Antifungal Activity of Titanium Dioxide Nanoparticles against *Candida albicans*

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The unregulated release of titanium dioxide nanoparticles into the environment has raised concern, in particular due to the impact of the nanoparticles on indigenous micro-biome in our ecosystem. This paper reports a study on antifungal activity of titanium dioxide nanoparticles on a healthy growing fungi species, *Candida albicans*, a known opportunistic pathogen. A quantification of the total cell death was performed using a direct staining method, Trypan blue exclusion assay. Exposure to nanoparticles not only altered the growth rate, but also affected the onset and length of *Candida albicans* growth phases. The log and the onset of the death phase were shortened and accelerated, respectively. Up to 65% of the *Candida albicans* were killed after exposure to 100 µg/mL of the anatase titanium dioxide nanoparticles, while only 33% were killed with rutile. A higher dosage and incubation time of the nanoparticles increased their toxicity. Cells suffered from morphological changes upon the nanoparticle exposure, which correlates well with the results showing an altered growth phase culture.

Keywords: Titanium dioxide; Trypan blue exclusion assay; Candida albicans; Antifungal activity; Growth rate

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INTRODUCTION

In recent years there has been a rapid development and application of nanotechnology in a wide range of fields such as biosciences, information of technology, computers, and medicine. With the rapid emergence of nanomaterial incorporated in consumer products, as well as its uncontrolled release into the environment, it is essential to identify the factors associated with its toxicity. Despite their distinctive application and advantages in the industrial and domestic sectors, the use of materials with nanometer dimensions has raised issues of safety for both consumers and the environment. Investigation on the exposure effect of discrete nanoparticles (NPs), one of the many forms of nanomaterials, and their toxicity is very important as their small size causes more inflammation than bulk counterparts when delivered at the same mass dose (Zhang *et al.* 2007; Nair *et al.* 2009; Liu *et al*. 2011; Raghupathi *et al.* 2011). Because of their small size and their unique characteristics, NPs have the ability to harm humans (Chang *et al.* 2011; Akhavan *et al.* 2012, 2013), microorganisms (Stoimenov *et al.* 2002; Baek and An 2011; Azam *et al.* 2012), and other wildlife (Wang *et al.* 2009; Akhavan *et al.* 2015; Chen *et al.* 2016) by interacting through various mechanisms (Liu *et al.* 2011; Xiong *et al.* 2011).

Titanium dioxide (TiO_2) is among the most widely used material with its broad

range of function, for both industry and consumer applications. There are three forms of TiO_2 crystal structures, which are anatase, rutile, and brookite. The first two forms play an important role in industrial applications, while the brookite form has no commercial value and is not being industrially produced (Diebold 2003). Each form has different chemical activities and properties, and hence different industrial uses. The rutile form is less reactive, has a greater ability to absorb ultraviolet (UV) radiation, and is thus suitable in the paints and cosmetic industries (Nowack and Bucheli 2007), while the anatase form is able to produce significant reactive oxygen species (ROS) levels under UV irradiation and hence is suitable for the water treatment industry (Ju-Nam and Lead 2008). TiO_2 NPs are organized either in fine (>100 nm) or ultrafine (<100 nm) sizes (Ma *et al.* 2010).

TiO_2 NPs also exhibited a value-added property of antimicrobial function. In previous studies, researchers reported that TiO_2 NPs exhibited significant toxicity effects against both Gram-positive (Adams *et al.* 2006; Yeung *et al.* 2009) and Gram-negative (Maness *et al.* 1999; Rincón and Pulgarin 2004; Fu *et al.* 2005) bacteria, as well as algae (Aruoja *et al.* 2009), zebrafish (Xiong *et al.* 2011), and mice (Ma *et al.* 2010). The usefulness of the antibacterial effect of TiO_2 NPs was used in the disinfection of water contaminated with *Escherichia coli (E. coli)* (Maness *et al.* 1999; Kubo *et al.* 2005). The already published studies on bacterial activity have demonstrated that TiO_2 NPs can kill bacteria even at low concentrations (Fu *et al.* 2005), in which the photocatalytic process of TiO_2 NPs causes fatal damage to microorganisms (Sunada *et al.* 1998).

