CHAPTER 4

Microbial Toxicology of Titanium Dioxide Nanoparticles

4.1 Background

Nano-titania is widely used nanomaterials in food and is known for its efficient antimicrobial activity but negligible studies are available on in-depth analysis to identify the microbial toxicity of nano-titania. For proper and safe use of nano-titania in food and drug as well as the efficient use of nano-titania against ecotoxicology, it is very urgent to identify the mechanism of microbial toxicology.

Recent years have witnessed the impact of nanotechnology in the field of various consumer goods. Due to their unique physicochemical properties, nanoparticles are currently the focus of intensive research (Ranjan et al., 2014). Titanium dioxide has been extensively used both in scientific research and industrial application. The chemical properties present in bulk titania such as photocatalytic activity, hydrophilicity, stability and good economic yield makes it useful for a variety of applications (Zimbone et al., 2015). These characteristics promote its use in diverse areas ranging from pharmaceuticals, cosmetics industry, anti-bacterial agents, water treatment, food and agriculture, imaging techniques, sensors, targeted drug delivery and many other fields.

The mechanism of antibacterial activity of nano-titania depends on upon its photocatalytic reaction. The reaction energy of OH, O\textsuperscript{2-}, and HO\textsubscript{2} formed during the process is higher than many organic bond energies, such as C-C, C-H, C-N, C-O, and H-O (Qiu et al., 2015). The intended end-use greatly depends on titanium dioxide nanoparticle structure and synthesis protocol. Despite their increasing application in a number of consumer goods, a detailed knowledge of their oral uptake, chemical fate, and toxicity is still lacking. Concerns have been raised that titanium dioxide nanoparticles challenge human health. It has also been classified as Group 2B carcinogen (possibly carcinogenic to humans) (Shi et al., 2013). Understanding the detailed mechanism of action is vital for its safer use.
On the other hand, TNPs are also well-known for its antimicrobial properties. Conversely, TNPs has been reported to interact with cellular membranes, cytoplasm as well as DNA of cultured mammalian cells (Gupta et al., 2013; Rai et al., 2013).

It has been noticed that the chemical approach using muffle furnace takes longer time (approximately 5 h) for the synthesis of TNPs (Rai et al., 2013) and also the activities of TNPs - mainly photocatalytic and photochemical activities – can be improved by varying the synthesis approaches (Bagheri et al., 2012; Gupta et al., 2013). Microwave assisted synthesis of TNP is greatly used to improve the photocatalytic activity (Jena et al., 2010; Arin et al., 2012; Xuyang et al., 2015).

It can be noted that most of the microbial evaluation discussed above have used sol-gel or air-annealed TNPs. While the other activities of TNPs – photocatalytic - are found to be increased when synthesized by microwave assisted synthesis, so, there is a need to analyze the dependence of micro- and cytotoxicity of TNPs with synthetic protocols followed. Additionally, it can be noted that the other microwave synthesis takes longer time and is tedious, therefore, there is a need of end-user friendly protocol which can be scaled up to industrial scale via pilot plant scale.

In this study, we give a detailed analysis of antibacterial mechanism by titanium dioxide nanoparticles. Titanium dioxide nanoparticles thus synthesized have shown improved antimicrobial activities than the existing synthesis methods. Though it shows improved antimicrobial activity, detailed studies should be performed to understand the antibacterial mechanism. Interestingly, up to our knowledge, no such detailed studies have been done to understand the mechanism of microbial toxicity for nano-titania synthesized by any approaches. Though authors of this manuscript have reported improved antimicrobial techniques but it also can be considered to have its ecotoxicology.
4.2 Materials and Methods

4.2.1 Chemicals

All reagents supplied were of analytical grade and were used without further purification. Throughout the procedures, double deionized (DI) water (with a measured resistivity of 18.2 MΩ cm$^{-1}$) was used. Nutrient media (Himedia Lab Ltd., Bangalore, India) was used for evaluating bacterial growth in liquid broth culture and was supplemented with a 2% bacteriological agar (Himedia Lab Ltd., Bangalore, India) to prepare the solid media used in plate culture studies.

