Comparative Study of Antimicrobial Activities of twotype of TiO_2Nanoparticles Against the Pathogenic Strain of *Escherichia coli*

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Abstract: The current study investigated the antibacterial properties0.25%, 0.50% and 1% of two different types of nano-TiO_2 against a selection of pathogenic bacteria (*Escherchia coli*) isolated from a sample of wastewater from Riyadh, for the purposes of further application to time and cost effective water purification in Saudi Arabia. A commercial sample of nanoparticles metal oxide containing 98% titanium dioxide (TiO_2) that was brown in colour – hereinafter (T2B). Another commercial sample of nanoparticles metal oxide was obtained containing 99% titanium dioxide (TiO_2) that was white in colour – hereinafter (T2W). Pathogenic bacteria were cultured in liquid nutrient medium to evaluate the antibacterial effects of 0.25%, 0.50% and 1% of both types of nano-TiO_2.Electron microscopy was also used to observe the effect of both nanoparticles on the pathogenic bacterial cells in the liquid media specimens. For both nano-specimens significant results were seen for 0.25%0.50% and 1% concentration. The bacterial number substantially decreased with 0.25%, 0.50% and 1% of both nanoparticles. However, better results were obtained with 0.50% and 1% of (T2B), where bacterial inhibition was greater in both media. With (T2B), bacterial clearance was observed in nearly half the time needed (T2W). This has been observed in both media. In the liquid medium, complete cell death was seen with 1% (T2B) after 4 hours compared with 6 hours with 1% (T2W). Electron microscopy showed bacterial samples completely destroyed with 1% (T2B). *E. coli* appeared to be sensitive bacteria to the presence of both (T2W) and (T2B) nanoparticles, as they experienced significant bacteria disruption and damage .

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1. Introduction

Clean and fresh water are essential for the very existence of life. Over 1 billion people worldwide mostly in developing countries have no access to clean potable water. Also, a further 2.6 billion people have no access to adequate sanitation (WHO 2004).A lot of attention has been attracted to inorganic materials such as metal and metal oxides due to their ability to tolerate harsh process conditions (Kursawe*et al.,* 2005; Makhluf*et al.,* 2005). TiO_2, ZnO, MgO and CaO metal oxides are generally considered as safe to human beings and animals and also have the ability to tolerate harsh process conditions (Stoimenov*et al.,* 2002). At very low concentrations, metal and metal salts are toxic to microbes by binding to intracellular proteins and inactivating them (McDonnell and Russell 1999). The varied uses and benefits of metal oxides are many and varied(Stoimenov et al., 2002).

There are various techniques for treating water in use today such as chemical and physical agents such as chlorine and it's derivatives, Ultraviolet light etc…(Droste, 1997), However, there have been problems with the direct use of traditional methods as bactericides because of that we must find new and innovative ways to solve the problem of water pollution , In the context of water purification, nanoparticles have been noted to have an important role (Stoimenov et al., 2002).Nanotechnology plays an important role in the industrial revolution. Nanotechnology is concerned with materials that exhibit significantly novel and improved physical, chemical and biological properties (Wang, 2000).

Heavy metal nanoparticles such as iron oxide and titanium dioxide have demonstrated that they were good sorbents for metal contaminants due to the effect of particle size on adsorption. In the field of water purification, spherical aggregates of nanoparticles that possess similar size and shape to resin beads are already being explored. Nanoparticles can be designed and synthesised to act as either separation or reaction media for pollutants. (Stoimenov et al., 2002).

Moreover, titanium dioxide, especially as nanoparticulateanatase is thought to be an interesting antibacterial agent with notable photocatalytic behaviour. However, ultrafine anatase has been observed to be cytotoxic. (Oberdörste, 2001; Ishibashi, 2000).For many year (TiO_2) Titanium dioxide used to different properties. In many

researchers told about titanium dioxide nano particle (TiO_2 NPs) has antimicrobial activities, and low toxicity. This size of nano make it have this important biological properties .TiO_2 NPs Works just like any other nanoparticle biomolecules influencing where it enters the environmental components such as water and soil by bacteria that caused it to toxicity or biotransformation. (RezaeiZarchi et al., 2009).