Only a few studies on the antifungal effect of NPs were found in scientific publications. Nevertheless, researchers only focused on the silver (Ag) (Kim *et al.* 2009; Panáček *et al.* 2009; Monteiro *et al.* 2013) and zinc (Zn) (Lipovsky *et al.* 2011) NPs and their antifungal activity against fungal species, *Candida albicans* (*C. albicans*). Both NPs exhibited a strong antifungal effect toward the *C. albicans* in a concentration-dependent manner. By comparison, zinc is less potent than silver NPs in reducing *C. albicans* growth with a dose of 0.1 mg/mL (Lipovsky *et al.* 2011) and a dose of 0.4 μg/mL to 3.3 μg/mL (Monteiro *et al.* 2012), respectively. To date, access to the antifungal activity of most manufactured NPs remains extremely limited. Hence, in this paper, the antifungal activity of TiO_2 NPs against *C. albicans*, a prevalent human pathogen, was investigated using a trypan blue exclusion assay, which is a simple quantification method. The study covers the effect of different forms (anatase versus rutile) of TiO_2, their exposure times, and dosage concentrations on *C. albicans* culture.

EXPERIMENTAL

Materials

The sample of *C. albicans* was a donation from the Faculty of Medicine, Universiti Putra Malaysia (Serdang, Malaysia). The TiO_2 powders ranging from 70 nm to 200 nm (anatase and rutile) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The potato dextrose medium, trypan blue, and glycerol were also purchased from Sigma-Aldrich and used directly without any further purification. All aqueous solutions were prepared with deionized water.

Characterization of TiO_2 NPs

For imaging of the TiO_2 NPs, anatase and rutile TiO_2 NPs powders were mounted onto the stub and coated with gold. Then, the samples were analyzed with a SEM

(scanning election microscope) (S-3400N, Hitachi, Tokyo, Japan) and EDX (energy dispersive x-ray) (S-3400N, Hitachi, Tokyo, Japan).

Preparation of TiO_2 Suspension

TiO_2 powder (50, 100, and 150 µg/mL) was suspended in deionized water. The suspensions were sonicated for 30 min to avoid any agglomeration. The suspension was freshly prepared, ensuring homogeneity when added to the growing *C. albicans* culture.

TiO_2 on *C. albicans* Culture

C. albicans was cultured in a potato dextrose medium at 37 °C and 180 rpm on an orbital shaker. When the culture reached a mid-log phase (approximately after 24 h of incubation time), the homogeneous TiO_2 suspension was added. The samples were incubated for 96 h. Sampling was done at 0, 6, 24, 48, 72, and 96-h time points. The numbers of viable and non-viable cells were counted and compared with a negative control (cell culture without TiO_2).

Quantification of Cell Viability using Trypan Blue Staining Method

Viable and non-viable cells were counted using a hemocytometer cell counter. An aliquot of the 100 µL sample was gently mixed with 100 µL of a 0.4% trypan blue dye and left to stand for 5 min at room temperature. Then, 20 µL of the cell suspension was loaded into each chamber of the hemocytometer and analyzed under a 40× magnification of light microscope (DM3000, Leica, Wetzlar, Germany). Both viable (unstained) and non-viable (stained) cells in each of the five quadrants (1, 2, 3, 4, and 5) as shown in Fig. 1 were counted.