4.2.2 Bacterial Toxicological Evaluation: Comparative Analysis

To evaluate the toxicological behavior of the synthesized TNPs - three bacterial strains have been selected which are Gram negative bacterial strain E. coli, and Gram-positive bacterial strains S. aureus and B. subtilis. Microbial toxicity of TNPs on growth has been evaluated and GrK, MIC, and MBC have been determined. TNPs antibacterial analysis has been evaluated by DIZ and DeK. The bactericidal experiments were carried out in nutrient media, composed of peptone (Loba Chemie Ltd., Mumbai) and NaCl (Merck Ltd Mumbai) 5 g l$^{-1}$ each and yeast extract (Central DrugHouse, New Delhi) and beef extract (S.D. Fine Chem Ltd Mumbai) 1.5 g l$^{-1}$ each.

Diameter of inhibition zone and activity index - Disk diffusion test is commonly used for the analysis of bacterial sensitivity to antibiotics by employing antibiotic impregnated disks (Case and Johnson, 1984). A similar test with the synthesized TNPs disks was used in this study. A 5 ml suspension of TNPs (5 mg ml$^{-1}$) was sonicated and filtered through a membrane filter (0.2 µm, 47 mm diameter Himedia Lab. Ltd., Bangalore, India). The filter paper laden by TNP was dried in an oven for 1 h and small disks of uniform size (6mm diameter) containing 100 ± 15 µg TNPs were punched out and stored in a desiccator at room temperature. 100 µl of a bacterial suspension containing $10^3$ - $10^4$ CFU ml$^{-1}$ microbial loads was spread uniformly on nutrient agar plate surface. The TNP disks were placed on the plate (2 per plate) and one standard Gentamicin disk has been placed per plate for
comparative analysis. After 24 h of incubation at 32 °C, the average DIZ was measured using a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for TNP with each microbial strain were based on six replicates.

MIC and MBC determination - The lowest concentration of material that inhibits the growth of an organism, widely known as MIC (Torrico et al., 2010; Jung et al., 2015; Dasgupta et al., 2016), was determined by batch cultures containing various concentrations of TNPs in suspension (10-100 µg l⁻¹) in sterile angled sidearm Erlenmeyer flasks (500 ml) containing 100 ml nutrient broth. The protocol followed was as described in our earlier analysis to determine antibacterial effects of nano-silver (Dasgupta et al., 2016).

The MBC, the lowest concentration of TNPs that kills99.9% of the strains was determined by assaying the live organisms in those flasks from the MIC test that showed no growth. A loop full from each of those flasks was inoculated on nutrient agar and observed for the signs of growth. The growth of bacteria demonstrates the presence of these germs in the original flask. In contrary, when no growths were observed in the original flask containing no living bacteria then the TNPs were considered being bactericidal at that concentration(Avadi, 2004; Hartshorn et al., 2013; Dasgupta et al., 2016).

GrK and DeK evaluation - Growth kinetics was observed for TNPs. It can be noted that the absorbance value represents the bacterial growth. Alternatively the death kinetics was observed only for MBC of the synthesized TNPs and it was measured by calculating CFU ml⁻¹ as described above. The MBC has been determined with time at which ≥99.9% bacterial killing achieves; which can be obtained by comparing absorbance of the negative control and the samples.

4.2.3 Mutagenic Potential Of Titanium Dioxide Nanoparticles

Ames test was performed as per Maron and Ames (Maron and Ames, 1983) following the principles of organization for economic co-operation and development (OECD) guidelines. Briefly, two Salmonella typhimurium histidine-auxotrophic
strains (TA 98 and TA 100) were used without S9 fraction for this test. Cultures of each test strain were re-cultured and further used in the late exponential growth phase. Each primary experiment included one negative control (distilled water), one positive control for each strain that is 2-Nitrofluorene for TA98 and sodium azide for TA 100 and varying concentrations of nano-titania (100-500 µg per plate). Further, the standard protocol has been used (Maria et al., 2014) and each test was done in triplicates.