Antibiotics have proved their ability to kill many of the bacteria in past few years Could nanoparticles that carry out such antibiotics or surpass them, recently nanoparticles have an impact pesticide and killer of bacteria as there are expectations of many dreams on the impact of nanoparticles in Future medicine.The important of nano-particles is having good size and characteristics that make it not resistance by bacteria, viruses or fungi. So it is a good treatment for bacterial resistance to antibiotics .The nanoparticles killed this microorganism within minutes if we but nano-particles with the growth media in the laboratory tests. This is instrumental to nanoparticles, where she entered with different microbes that are part of the ecosystem or part of the food chain.(Nel et al., 2006; Thill et al., 2006).

New properties of nano-materials is unique make thim an effective antibacterial activity as mentioned in many new studies. For example, many metal oxides like nano-silver, ZnO, CdO and nano-TiO_2 also mention for good antibacterial activity properties .CdS and TiO_2 NPs are metals oxides Favorites as anti-bacterial than silver nano because it is less expensive (Economical) , toxic and chemically stable under high exposure and temperatures .Antibiotics are widely used throughout the agricultural industry as a prophylactic or treatment against infections disease. (Nel et al., 2006; Thill et al., 2006).

The resistancing of bacteria for antibiotic make five million people dying every year from infections not responding to antibiotics. (Nel et al., 2006; Thill et al., 2006).This study aimed to investigate the potent long-lasting antibacterial activity of nano Tio2 to ward the gram negative bacterium *E.coli*. (RezaeiZarchi et al., 2009).TiO_2 is reputed to be toxic to Gram-negative& positive bacteria. (Adams et al., 2006).Moreover, titanium dioxide, especially as nanoparticulateanatase is thought to be an interesting antibacterial agent with notable photocatalyticbehaviour. However, ultrafine anatase has been observed to be cytotoxic. In vivo studies demonstrated that can be severely toxic in the respiratory system (Oberdörste, 2001; Ishibashi, 2000). New possibilities for drug delivery, gene therapy, medical diagnostics, and antimicrobial activities may involve the use of nanocapsules and nanodevices.

Study Aims: The anti-bacterial effects of two different types of TiO_2 nanoparticles concentrations were compared, analysed and evaluated for gram-negative bacterium *E. coli*.

2. Materials and Methods

Nanoparticles Used

A commercial sample of nanoparticles metal oxide containing 98% titanium dioxide (TiO_2) – kindly, given by Dr Hassan El-Dessouki, From the University of Leeds – hereinafter (T2B). This was received as a fine powder of particle size ranging from 60 nm to 200 nm and light brown in colour. Another commercial sample of nanoparticles metal oxide was obtained containing 99% titanium dioxide (TiO_2) – hereinafter (T2W). This was also received as a fine powder of particle size of 200 nm, white in colour, and with a bulk density of 0.46g/ml.

Media Used

Luria bertani broth medium (LB) was prepared by dissolving 13 g of nutrient in 1000 ml of distil-water. The above solution was autoclaved subsequently at 121^{o}C, 15 lbs for 30 min.

Isolation of Bacteria

Seven samples of wastewater were obtained from different areas in the Kingdom of Saudi Arabia. Wastewater was concentrated by centrifuging 50 ml of wastewater for 20 minutes at 4000 rpm. The supernatant was removed and the cells were re-suspended in 1 ml of distilled water. Concentrated wastewater (100 microliters) was plated on LB agar medium. Plates were incubated overnight at 37 ^{o} C and 16 colonies picked and were streaked in LB agar plates for isolation and identified by gram stain and API. Fresh colonies of each bacteria were obtained and then were cultured into LB broth for further experiments.