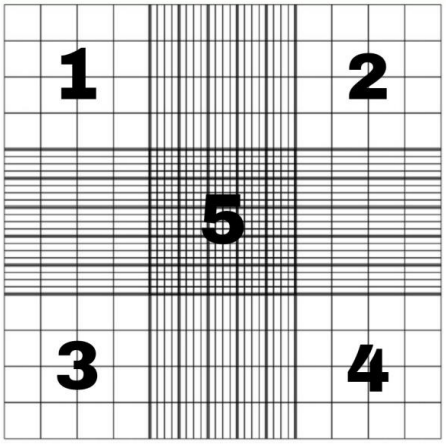

Fig. 1. Hemocytometer grid

The averages of these five readings were calculated and multiplied by 10^4 to obtain the number of cells per mL in the sample that were applied to the hemocytometer. The readings were multiplied by a ratio of trypan blue used in the solution and the dilution factors used in the original sample preparation on the cell suspension. The number of cells was calculated by Eq. 1.

$$\text{Cell concentration (cell/mL)} = \frac{\text{Cell number in five quadrants}}{5} \times 10^4 \times$$

$$2 \times \text{Dilution Factor} \quad (1)$$

Cell Morphology Observation

The cell culture was harvested *via* centrifugation. The samples were fixed in 2.5% glutaraldehyde for 6 h at 4 °C. The samples were centrifuged to remove the liquid form and washed three times with 0.1 M of a sodium cacodylate buffer. It was incubated for 10 min after each washing process. The samples were then post fixed in 1% of an osmium tetraoxide solution for two h at 4 °C and washed with 0.1 M of a sodium cacodylate buffer three times. Again, it was left to sit for 10 min after each washing process. It was then put into a critical dyer with CO_2 at 35 °C (EM CPD030, Leica) for 30 min, mounted onto the stub, and coated with gold. The samples were analyzed with a SEM (S-3400N, Hitachi, Tokyo, Japan). For the TEM (transmission electron microscope) analysis, the specimen were infiltrated with an acetone and resin mixture, placed into a beam capsule, and filled up with resin. It was then polymerized in an oven at 60 °C for 24 h. The specimen was cut and stained with uranyl acetate for 15 min before observation under a TEM (JEM-2100F, JEOL Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

The Effect of TiO_2 NPs on *C. albicans* Growth

The study on antifungal activity of TiO_2 (in a form of rutile and anatase) against *C. albicans* growth was performed by dosing the culture at their log phase of growth (Fig. 2) with 100 ppm of TiO_2. In the absence of TiO_2 (indicated as a control in Fig. 2), the culture showed a typical growth curve in which the sample continued to grow at an exponential phase pattern until 48 h, followed by a stationary phase at 48 h to 72 h, and subsequently entered the death phase with a gradual decrease in the viable cell count. The calculated specific growth and death rate for this control culture was 0.0146 h^{-1} and 0.0188 h^{-1}, respectively (Table 1). The culture dosed rutile experienced some reduction in their log phase with until 48 h of incubation time, before entering the death phase. Anatase NPs were the most toxic toward the *C. albicans* culture as it had the highest death rate of 0.0223 h^{-1} when compared to rutile (0.0119 h^{-1}) NPs. At the end of the incubation time, rutile TiO_2 NPs inhibited 33% of the *C. albicans* culture as compared to the control, while the anatase TiO_2 caused 65% of the growth inhibition.

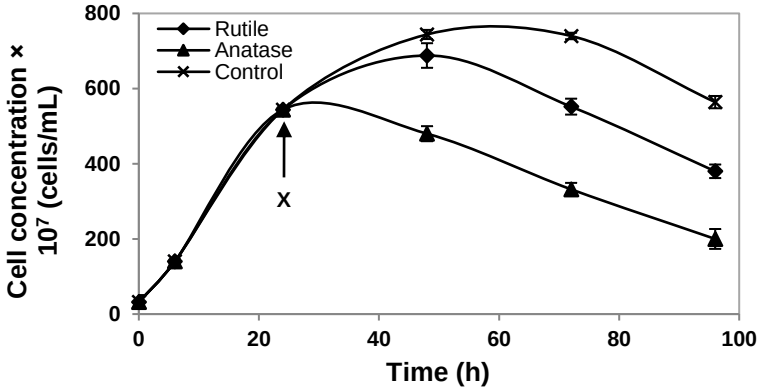

Fig. 2. Growth of the *C. albicans* cells without and with anatase and rutile TiO_2 NPs exposure at 37 °C for 96 h and at a 180-rpm shaking speed; growth inhibition was determined by Trypan blue staining using a hemocytometer cell counting method and expressed as a percentage of the control; the variable, *X,* was defined as the time of the NPs dosage; error bars represent the standard error of the mean.