4.2.4 Detection of Reactive Oxygen Species Generation

Reactive oxygen species generation was measured by the method discussed by Das et al. (Das et al., 2015). The intracellular reactive oxygen generation was measured by using 2,7-dichlorofluorescein diacetate (DCFH$_2$-DA) as the DCFH$_2$-DA passively enters the cell and reacts with reactive oxygen species to form the highly fluorescent compound 2,7-dichlorofluorescein. The testing protocol has been followed as discussed earlier (Janardan et al., 2016). It can be noted that dose dependence reactive oxygen generation has been analyzed using fluorescent spectrophotometer (Hitachi F-1700) and fluorescent microscopic analysis has been performed at minimum inhibitory concentration.

4.2.5 Fluorescence Microscopy and SEM

*Escherichia coli* (*E.coli*) cells were grown in Luria broth at 37 °C overnight. From the overnight culture 2% inoculums were added to 5 mL of Luria broth and shaken at 37 °C until the optical density was reached 0.5. Then nano-titania of minimum inhibitory concentrations were added and incubated at 37 °C for 1 h. The control was maintained under the same condition without nano-titania. The further method was followed as discussed by Li et al (Li et al., 2012). Each slide was then viewed by Zeiss Axiovert 200 M fluorescence microscope under 40× magnification.

4.2.6 Integrity of Bacterial Cell membrane

If the bacterial membrane is disrupted, the release of cytoplasmic constituents of the cell can be examined by UV-visible spectroscopy at 260 nm. It mainly estimates the amount of nucleic acid released from the cytoplasm (Deokar et al., 2013). After 1 h
incubation with varying concentrations of nano-titania, the bacterial suspensions were immediately collected and passed through a 0.22mm syringe filter to remove the bacteria. The supernatant was then diluted appropriately, and the optical density at 260nm was recorded.

4.2.7 Determination of Superoxide formation

The tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H tetrazolium hydroxide, sodium salt) when exposed to superoxide radicals (O$_2^-$), is reduced to a water-soluble formazan that has an absorbance peak within the visible range with a maximum peak at $\lambda = 490$nm. Development of the characteristic formazan peak in aqueous media provides quantitative evidence for O$_2^-$ production. At the end of exposure of bacterial cells with varying concentrations of nano-titania, 25 µl of 100 mM XTT was added. After an incubation period of 4 hrs, absorbance was measured at 490nm (Priester et al., 2014).

4.2.8 Membrane Permeabilization Assay

Membrane permeabilization activity of nano-titania was determined by the 1-N-phenylnaphthylamine assay as per Xing et al., 2009 (Xing et al., 2009). E.coli cultures grown in nutrient broth at an optical density of 0.3-0.5 at 610nm were harvested by centrifugation at 11,000 g for 10 min, washed and resuspended in 0.5% NaCl solution. The solutions of varying concentrations of nano-titania interacted with bacterial suspension. 1 ml of this suspension was mixed with 20 µl of 1 mM 1-N-phenylnaphthylamine. The fluorescence was recorded with a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan), with an excitation wavelength of 350nm and an emission wavelength of 420nm, respectively.

4.2.9 Statistical Analysis

All statistical analysis was performed and expressed as the mean of parallel duplicates of ANOVA correlation. P<0.05 was considered as statistically significant.
4.3 Results and Discussion

4.3.1 Bacterial Toxicological: Comparative Analysis

Two Gram positive and one Gram negative bacterial strains have been analyzed for DIZ, MIC, MBC, GrK and DeK. *E. coli* has shown more susceptibility (Fig. 4.2), while the least was observed in *B. subtilis*. The MIC and MBC ranges from 15 to 30 and 20 to 35 µg ml$^{-1}$ respectively. GrK was studied at MIC and MBC was opted for DeK analysis. The pattern for GrK and DeK matches with the findings from DIZ, MIC, and MBC i.e. TNP was observed to be more toxic for *E. coli* followed by *S. aureus* and *B. subtilis* (Fig. 4.3; Fig. 4.4).

The comparative antibacterial activity profile has been determined for hybrid method synthesized TNP with Gram positive (*S. aureus* and *B. subtilis*) and Gram-negative (*E. coli*) bacterial strains by different factors – DIZ, MIC, MBC, GrK, and DeK. The microbial susceptibility magnitude is reflected by DIZ; the less susceptible microbial strains exhibit less DIZ and vice versa. The disks impregnated with nano-titania were surrounded by larger DIZ compared to Gentamicin for *E. coli* while the *S. aureus* strain has lesser susceptibility followed by *B. subtilis*. We have provided the representative image showing the DIZ for Gentamicin, TNP impregnated disks for *E. coli* strain (Fig. 4.5). It can be noted that, since 1 mm resolution ruler was used to measure the DIZ, the chances for measurement errors exist; though the method exemplifies the potential bactericidal activity of nano-titania to different strains. Additionally, the relative efficacy of bacterial sensitivity against nano-titania was found to be more than the earlier reports which will be comparatively described later in this article.

