size TiO$_2$ treated $E$. coli cells compared to 8–10 nm size TiO$_2$ treated $E$. coli cells. While in case of $S$. aureus, percent of intermediate cells are similar in both sizes treated $S$. aureus cells (E: 8–10 nm size TiO$_2$ treated, F: 90–100nm size TiO$_2$ treated $S$. aureus cells).
cells that maintain $\Psi$ and appears red. If the $\Psi$ across the membrane is compromised, e.g., in presence of CCCP (strong proton gradient inhibitor) cells appear green which is supported by confocal microscopy (figure 3).

A quantitative and qualitative measurement of the depolarized populations of cells was performed by high throughput technique like flow cytometry. In flow cytometer, control population of E. coli showed low values of permeability and relatively high values of $\Psi$, appearing a red colored population. However, in presence of TiO$_2$ NPs, population of E. coli have lost $\Psi$ and appeared green colored populations. On contrary, the $\Psi$ in S. aureus remained unaffected, indicating size dependent antibacterial mechanism of TiO$_2$ NPs. In an interesting study, the effect of functionalized polystyrene NPs of varied sizes were investigated on $\Psi$ of CHO and HeLa cells, and it was found that the amine-modified surfaces lead to significant depolarization of $\Psi$ of both cell types, whereas carboxylate-modified NPs do not cause depolarization [14], an observation similar to our study but those were animal cells. As hypothesized previously, cell lysis may be attributed to murein hydrolyses enzyme. It has been reported that in a depolarized cell, the murein hydrolyses are activated and the intact peptidoglycan is degraded, compromising the structural integrity of the cell wall and resulting in cell lysis [15]. The respiring cell produces a proton gradient outside of the cytoplasmic membrane that causes a localized reduction in the pH within the cell wall. This acidic pH suppresses the activity of murein hydrolydases associated with the teichoic acids and lipoteichoic acids by promoting the protonation of the D-Ala ester linkages. Upon dissipation of the membrane potential, the pH in the cell wall increases and destabilizes the D-Ala ester linkages, thus depressing the murein hydrolases [16]. Hence, there is a co-relation between cell death by murein hydrolysates and depolarization of membrane potential.

As mentioned earlier, membrane damage may also be attributed to change in membrane permeability. Hence, we started to investigate the permeability of membrane by flow-cytometry. Propidium iodide (PI) solution is a bacterial fluorescence staining dye and can be applied for microbial cell viability assay in different principles. It is an ethidium bromide analog that emits red fluorescence upon intercalation with double-stranded DNA. Though PI does not permeate viable cell membranes, it passes through injured cell membranes and stains the nuclei. It’s a tool for staining of viability and membrane injury. Measurement of membrane permeability of E. coli cells treated with TiO$_2$ NPs by flow cytometry showed decreased in membrane permeability in cells but presence of intermediate population of cells (cells showing combined fluorescent population) are due to the unavailability of DNA inside dead cells due to cytoplasm leakage of DNA (figure 2) but intermediate population is also because PI also has affinity to RNA but gives weak response also indicates cell death. Recently, silver NPs were tested on E. coli, and it was observed that bacterial plasma membrane was the main target of silver NPs. Membrane depolarization was reason to induce permeability and membrane leakage [17].

Recently bacterial cell lysis reported due to depolarized membrane potential by peptidoglycan recognition proteins (PGRPs) - Peptidoglycan interaction activates bacterial two-component systems in Gram-positive and Gram-negative bacteria, respectively that induce bacterial lysis by membrane depolarization and the simultaneous induction of oxidative, thiol, and metal stresses, which produce bacterial killing [18, 19]. These finding and correlation support current results about membrane depolarization of small sized treated cells and lysis of E. coli cells. Our study clearly indicates that there exists a strong co-relation between death of bacterial cells and depolarization of membrane potential due to TiO$_2$ NPs. Cytoplasm leakage (protein, DNA and K$^+$ analysis) also support the observation that there is a co-relation between antimicrobial activity of TiO$_2$ NPs and changes in membrane potential and membrane permeability. In summary, it is observed that whenever there is inhibition of bacterial cell due to interaction TiO$_2$ NPs, there exists two co-relations (1) reduction in membrane potential and (2) increase in membrane permeability and impact of inhibition of cells is proportional to decrease in membrane potential and increase in membrane permeability.

5. Conclusions

The present study explored the nanoparticle–cell interactions of two different size TiO$_2$ NPs 8–10 nm and TiO$_2$ NPs 90–100 nm on *Escherichia coli* and *Staphylococcus aureus* by using traditional methods of antibacterial assays and modern fluorescent-based analytical techniques and formulated a hypothesis that there exists a co-relation between antibacterial activity of TiO$_2$ NPs and membrane potential and membrane permeability of bacterial cells (this hypothesis is also supported by cytoplasm leakage test) which is found to be size and species dependent. Membrane permeability and membrane potential studies have shown that, TiO$_2$ NPs 8–10 nm have profound action on *E. coli* cells while *S. aureus* cells were not affected. TiO$_2$ NPs 90–100 nm have very less effect on both organisms.
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Conflict of interest

The authors declare that there is no conflict of interest.

Ethical statement

We have not used any materials that require ethical clearance.

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