Bacterial Susceptibility to Nanoparticles

To examine the susceptibility of bacterial isolates, (*Escherichia coli)* to two different types of nano-TiO_2, different estimation methods were used with three repetitions.

Bacterial Growth in the Presence of Nano-TiO_2 in Liquid Medium

Test organism of bacteria were grown separately in 50 mL sterilized (LB) broth medium and kept in shaker incubator at 37°C for 16 hour (overnight incubation). On the subsequent day test organism cultures were transferred at the rate of 1% in 10 mL LB broth. Various concentrations of nanoparticles (0.25, 0.5 and 1% of TiO_2) were carefully placed into each tube, leaving one as a control to track the normal growth of the microbial cells without nanoparticles. Experiments were

performed using both a negative control (tube containing cells plus media) and a positive control (tube containing nanoparticles plus media). The tubes were incubated at 37°C. Optical density measurements from each tube were taken every two hour to record the growth of the microbes in a spectrophotometer at 600 nm. The growth of microbial cells interacting with the nanoparticles was determined from a plot of the optical density versus time.The data obtained in all tests were compared with the control. Student's *t*-test was used to evaluate the significance of experimental results ($P<0.05$). Statistical analysis was performed using Microsoft Excel with the Analysis ToolPak Add-In.

The Detection and Analysis by scanning electron microscope (SEM) and a transmission electron microscope (TEM)

All samples for imaging were prepared by Central Laboratoryof the King Saud university Female Science and Medical Colleges.

3. Results

Effect of Nano-TiO2 on *E. coli* Bacterial Growth in Liquid Medium

The effect of T2W and T2B Nano-TiO_2 on bacterial growth in liquid medium was measured at different concentrations of Nano-TiO_2 and at different time intervals.In Figure 1:all concentrations 0.25 , 0.5 and 0.1% of tow type of nano-TiO_2 inhibited the bacterial growth as compared to the control .The data obtained demonstrated that the highest concentration of Nano-TiO_2 at 1% was the most effective in reducing the number of bacteria . With bacteria studied, T2B was more effective at reducing bacterial growth than T2W at any given concentrations (P<0.05).

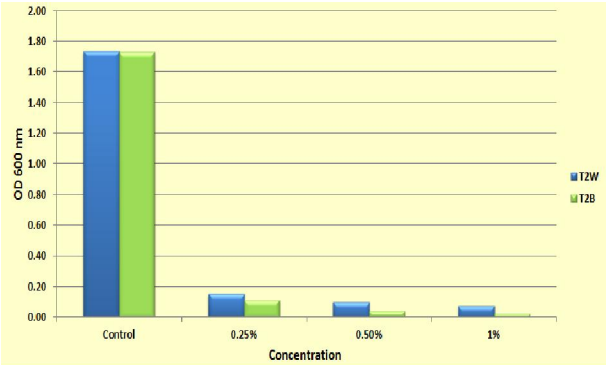

Figure 1. Effect of Different Concentrations of T2W and T2B Nano-TiO_2 on *E. coli* after 16 Hours.

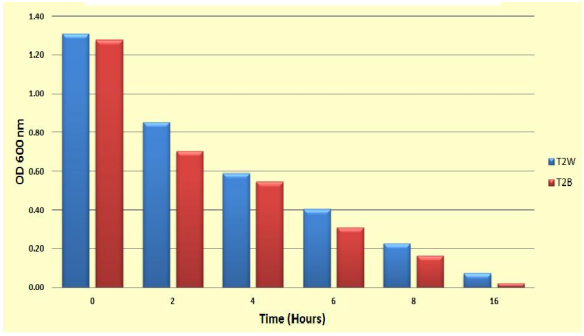

Figure 2. Effect of 1% of T2W and T2B Nano-TiO2 on the Growth of *E. coli* Bacteria in Liquid Medium during 16 Hours.