To demonstrate the antibacterial activity of nano-titania different bacterial strains have been selected (one Gram-negative and two Gram-negative strains). With the initial bacterial concentration of $10^3$– $10^4$ CFU ml$^{-1}$, MIC and MBC represent the bacteriostatic and bactericidal effects of nano-titania respectively.
Fig. 4.1 Representative Image of Agar Plates Showing DIZ for Gentamicin, and TNP impregnated Disk for *E. coli* Bacterial Strain

![Fig. 4.1](image1.png)

*E. Coli* - Growth Kinetics

![E. Coli - Growth Kinetics](image2.png)

Fig. 4.2 *E. coli* Growth Kinetics of Negative Control; MIC and MBC of TNP
Fig. 4.3 *B. subtilis* Growth Kinetics of Negative Control; MIC and MBC of TNP

Fig. 4.4 *S. aureus* Growth Kinetics of Negative Control; MIC and MBC of TNP
**E. coli - Death Kinetics**

![E. coli Death Kinetics](image1.png)

Fig. 4.5 *E. coli* Death Kinetics of Negative Control and MBC of TNP

**B. subtilis - Death Kinetics**

![B. subtilis Death Kinetics](image2.png)

Fig. 4.6 *B. subtilis* Death Kinetics of Negative Control and MBC of TNP
To best of our acquaintance, this is the first study providing a systematic and detailed study regarding, the complete antimicrobial profiling for TNPs synthesized by microwave-assisted hybrid chemical approach which also includes MIC, MBC, GrK and DeK analysis (Fig. 4.6; Fig. 4.7). The MIC and MBC range from 15 to 30 µg ml\(^{-1}\) and 20 to 35 µg ml\(^{-1}\) respectively for TNPs against different Gram positive and Gram negative bacterial strains. In this experiment - to find the dose dependency - we varied the concentration of the nano-titania and observed variations (data not shown). From the above observations it can be concluded that nano-titania is having broad-spectrum of bactericidal activities which were found to be dose-dependent, it can be noted that for comparative analysis, the concentration of TNP was considered at their respective MIC and MBC concentration for a particular bacterial strains, from which bacterial growth and death curve was inhibited. The above mentioned initial concentration was adjusted in 50 ml nutrient broth and the antibacterial activity was evaluated up to 24 h and 3 h for GrK and DeK respectively since the minimum time to achieve bacteriostatic and bactericidal effects is likely to fall within said period. The growth profiles of bacterial strains with varying concentration of TNP are depicted in Fig 4.6. Similarly, the death
kinetics is shown in Fig. 4.7. In batch culture studies, as the concentration of TNP increases, the lower maximum absorbance (at 600 nm) and greater lag phase was observed. Notably, for *E.coli* strain, TNPs show the maximum bacteriostatic effect as there is a sharp decrease in absorbance for a particular concentration of TNP also, the increase in greater lag phase. Additionally, the graphs represent TNP has more bacteriostatic and bactericidal activities for *E. coli* which establish the fact for its lower MIC and higher MBC. Very few studies are available for the complete antibacterial activity of nano-titania. Recently, DIZ and MIC has been estimated for TNPs of particle size 17 nm synthesized by sonochemical approach (Kasap et al., 2011; Picskin et al., 2013). It can be noted that the initial bacterial volume taken was in the range of µl and determined by 96-well micro-dilution plates assay and also the nano-titania was not microwave-assisted. In this study, $10^3$-$10^4$ CFU ml$^{-1}$ initial bacterial concentration has been taken irrespective of microbial strains and nano-titania concentration. No growth was observed in the flask as the concentration of TNPs increased to MIC. To analyze the complete bacterial toxicological profile, the growth and death kinetics of nano-titania against the three bacterial strains used in earlier studies was represented in Fig. 4.5 and Fig. 4.6 respectively. In the case of *E. coli* nano-titania showed bacteriostatic activity till 6 h at MIC and bactericidal activity in 60 min at MBC while for *B. subtilis* the bacteriostatic and bactericidal time is 4 h and 90 min respectively. Notably, TNP is found least toxic for *S. aureus* which can be depicted by the growth and death kinetics as 2 h and 120 min for bacteriostatic and bactericidal activities respectively. This shows that TNP was found to have a best antibacterial activity for *E. coli*, *B. subtilis* and *S. aureus* but the bacteriostatic and bactericidal activity time increases (Fig. 4.1 to Fig. 4.7). Although, out of the three bacterial strains studied, *S. aureus* was found to be the least sensitive strain against nano-titania, but subsequently TNPs showed antibacterial activity for *S. aureus* too. This can be explained on the basis of DIZ, MIC, MBC and the time taken for bacteriostatic and bactericidal activity. Notably, it can be expected that complete killing might be possible if the analysis time can be extended.
The TNP synthesized by microwave assisted *hybrid* chemical approach established more antibacterial as well as cytotoxicity though it has the comparatively higher size than the previous analysis reported using almost same bacterial and cellular concentrations and their types (Jin et al., 2008; Wagner et al., 2009; Gupta et al., 2013; Picskin et al., 2013; Carrière et al., 2014; Yurong et al., 2014). Although, Picskin et al., have demonstrated the effective antimicrobial activities for TNPs of average size \( \approx 17 \) nm size and Gupta et al., have demonstrated the same for \( \approx 10 \) nm undoped TNPs which is even smaller than the TNP size used in this experiment. Additionally, they have used higher TNP concentration – 200\( \mu g \) ml\(^{-1}\), 100 \( \mu g \) ml\(^{-1}\) respectively. Even the higher TNP concentration was not able to provide complete growth inhibition, it can be noted that the colony forming unit (CFU) is not mentioned in these studies (Gupta et al., 2013; Picskin et al., 2013).