Table 1. Growth Phase and Kinetics of *C. albicans* Culture

NPs	Log phase (h)	Stationary phase (h)	Death phase (h)	Growth rate, μ_g (h^{-1})	Death rate, $-k_d$ (h^{-1})	Final cell concentration $\times 10^7$ (cell/mL)	Percentage of viable cell reduction upon TiO_2 exposure (%)
Control	24-48	48-72	72-96	0.0146	0.0188	564 ± 1.732	0
Anatase	-	24-26	26-96	-	0.0223	200 ± 1.000	65
Rutile	24-48	-	48-96	0.0100	0.0119	380 ± 1.732	33

Both the anatase and rutile TiO_2 NPs induced a toxicity effect at different magnitudes, leading to a reduction in the viable cell. Based on the result (Table 1), it can be clearly seen that anatase was the most toxic toward *C. albicans* as compared to rutile. Anatase had the least number of viable cells with a final cell concentration of 200±1.000 $\times 10^7$ cells/mL. Few researchers reported that anatase NPs tend to be more toxic than rutile when tested on different cell cultures, such as mammalian (Gurr *et al.* 2005), *E. coli* (Sunada *et al.* 1998)**,** and *Bacillus megaterium (B. megaterium)* (Sunada *et al.* 1998). This statement agreed well with the findings in this study.

When comparing the toxicity of anatase and rutile, several factors can be considered. The SEM image (Fig. 3) showed the morphology of anatase and rutile NPs. It was found that the size of anatase NPs (70 nm to 130 nm) was generally smaller than the rutile (90 nm to 200 nm) particles. A similar observation was made by Reyes-Coronado *et al.* (2008) and Gopal *et al.* (1997), in which they observed that anatase was smaller than rutile NPs. Anatase NP had a mean particle diameter of 13 nm and a relatively narrow size distribution; whereas, rutile consisted of relatively large particles that can form spheroids, which were upwards of 200 nm. Note that, smaller particles have higher surface areas and particle numbers per unit mass, and this may contribute to the higher antifungal activity observed in this study. Previous work by Pal *et al.* (2007) found that smaller particles caused significant reduction in cell viability due to a larger contact surface area, and therefore have a higher tendency to induce oxidative damage (Gurr *et al.* 2005), causing more cell death. In a different study using a different organism, it was also found that the smaller size of TiO_2 NPs showed significant toxicity against nematode *Caenorhabditis elegans*, compared to the bulk particles which did not show any toxicity (Wang *et al.* 2009).

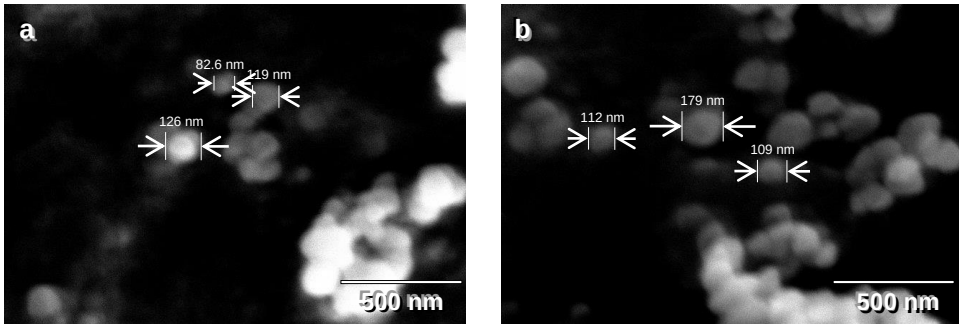

Fig. 3. Particle size of (a) anatase and (b) rutile TiO_2 NPs

Larger particles that consist of the aggregation of two or more NPs together might reduce the direct interaction between bacteria and NPs (Zhang *et al.* 2007; Das *et al.*

2011); hence they have fewer chances to mingle with cells, and as a consequence, reduce the cell wall penetration or membrane damage. An increase in the aggregation of the NPs will lead to a reduction in the surface area, which would reduce the surface available for the interaction of cells and NPs, resulting in a lower toxicity effect. This statement was in agreement with work presented by Zhang *et al.* (2007) and Das *et al.* (2011). Rutile was observed to form larger aggregates with relatively smaller surface areas, whereas anatase was indicated to have lower aggregates particles (Reyes-Coronado *et al.* 2008). This finding explained why anatase was more toxic to microbes in comparison to rutile under the same incubation conditions and agreed well with the current work.