Bactericidal effect of nano-TiO2 on *E.coli* in liquid medium

Figure 2: shows the Effect of 1% of T2W and T2B Nano-TiO_2 on the Growth of *E. coli* Bacteria in Liquid Medium during 16 Hours. In all different time intervals of tow type of nano-TiO_2 inhibited the bacterial growth as compared to the control .The data obtained demonstrated that the most effective in reducing the number of bacteria of Nano-TiO_2 at 1% was 16 Hours .The tow type of nano-TiO_2 began to reduce the number of bacteria after 4 hours of incubation, and gave the best inhibited the bacterial growth after 16 hours as compared to the control .With bacteria studied, T2B was more effective at reducing bacterial growth than T2W at any different time intervals given.The data obtained demonstrated that the Best decrease of Nano-TiO_2 at 1% was the most effective in reducing the number of bacteria in a short period of time less than 1days.

In Table 1: Statistical analysis carried out to determine whether there had been a significant result in the use of T2W and T2B Nano-TiO_2 in liquid medium showed that the results obtained were significant. The null hypothesis may be rejected and there is a 95% confidence level that the parameters are not the same. P values are all (p= <0.05) – meaning that they are significant.

Table 1. Table to Show Statistical Significance of Results Obtained forTreating Bacteria with Both T2W and T2B Nano-TiO_2 in Liquid Medium

Concentration/Bacteria	***E. coli***
1.00%	0.01009
0.50%	0.00056
0.25%	0.02479

Nanoparticle Agglomerate Shifts in Growth Experiment:

Scanning Electron Micrographs Showing Interaction of Aggregated Nano-TiO_2 with Bacterial Cells

Experiments were carried out under conditions of 37^0C, and in the absence of UV light. TiO_2 nanoparticles were highly agglomerated in the LB broth medium. After 4 hours under abiotic conditions, bacterial cells were shown eliminated by both the T2W and T2B nanoparticles in the SEM images taken. In these micrographs, the bacteria are shown to be killed and destroyed by the nanoparticles. Both T2W and T2B formed large aggregates after eliminating bacteria (e.g. Figures (d)+(e) in 3).TiO_2nanoparticles (T2B) after 4 hours.

Transmission Electron Micrographs Showing Interaction of Nano-TiO2 with Bacterial Cells

The effect of T2W and T2B Nano-TiO_2 on E. coli bacteria selected in this study were examined via Transmission Electron Microscopy (TEM). The micrographs revealed the attachment of the T2W and T2B nanoparticles to the surface of the bacteria causing cell damage. After 4 hours partial and complete bacterial cell disintegration was visible. T2B nanoparticles were much more effective at destroying bacterial cells than T2W during 4 hours. T2B nanoparticles caused rapid bacterial cell disintegration and wider bacterial damage (e.g. Figure 4.(d)).

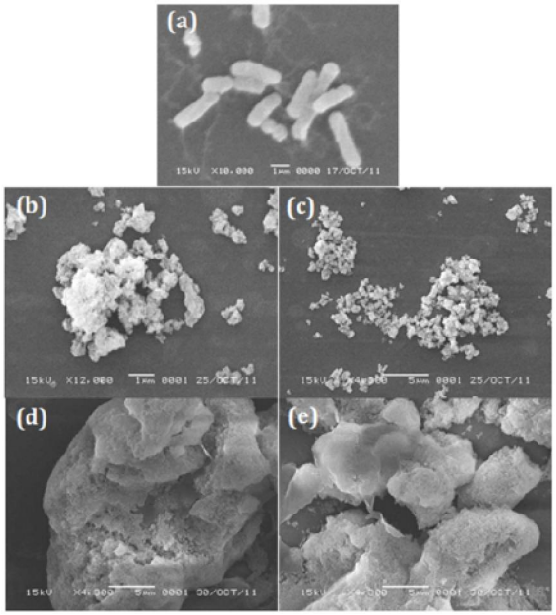

Figure 3. (a) SEM of *E. coli* growing on the LB broth after 4h without TiO_2 nanoparticles. (b) and (c) *E. coli* growing on Liquid medium with 1% TiO_2 nanoparticles (T2W) after 4 hours. (d) and (e) *E. coli* growing on Liquid medium with 1% TiO_2 nanoparticles (T2B) after 4 hours.