Initial strain concentration used in this study is in the average of \( 10^3 – 10^4 \) CFU ml\(^{-1}\), TNP depicted MIC values of 15, 25 and 30 \( \mu g \) ml\(^{-1}\) for *E. coli*, *B. subtilis* and *S. aureus* strains respectively, simultaneously, it depicted MIC values of 20, 30 and 35 \( \mu g \) ml\(^{-1}\) for the respective bacterial strains which is much lesser value than the AgNPs analyzed in our earlier studies. Interestingly, similar antibacterial analysis was performed by Gupta et al., and Picskin et al., with air-annealed/sol-gel synthesized TNP for the same bacterial species and reported almost similar DIZ and MIC for relatively smaller nanosilver (17 nm and 10 nm) but they have not studied MBC, GrK and DeK (\( 10^5 \) to \( 10^6 \) CFU ml\(^{-1}\)); while in this study similar antibacterial profiling was established for even higher sized TNPs – 28.3 ± 3.1 nm – with lower initial strain concentrations for TNP synthesized by *hybrid* chemical approach.

Referring to the cytotoxicological evaluation in this study, we have reported the relatively lower concentration of TNP as a potential cytotoxic substance with respect to the comparative IC\(_{50}\) values than Carrière et al.,(Carrière et al., 2014). It can be noted that, nano-titania synthesized by Pişkin et al., achieved higher toxicity for Gram negative bacterial strain i.e. *E. coli* which correlate with our observation (Picskin et al., 2013). Studies by many researchers have established that the many physical and chemical factors of TNP – TNP nanocomposite with metals or metal compounds or organic compounds, radiations like UV or microwave, crystallizing agents, size shapes of TNP etc. - have a predominant role on microbial and cyto-
toxicological activities (Kui et al., 2013). Thus, the antibacterial activity of nano-titania can’t be explained based on the size, since the possibility of significant effects of shapes of the AgNPs also exists. It can be noted that the earlier researchers have concluded that the dispersion state and dispersion method of TNP also vary the toxicological activities (Carrière et al., 2014).