The size of NPs alone is not the only factor contributing to the toxicity and explains why the rutile and anatase forms of TiO_2 behave differently. It seems likely that the particle chemistry played an important role in affecting the toxicity of the TiO_2 NPs, based on some findings by other researchers. Anatase with a band gap of ~3.2 eV (Luttrell *et al.* 2015) exhibited higher photocatalytic activity compared to rutile (Gurr *et al.* 2005) with a band gap of ~3.0 eV (Luttrell *et al.* 2015). The photocatalytic activity of the anatase was approximately 1.5 times higher than that of the rutile form (Kakinoki *et al.* 2004).

Concentration-dependent Antifungal Activity of TiO_2 NPs

TiO_2 NPs at different concentrations (50, 100, and 150 μg/mL) were dosed into *C. albicans* culture, and their effect was measured after 48 h. Figure 4 showed an increase in growth inhibition with the increase in the TiO_2 NPs dose for both anatase and rutile. At a low dosage (50 μg/mL) of rutile TiO_2, the cell exhibited tolerance with no significant cell loss at only 3%. Similarly, acceptable tolerance was observed for the culture dose with anatase TiO_2, with a slightly higher cell loss at 17%. However, at higher doses (100 and 150 μg/mL), both anatase and rutile TiO_2 caused a significant toxicity effect on the cells. Anatase at 100 μg/mL and 150 μg/mL resulted in a 35% and 57% cell loss, respectively. Whereas, for rutile, the dose of 100 μg/mL and 150 μg/mL lead to a lower cell loss as compared to anatase, at 8% and 48%, respectively.

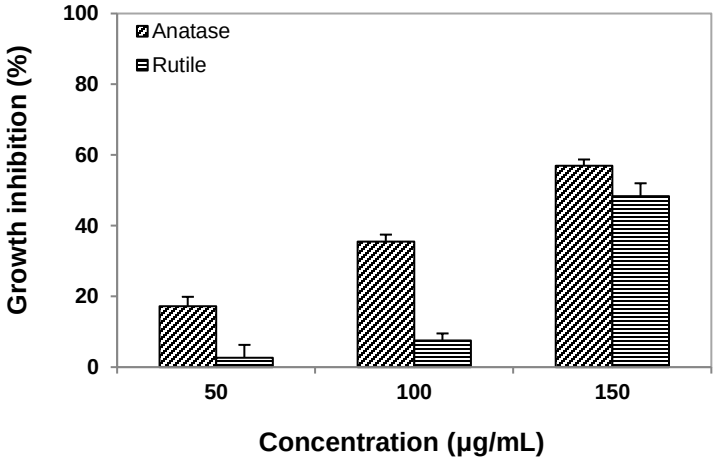

Fig. 4. Growth inhibition of *C. albicans* treated with anatase and rutile TiO_2 NPs at 37 °C for 24 h at a 180-rpm shaking speed; growth inhibitions were determined by Trypan blue staining using a hemocytometer cell counting method and expressed as a percentage of the control; error bars represent the standard error of the mean.