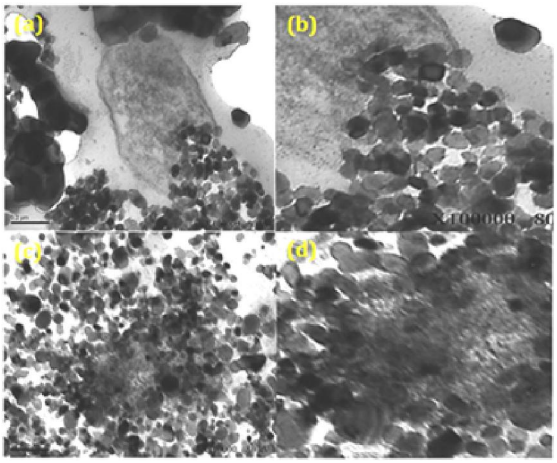

Figure 4. (a) and (b) *E. coli* bacteria in the presence of TiO2 nano-particles (T2W) after 4 hours, revealing the attachment of the particles to the surface of the bacteria. (c) and (d) E. Coli bacteria in the presence of TiO_2 nanoparticles (T2B) after 4 hours, revealing the attachment of the particles to the surface of the bacteria and causing bacterial damage.

4. Discussion

The antibacterial activities of different concentrations of two different types of Nano-TiO_2were investigated in this study. The bacteria isolated from wastewater samples in

Riyadh, Saudi Arabia (*Escherchia coli*) were used as test organisms during the experiments. Good growth inhibition results were observed when the bacterial cells were incubated with both kinds of nanoparticles during the liquid cultures, with T2B nanoparticles proving to be more effective at reducing bacterial growth and eliminating bacteria in a shorter period of time than T2W. (Jaiswal and Simon, 2004) .

The present study investigated the effect of different concentrations of nano-scale TiO_2 to determine the most effective concentration that could have the best possible antibacterial properties against the bacterial strains used in this study. The results in this study are consistent with previous researches examining the antibacterial effects of nano-materials (Clement and Jarrett, 1994; Zhang and Chen, 2009; Cook and Costerton, 2000). As the sizes of nanoparticles are similar to that ofinternal cell organelles, they may be used in manipulating or sensing biological systems (Jaiswal and Simon, 2004) .

The surface area to volume ration increases as the size of the particle decreases. This allows a greater number/proportion of atoms or molecules to be displayed on the surface rather than the interior of the material. The potential reactive groups on the particles' surface are determined by the increase in

the percentage of atoms on the surface (Nel et al, 2006) . This could generate adverse biological effects in living cells that would not be otherwise possible with the same material, but in bulkier form, as the small sizes can modify and alter the physiochemical properties of the material and can increase the uptake and interactions with biological tissues.(Zhang and Chen, 2009).

In contrast to their bulkier counterparts, oxide nanoparticles possess a greater surface area and reactivity which grants them superior performance. However, they may be associated with higher environmental and health risks. (Zhang and Chen, 2009). This has however raised concerns with respect to the possible harmful interactions that they may give rise to in the environment (Nel et al., 2006; Thill et al., 2006). Various investigations have presented possible theories into the mechanisms that may be involved in the interaction between nanoparticles and cellular biological macromolecules. It has been suggested that microorganisms carry a negative charge whilst metal oxides carry a positive charge, thereby establishing an "electromagnetic" attraction between the microbe and treated surface(Zhang and Chen, 2009).

Following contact with a nanoparticle, the bacterial cell is oxidised and cell death immediately follows. It has been suggested that nanoparticles release ions that react with the thiol groups (-SH) of the proteins found on the surface of the bacterial cell. These proteins protrude through the membrane of the bacterial cell and thus permit the transport of nutrients through the cell wall. These proteins are deactivated by nanoparticles, and this reduces membrane permeability resulting in eventual cell lysis and death (Zhang and Chen, 2009). Nano-materials also retard the bacterial adhesion and bio-film formation (Raad et al., 2005).