Referring to the earlier findings for air-annealed TNPs it can be concluded that DNA damage and reactive oxygen species generation of microbial and cell lines might be the major factor for its toxicological activity (Quaiser et al., 2012; Kansara et al., 2015). Interestingly, most of the mechanism of action analyzed in the earlier reports is for the air-annealed or sol-gel synthesized nano-titania. Negligible reports are available to determine the higher toxicity for microwave-assisted synthesized TNPs and no report is available for the hybrid method synthesis of TNP.

4.3.2 Ames Test

Ames test or bacterial reverse mutation assay is the first and foremost screening test to identify the genotoxicity of any chemical compound. Different concentrations of nano-titania were used on Salmonella typhimurium histidine-auxotrophic strains (TA 98 and TA 100) without the S9 fraction. TA 98 was used to assess frameshift type and TA 100 for base-pair substitution type The result obtained is summarized in Table 4.1. Growth inhibition by titanium dioxide nanoparticles was observed in both the test strains at a concentration of 100 μg per plate or higher. Large increase in the number of revertant colonies was seen for the positive controls in all cases, indicating that the test system responded appropriately. However, nano-titania mutagenicity was not detected in both strains. Most of the reports published have observed weakly positive or negative results for Ames test on various nanomaterials (Maenosono et al., 2007; Landsiedel et al., 2009; Yoshida et al., 2009; Akyil et al., 2015). Woodruff et al have reported that 10 nm TiO₂-nanoparticles had no mutagenetic ability in any of the five different bacterial tester strains, even at a concentration of 4915.2 mg per plate, which is close to the highest concentration (5mg per plate) recommended by OECD Guideline no. 471 (OECD 1987) for a negative result (Woodruff et al., 2012).
Table 4.1 Mutagenicity Potential of Nano-Titania Against *Salmonella typhimurium* Tester Strains (TA 98 and TA 100) with Varying Concentration

<table>
<thead>
<tr>
<th>Dose (µg)</th>
<th>Number of Colonies Per Plate (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA 98</td>
</tr>
<tr>
<td>0</td>
<td>24±1</td>
</tr>
<tr>
<td>100</td>
<td>26±4</td>
</tr>
<tr>
<td>200</td>
<td>28±3</td>
</tr>
<tr>
<td>300</td>
<td>31±4</td>
</tr>
<tr>
<td>400</td>
<td>23±2</td>
</tr>
<tr>
<td>500</td>
<td>22±3</td>
</tr>
<tr>
<td>Positive Control (2-Nitrofluorene)</td>
<td>719±47</td>
</tr>
<tr>
<td>Positive Control (Sodium Azide)</td>
<td>-</td>
</tr>
</tbody>
</table>

The negative mutagenicity of nanomaterials can be due to the antibacterial effect on bacteria. The antibacterial effect may have reduced the sensitivity of the Ames test in determining the mutagenicity. Also, the Ames test is not recommended for detecting genotoxins that induce large-scale deoxyribonucleic acid (DNA) damage (Kim et al., 2013). The insensitivity of most of the tester strains to oxidative DNA damage can be another reason for negative response in Ames test. Kurokawa et. al., have observed that a strong oxidizing agent, potassium bromate was negative in the Ames test with *S. typhimurium* strains TA98, TA1535, TA1537, and TA1588, and only weakly positive in TA100 at doses of 2-4 mg per plate. However, when tested in *Hprt* and mouse lymphoma *Tk* gene mutation assays, the results were strongly positive (Kurokawa et al., 1990; Li et al., 2012). 4-methylimidazole, a compound formed by the interaction of ammonia with reducing sugars also showed negative results in Ames test but has been reported to induce alveolar/bronchiolar tumors in mice.(Beevers and Adamson, 2016). Kim et al. have suggested that as the cells take nanomaterials, it can generate oxidative stress by activating membrane-bound NAD(P)H oxidase in the cells. So, the bacterial strains might not be sensitive to this type of oxidative species (Kim et al., 2011).
4.3.3 Reactive Oxygen Species Generation

Generation of reactive oxygen species is one of the possible mechanisms by which nano-titania exert its toxicity. To study the release of intracellular reactive oxygen, a cell-permeable non-fluorescent dye 2,7-dichlorofluorescin diacetate (DCFH$_2$-DA) was used which can be de-esterified intracellularly and turns to highly fluorescent 2′,7′-dichlorofluorescein (DCF$^+$) upon oxidation (Wang et al., 2015). Reactive oxygen in bacteria is generated from the autoxidation of reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase II in the respiratory chain (Dwivedi et al., 2014).