The results agreed with findings previous research using different varieties of NPs on different living organism (Rincón and Pulgarin 2004; Brayner *et al.* 2006; Hu *et al.* 2010; Das *et al.* 2011; Tu *et al.* 2013; Akhavan *et al.* 2015). It was concluded that the antimicrobial activity of NP was concentration-dependent. Fu *et al.* (2005) reported that by increasing the concentration of anatase TiO_2 from 0.5 mM to 5 mM, the growth of both *E. coli* and *B. megaterium* were effectively inhibited. Research by Maness *et al.* (1999) revealed that in the presence of 0.1 mg of TiO_2, NPs killed 98% of *E. coli* cells, but lower concentrations did not kill the cells effectively. As suggested earlier, regarding the factors affecting the antibacterial activity of NPs, a generation of ROS also played an important role. Xiong and coworkers (2011) reported that the rate of hydroxyl (OH) radical generation by NPs increased with an increasing NP concentration. At a concentration of 50 mg/L, TiO_2 NPs did not produce mortality in zebrafish. However, 300 mg/L of TiO_2 NPs caused 100% mortality of the zebrafish.

In other research, *C. albicans* had an antifungal effect toward various NPs at different magnitudes (Kim *et al.* 2009; Panáček *et al.* 2009; Lipovsky *et al.* 2011; Monteiro *et al.* 2012; Li *et al.* 2013). The Ag NP exerted a strong antifungal effect against *C. albicans* cells at very low concentrations (0.4 μg/mL to 3.3 μg/mL) (Monteiro *et al.* 2012). Additionally, 97.5% of cell reduction was observed when *C. albicans* was exposed to 100 μg/mL of the zinc oxide (ZnO) NP, while almost complete cell reduction was recorded at 1000 μg/mL ZnO NP exposure (Lipovsky *et al.* 2011). When comparing the previous result with our findings, TiO_2 had the least antifungal activity as compared to Ag and ZnO because at 100 μg/mL, both anatase and rutile TiO_2 NPs only killed 35% and 8% of the *C. albicans*, respectively. Based on the previous findings, it was demonstrated that other materials may exert the same effect in different magnitudes towards the *C. albicans* cell, while some researchers also recorded similar trends over various types of cells using different NPs.

Time-dependent Antifungal Activity of TiO_2 NPs

The time-dependent antibacterial activity of NPs was evaluated by incubating the *C. albicans* culture with the addition of 50 μg/mL TiO_2 (anatase and rutile) NPs at the mid log phase of their growth (24 h) and leaving the sample to further incubate for 96 h. Figure 5 shows the percentage of cell viability versus time for *C. albicans* dosed with anatase and rutile NPs.

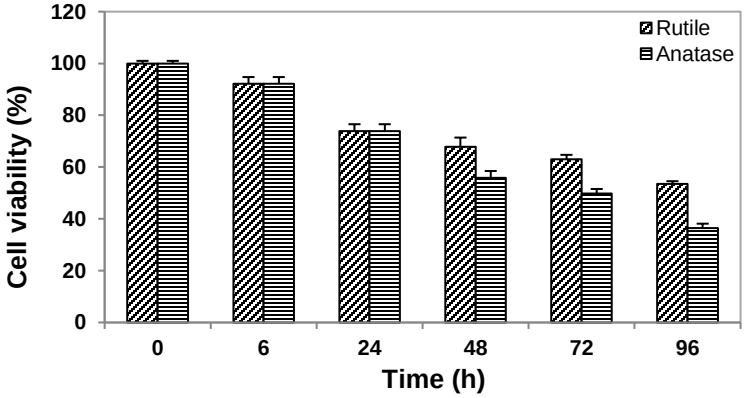

Fig. 5. Percentage of *C. albicans* remaining after treatment with rutile and anatase TiO_2 NPs at 37 °C and a 180 rpm shaking speed; cell viability was determined through a hemocytometer cell counting method and expressed as a percentage of the control; error bars represent the standard error of the mean.

After exposure of anatase in the *C. albicans* culture for 24 h of incubation time, only 56% of the cells remained viable. Prolonged incubation (72 h) led to further reduction in the cell viability to 36%. It was observed that this trend was also similar when *C. albicans* cells were exposed to rutile TiO_2 NPs. Cell viability reduced from 74% to 68% after 24 h of NP exposure. Cell viability continued to drop to 63% and 54% after 48 h and 72 h of NP exposure, respectively.