TiO_2 nanoparticles possess good inhibitory effect on bacteria. Both T2W and T2B exhibited signs of toxicity to the tested bacteria compared to the control. The toxicity of nanoparticles is not only considered in terms of the dissolved metal ions, but also from their tendency to attach to the bacterial cell walls instead of aggregating together. Each type of bacteria had a different degree of sensitivity to the nanoparticles used. TEM images obtained throughout this study confirmed the attachment of TiO_2 nanoparticles to the surface of the bacteria. The bacteria studied seemed to have a higher degree of sensitivity to T2B than T2W nanoparticles as the former was quicker at destroying and eliminating bacterial cells in a shorter period of time. Both E. coli (Figures 3 and 4) appeared to be the sensitive bacteria to the presence of both T2W and T2B nanoparticles, as they experienced the significant bacterial disruption and damage. Previous studies have already discussed the microbial cytotoxicity of nanoparticles (Adams et al., 2006; Brayner et al., 2006; Thill et al., 2006; Huang et al., 2008).

In this study the toxicity of the TiO_2 nanoparticles appeared to be derived from the ability of the nanoparticles to attach on the bacterial cell envelope. From the suspension, the bacteria would be able to attract small aggregates and individual particles . This attraction would cause the nanoparticles aggregation-dispersion equilibrium to move towards the dispersion direction . The toxicity of metallic nanoparticles overall depends on the chemical stability and aggregation of particles, and chemical speciation (Kahru et al., 2008; Auffan et al., 2009; Dasari et al., 2013). To understand the mechanisms of toxicity, the location of nanoparticles toxicity, whether it occurs inside the bacterial cells or on the cell surface, is an important consideration. Although it has been reported that nanoparticles can be found inside the bacterial cells, it is unlikely that nanoparticles pass across intact membranes (Neal, 2008). Nanoparticle accumulation in the cytoplasm is most likely to be observed following membrane disruption (Brayner et al., 2006; Huang et al., 2008). It was reported that the adsorption of nanoparticles to bacterial surfaces was associated with significant bacterial cytotoxicity (Thill et al., 2006) .

Bacteria properties and the methods for their destruction are highly specific and depend on the respective bacterial strain and the type and role of the bacterial cell wall. The cell wall for bacteria are vital as they provide strength, rigidity, and shape and also protect the cell from osmotic rupture and mechanical damage. The bacterial cell wall is divided into two types in accordance to their structure, components, and functions: Gram positive (+) and Gram negative (–) . Gram-positive bacterial cell wall contains a thick layer of peptidoglycan (PG) that is around 20-50nm thick, and that is attached to teichoic acids that are unique to the cell wall of gram-positive bacteria. Contrastingly, the cell wall of gram-negative bacteria is more complex in terms of structure and chemical composition. The cell wall in gram-negative bacteria contains a thin layer of PG and an outer membrane that covers the entire surface of the membrane. Resistance is conferred to hydrophobic compounds by the outer membrane of gram-negative bacteria including detergents. The cell wall contains lipopolysaccharides, which are a unique component that increases the negative charge of the cell membranes and are vital for maintaining the structural integrity of the bacteria and its viability . (Jucker et al., 1998; Omoike and Chorover, 2004; Parikh and Chorover, 2006).

It has been observed that nanoparticle attachment to the cell envelope was necessary to induce bacterial cytotoxicity . The adhesion process to the surface of the bacteria is essential in any discussion of nanoparticle bacterial cytotoxicity. The manner in which nanoparticles attach to the surface of the bacteria and affect the biomolecules located on the cell surface is of significant interest. The highly-charged structure, functional groups, and/or bridging effect to the surface of biomolecules found on the bacterial envelope determine cell adhesion (Jucker et al., 1998; Omoike and Chorover, 2004; Parikh and Chorover, 2006). The biomolecules are important for maintain normal cell physiological activities and also function as adhesins. Nanoparticle microbial toxicity may be induced by the adhesion of the nanoparticle onto bacteria resulting in damage and change in the physio/chemical properties of the surface biomolecule(Neu and Marshall, 1990).