Bacteria strain was stained with DCFH$_2$-DA for 30 min, after exposure of nano-titania. It can be noted that the dose of nano-titania has been varied in the range of minimum inhibitory concentration and minimum bactericidal concentration i.e. 10 to 25 ppm and the same doses have been followed in all dose dependent tests. With the increase in nano-titania concentration, the fluorescent intensity increases at 520 nm using 485 nm excitation (Fig. 4.8) which depicts the dose dependent reactive oxygen species generation. The negative control shows negligible fluorescence activity. during the growth phase of *E. coli* in oxygen rich glucose medium, the reactive oxygen species is normally generated i.e. H$_2$O$_2$ at the rate 10–15 $\mu$ M/s, which is further counterbalanced by several scavenging enzymes such as superoxide dismutase for *E.coli* (Arakha et al., 2015). The microscopic study also revealed that *E. coli* became the similar color of DCF$^+$ after interaction with nano-titania (Fig. 4.9 and Fig. 4.10). The generation of reactive oxygen species could be caused by the impeded electronic transport along the respiratory chain in the damaged plasma membrane (Das et al., 2015).

The reactive oxygen species thus generated is responsible for depolarization of the bacterial membrane which ultimately damages the cell wall. Reactive oxygen species generation mediated membrane damage and cell death by nanomaterials interaction has been reported by many studies (Dwivedi et al., 2014; Arakha et al., 2015; Das et al., 2015).
**Fig. 4.8** Fluorescent Spectrophotometric Peaks Increase with the Dose Concentration Showing Dose Dependent Reactive Oxygen Generation while Treating *E. coli* with Different Concentration of Nano-
Titania

**Fig. 4.9** Fluorescent Microscopic Image of Negative Control Showing Negligible Generation of Reactive Oxygen Species
Fig. 4.10 Fluorescent Microscopic Image of *E. coli* Treated with Nano-Titania at Their Minimum Inhibitory Concentration Showing Reactive Oxygen Species Generation

Fig. 4.11 Scanning Electron Microscopic Images of Negative Control of *E. coli*
4.3.4 Scanning Electron Microscopic Analysis

The change in morphology of the bacterial cell was analyzed by scanning electron microscope. Cells with and without exposure to nano-titania were observed for any damage. The untreated cells were rod-shaped as depicted in Fig. 4.10 while the treated cells were highly damaged (Fig. 4.11). The shape and size of cells were also damaged dramatically indicating the death of the cells. The change in morphology has been observed in most of the reports using few other nanomaterials. Li et al. have reported that Catechin-Copper nanoparticles disrupt the cell membrane causing leakage of intracellular material and thus shrinkage of the cell (Li et al., 2015).

4.3.5 Integrity of Bacterial Membrane

The major outer structural component of bacteria is the bacterial membrane. The bacterial membrane may be compromised during biocidal challenges, such as exposure to the drugs of toxins, by losing the membrane integrity lead to releasing the cytoplasmic contents.
**Fig. 4.13** Release of 260 nm Absorbing Material from *E. coli* Suspensions Treated with Varying Concentration of Nano-Titania

**Fig. 4.14** The Uptake of 1-N-Phenylnaphthylamine (Measured as Fluorescence Intensity) by *E. coli* with Addition of Varying Concentrations of Nano-Titania
Fig. 4.15 Depicts the Probable Mechanism of Action for Microbial Toxicity of Nano-Titania Synthesized from Microwave-Irradiation Assisted Hybrid Chemical Approach

In such cases, small ions – potassium and phosphate – have a tendency to leach out first followed by biomolecules like DNA, ribonucleic acid (RNA) and others. Since these nucleotides have strong absorption at 260nm, they are termed as “260nm absorbing materials” (Dongdong et al., 2015). The UV absorption of the supernatant obtained from the interaction is represented in Fig. 4.13. As observed from the graph there is an absorbance at 260nm states that the cell membrane is damaged and release of cytoplasmic constituents have occurred. Initially, up to 5 min, no absorbance was observed as *E.coli* has the outer membrane to prevent the influx of foreign molecules.