The viability of cells treated with NPs decreased with increasing incubation time. By increasing the incubation time, NPs had more of a chance to have contact with the cells, attach to the cell membrane, and therefore caused more membrane damage leading to cell death (Stoimenov *et al.* 2002). This trend was similar with the findings of other researchers (Liu *et al.* 2011; Raghupathi *et al.* 2011; Gurunathan *et al.* 2012; Li *et al.* 2013), which suggested that the antibacterial activity of NPs is time-dependent. Rincón and Pulgarin 2004 reported that for high cell mass populations, longer exposure time is required for bacterial inactivation.

Morphological Assessment of Cell Morphology When Exposed to NPs

Morphological changes in all samples were monitored after treatment with 100 µg/mL of NPs for 96 h. For normal *C. albicans* (Fig. 6a), its morphology consists of mycelia and rod shapes. Cell-treated NPs became abnormal in size, displaying cellular shrinkage, and had irregular shape. Cells treated with anatase TiO_2 (Fig. 6b) suffered from chronic cell membrane damage due to the high toxic impact that took place. Most of the cells become flattened and lost their cellular integrity after being exposed to anatase TiO_2. Meanwhile, rutile TiO_2 (Fig. 6c) had a minimal effect towards the *C. albicans* culture because some of the cells remained integral. Size of the treated *C. albicans* culture did not change, but only surface breakage occurred. To confirm this phenomenon, TEM analysis was conducted to examine the membrane defects upon treatment with NPs. From TEM analysis, untreated *C. albicans* consist of a rod shape with a smooth surface structure (Fig. 6d); whereas, cell-treated anatase NPs (Fig. 6e) cannot retain its original shape as compared to untreated cells. Direct contact of NPs could influence the cellular integrity of the cells and cause cell burst, with the cell membrane being severely destroyed, and the cytoplasm and cell constituents flowing out (Stoimenov *et al.* 2002; Kang *et al.* 2008; Tu *et al.* 2013). The degradation of the cell wall and the loss of intracellular materials were observed after the exposure of anatase NPs to the cell culture.

The presence of elemental titanium on the bacterial cells was shown on the EDX analysis as a green colored region on the image (Fig. 7). It was found that a strong physical attachment of TiO_2 was evident, even after an attempt to dissociate the NPs from the cell surface via a vigorous rinsing of the cell suspension took place. TiO_2 failed to dissociate the later from the cell surface due to opposite charges between the bacteria and NPs. One possible reason is that opposite charges caused adhesion of NPs to the cell surface. NPs were tightly bound with the cell surface due to the electrostatic force (Stoimenov *et al.* 2002; Hu *et al.* 2009; Nair *et al.* 2009). This result was correlated well with a study by Morones *et al.* (2005) in which the difference in charge between bacteria and NPs might have caused the strong interaction between them. However, based on the EDX mapping and analysis, the percentage of attachment for both of the NPs were not significant even though anatase exerted a stronger antifungal effect compared to the rutile TiO_2.