LPS is amphiphilic biopolymers with a hydrophobic side embedded in the membrane and a hydrophilic side extending into the aqueous solution from the intact cell . LPS is most likely to be the first to contact with the surface or particles when a bacterial cell approaches a surface or a small particle is attracted to it. In this study, this biopolymer may have adsorbed to the nanoparticles used in this study. The adsorption of nanoparticles to the biomolecules does not seem to damage the molecules or result in direct cytotoxicity as it is similar in concept to the bacteria adhering to surfaces via the LPS polymer in the natural environment (Neu and Marshall, 1990). Nanoparticles adsorb to the LPS via hydrogen bonding. The interaction with the cell surface biopolymers may increase due to the small size and large surface area of nanoparticles as opposed to surfaces found in the natural environment (Nel et al., 2006).

Damage to proteins and phospholipids most probably form the basis for the cytotoxicity-related changes. In cell physiological activities, an important role is played by the different types of proteins found on the outer membrane and cell surface. Outer membrane proteins and some cell surface proteins are unlikely to interact with large particles as they are hidden behind the O-antigen layer of the LPS (Jucker et al., 1997). As nanoparticles are small in size with the ability to enter the gaps between the long biopolymer chains, the chances of exposure increase significantly. The secondary structures of proteins may change should they adhere to particles or solid surfaces (Buijs and Norde, 1996; Vertegel et al., 2004; Strehle et al., 2004) possibly giving rise to partial protein unfolding (Wu and Narsimhan, 2008).

Following exposure to nanoparticles, bacterial proteins may show a decrease in β-sheet content. Outer membranes or surface proteins in such conditions may be damaged and cell physiological activities may be affected. This is a possible explanation for nanoparticle cytotoxicity. Cell surface biopolymers protect the phospholipid membrane and so the latter does not generally attach to the particles or surface in the environment during normal bacterial adhesion processes. Cell death may occur if the phospholipid structure is damages as many important cell physiological activities occur in the periplasm. During the exposure to nanoparticles, vital bacterial biomolecules can adsorb on the surface of the oxide nanoparticle. (Kahru et al., 2008; Auffan et al., 2009; Dasari et al., 2013(.

LPS has good adhesins that can bind to the oxide nanoparticles in a way that is similar to how this biomolecule would allow bacteria to bind to a solid surface in the environment. Proteins and phospholipid can suffer from function-involved or devastating changes induced by nanoparticles attaching on the cell surface. When selecting or designing safe nanoparticles for biological and biomedical applications, these factors should be taken into consideration. The difference in nanoparticle bacterial toxicity has important antimicrobial implications. Knowledge of nanoparticle toxicity and the adhesion properties of bacterial amphiphilic biomolecules and the interactions between them is useful to permit the effective selection and modification of nanoparticles to achieved desired properties and results. (Kahru et al., 2008; Auffan et al., 2009; Dasari et al., 2013).

Chemical structure changes may also be induced in bacterial surface biomolecules. The damage caused to the structure and the change in the physio/chemical properties of surface biomolecules arise out of the cytotoxicity of nanoparticles by the necessary attaching to the cell envelope. (Kahru et al., 2008; Auffan et al., 2009; Dasari et al., 2013). The toxicity of metallic nanoparticles was found to not be caused solely by dissolved metal ions, but also from their ability and tendency to attach to bacterial cell walls (Jiang et al., 2009; George, et al., 2011) observed that nanoparticle toxicity occurs when the nanoparticle is associated with cell membrane or in near vicinity to cells. The results in this study confirm these findings.

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