4.3.6 Superoxide Formation

Superoxide formation was not detected when *E.coli* was exposed to nano-titania. No absorbance change was observed at 490nm. Priester et al have reported the negative formation of superoxide radicals in *E.coli* and *P. aeruginosa* by cysteine-capped silver nanoparticles (Priester et al., 2014). Meghana et al have also reported negative results for superoxide by copper oxide nanoparticles (Meghana et al., 2015). However, Ivask et al have reported superoxide formation in *E.coli* when exposed to nano-silvers even at low concentrations (<1 mg/L). However, they have
used a bioluminescent *E.coli* reporter strain which is more sensitive than the XTT assay (Ivask et al., 2010).

4.3.7 Membrane Permeabilization

Being a Gram negative bacteria - *E.coli* has two cell envelope membranes. We examined the ability of nano-titania to interact with bacterial membranes. 1-N-phenylnaphthylamine, a hydrophobic probe, is normally excluded by an intact out membrane of Gram-negative bacteria. Increased fluorescence uptake of 1-N-phenylnaphthylamine occurs in bacterial suspensions containing cells whose membrane is damaged and functionally invalid, Based on this principle, the 1-N-phenylnaphthylamine uptake by nano-titania-treated *E.coli* is shown in Fig. 4.14. As soon as nano-titania was mixed with *E.coli* suspensions, there was the immediate uptake of 1-N-phenylnaphthylamine with the amount released being maximal after about 6 min and caused a time-dependent increase in fluorescence. Thereafter the 1-N-phenylnaphthylamine uptake was almost unchanged until 10 min. The fluorescence increase was dose-dependent so that the maximum fluorescence was greater with the higher than the lower concentration of nano-titania, which was in accordance with the cell membranes integrity results. In control suspensions, there was almost no 1-N-phenylnaphthylamine uptake in 10 min. Thus, Fig. 4.15 depicts the probable mechanism for antimicrobial activity of nano-titania synthesized from thermal co-reduction approach.

4.4 Summary

The results showed that nanotitania synthesized by this method has the best antibacterial activity in the order of *E. coli*, *B. subtilis*, and *S. aureus*, which can be observed by higher DIZ and lower MIC and MBC for the corresponding bacterial strains. Therefore, the study determines that the microwave-assisted hybrid chemical approach is time-saving. Moreover, the study provides a detailed understanding of the micro- and cytotoxicological profiles for various cell lines and microbial strains. The toxicity study raises concern regarding the safety associated with the applications of TNP in consumer products. Moreover, it is necessary to find the
action mechanism at the molecular level to establish the reason for greater bacterial toxicity.

This is first of its kind study in which many facts have been established regarding the mechanism of action of nanotitania. Dose-dependent mutagenicity has been shown by nano-titania but at higher dose nano-titania has shown antimicrobial activities against the microbial strains specified for mutagenicity test—Ames test. More amount of intracellular reactive oxygen generation has been observed using DCFH dye test, which was hypothesized to be produced from autoxidation of NADH dehydrogenase II in stress condition. Microbial cells treated with nano-titania have been observed under scanning electron microscope, and ruptures in cell membranes can be easily seen this rupture may result because of generation of reactive oxygen species. Further, membrane integrity tests confirm the membrane ruptures after treatment with nano-titania.

Further to confirm that reactive oxygen generation is the main reason for cell death, the generation of superoxide has been analysed by the tetrazolium salt XTT assay—and no superoxide generation has been observed after treatment of cells with nano-titania. Further, an increase in membrane permeabilization has been observed. Conclusively, the one reason which can be assured for microbial cell death after treatment with nano-titania is reactive oxygen generation and increase in membrane permeabilization, not the superoxide generation. Further, the reactive oxygen generation and membrane permeabilization are dose dependent on showing the microbial toxicity. Knowing the exact mechanism of action for microbial toxicity of nano-titania synthesized by microwave-irradiation-assisted hybrid chemical approach will lead to its potential applications in antimicrobial drug discovery or controlling the nanoecotoxicology.