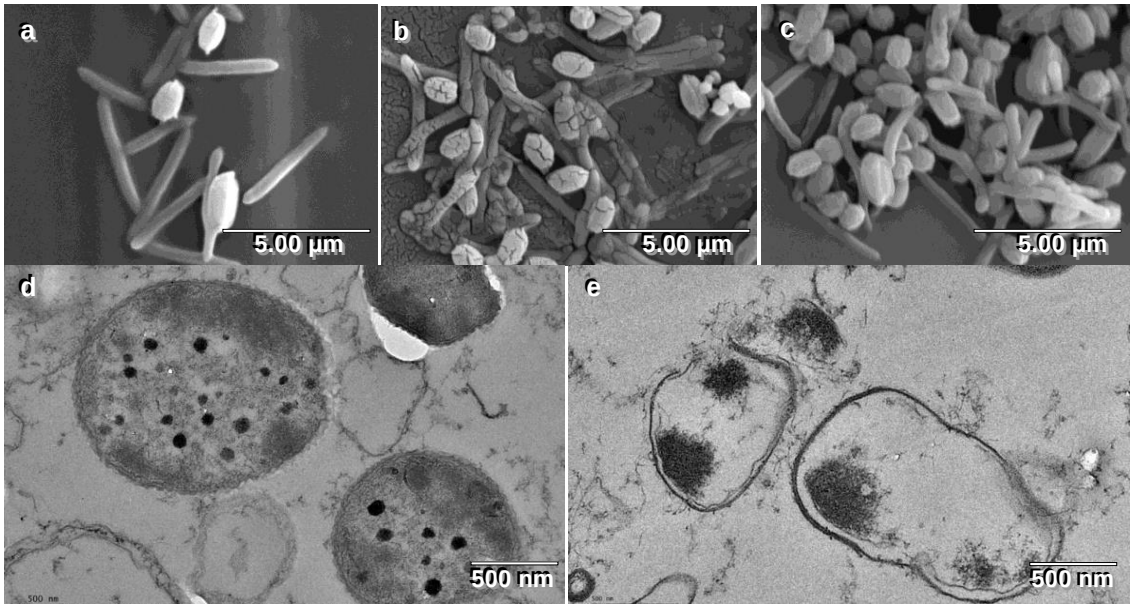

Fig. 6. Electron micrographs for a) surface scanning image of untreated *C. albicans*; b) surface scanning image of *C. albicans* after exposure with anatase TiO_2 NP; c) surface scanning image of *C. albicans* after exposure with rutile TiO_2 NP; d) transmission cross-sectional image of untreated *C. albicans* cell, and e) transmission cross-sectional image of *C. albicans* after exposure with anatase TiO_2 NP

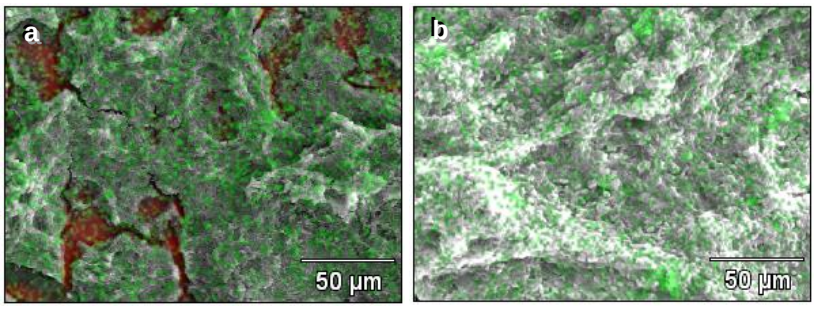

Fig. 7. EDX mapping and spectrum of *C. albicans* exposed to (a) anatase and (b) rutile TiO_2 NPs

The mechanism by which the NPs are able to cause the cell membrane damage and lead to cell death is not fully understood, but the present study suggests that when cells were treated with NPs, changes took place in its membrane, leading to a major increase in its permeability. Nevertheless, different mechanisms of action taking place within the cells upon exposure to TiO_2 has been suggested by many researchers (Cho *et al.* 2004; Rincón and Pulgarin 2004; Yeung *et al.* 2009; Xiong *et al.* 2011). Cells were reported to generate higher amounts of ROS (Hussain *et al.* 2005; Yeung *et al.* 2009) and OH radicals (Cho *et al.* 2004; Rincón and Pulgarin 2004) when exposed to NPs. This lead to an increment in lipid peroxidation (Ma *et al.* 2010), inflammatory cells (Ma *et al.* 2010), and a reduction in the mitochondrial function of the cell (Hussain *et al.* 2005), eventually causing cell death (Cho *et al.* 2004).

CONCLUSIONS

1. This study demonstrated TiO_2 nanoparticles (NPs) in the form of anatase and rutile displayed excellent antifungal potential towards *C. albicans.*
2. The loss of cell viability was concentration- and time-dependent.
3. Morphological analysis suggests a direct interaction between TiO_2 and cells as the main mechanism causing cell death.
4. Although the findings of this study suggested that TiO_2 NPs had antifungal activity against *C. albicans*, their uncontrolled released to the environment may harm the ecosystem.

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