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Size of TiO₂ nanoparticles influences their phototoxicity: an in vitro investigation

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Abstract

To uncover the size influence of TiO₂ nanoparticles on their potential toxicity, the cytotoxicity of different-sized TiO₂ nanoparticles with and without photoactivation was tested. It was demonstrated that without photoactivation, TiO₂ nanoparticles were inert up to 100 µg/ml. On the

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contrary, with photoactivation, the toxicity of TiO₂ nanoparticles significantly increased, which correlated well with the specific surface area of the particles. Our results also suggest that the generation of hydroxyl radicals and reactive oxygen species (ROS)-mediated damage to the surface-adsorbed biomolecules could be the two major reasons for the cytotoxicity of TiO₂ nanoparticles after photoactivation. Higher ROS generation from smaller particles was detected under both biotic and abiotic conditions. Smaller particles could adsorb more proteins, which was confirmed by thermogravimetric analysis. To further investigate the influence of the generation of hydroxyl radicals and adsorption of protein, poly (ethylene-*alt*-maleic anhydride) (PEMA) and chitosan were used to coat TiO₂ nanoparticles. The results confirmed that surface coating of TiO₂ nanoparticles could reduce such toxicity after photoactivation, by hindering adsorption of biomolecules and generation of hydroxyl radical ($\cdot\text{OH}$) during photoactivation.

Keywords

Titanium dioxide nanoparticles; Phototoxicity; Cytotoxicity; Nanotoxicity; Surface coating

Introduction

Nanomaterials possess unique properties, arising from their minute sizes, large surface areas and high surface reactivity, and have thus been explored for a wide range of applications such as in sporting goods, cosmetics and electronics (Maynard et al. 2006; Xia et al. 2006; Nel et al. 2006). It was estimated that there are at least 1,300 commercially available products that contain nanomaterials (McCall 2011). Because of this, direct or indirect human contact with nanomaterials becomes inevitable, ultimately raising issues pertaining to their safety (Oberdörster et al. 2005; Nel et al. 2006; Morris et al. 2011; Meng et al. 2009). For example, zinc oxide (ZnO) and titanium dioxide (TiO₂) nanoparticles are known active components in most sunscreens. Studies have therefore focused on their cytotoxicity and safety, of which there are studies reporting that ZnO nanoparticles can cause cytotoxicity, resulting from the generation of ROS, mitochondrial depolarization and intracellular calcium ion disturbance (Ng et al. 2011; George et al. 2009; Heng et al. 2010a, b). ZnO nanoparticles have also been shown to induce genotoxicity, which have been reported to be likely due to the release of Zn²⁺ ions (George et al. 2009; Ng et al. 2011).

TiO₂, which is considered as non-soluble, is widely used in the form of nanoparticles across industrial and consumer goods, including cosmetics, paints and food additives, mainly due to their ability to confer opacity and whiteness in different applications (Skocaj et al. 2011; Araujo and Nel 2009). In contrast to ZnO, TiO₂ was classified as a biologically inert material, as reported in some studies (Lindenschmidt et al. 1990; Ophus et al. 1979). TiO₂ was even considered as a “natural” material and is generally positively accepted by the public (Skocaj et al. 2011). However, since 1985 it has been shown that photoactivated TiO₂ nanoparticles do possess antimicrobial properties (Skocaj et al. 2011). TiO₂ (anatase) can be excited by light with wavelength shorter than 385 nm (Maness et al. 1999). The photons, with sufficient energy, can excite the electrons from the valence band to conduction band, thus generating electron–hole pairs, which will actively react with adsorbed water or oxygen to produce cell-damaging ROS (Bar-Ilan et al. 2011; Hoffmann et al. 1995). ROS

generation is detrimental to many species such as fish and its role in many pathological conditions in human beings is also well documented. Reeves et al. (2008) observed increased damage in TiO₂-treated fish cells after UVA activations, whereby the hydroxyl radicals generated (extracellular and intracellular) were likely responsible for this damage. Nakagawa et al. (1997) also pointed out the potential photogenotoxicity of TiO₂ nanoparticles to Chinese hamster cell line. In another study, Gopalan et al. (2009) reported that TiO₂ tend to introduce dose-dependent photogenotoxic effect to human lymphocytes but not to human sperms.

However, how particle properties such as size, shape and crystal structure could influence the potential phototoxicity of TiO₂ nanoparticles has not been sufficiently investigated and the possible mechanism has not been fully understood. Jang et al. (2001) observed an inverse relationship between TiO₂ particle size and antimicrobial effect after photoactivation, but did not provide the probable mechanism behind this phenomenon. By understanding how a nanomaterial property has an influence on biological activity will provide us an insight on the possible mechanisms behind the toxicity of nanoparticles, and with this understanding find ways to modify their properties and design safer nanomaterials.

In this study, we tested the cytotoxicity of different-sized TiO₂ nanoparticles with and without photoactivation. The particles were photoactivated with UV and near-Vis light, ranging from 280 to 450 nm, for 5 min. The short irradiation time was chosen to minimize the toxicity due to light inhibition. Characterization of the nanoparticles, cell viability, generation of ROS and mitochondrial depolarization were conducted to understand the relationship between the nanoparticle properties and their related toxicities. A potential method to decrease such phototoxicity of TiO₂ nanoparticles was also proposed and examined.

Materials and methods

Preparation of nanoparticles

TiO₂ nanoparticles with primary particles size of 10 nm (T10), 20 nm (T20) and 100 nm (T100) were purchased from SkySpring Nanomaterials Inc., Evonik Industries and MKnano Inc., respectively. All PEMA-coated TiO₂ (T20-PEMA) and chitosan-coated TiO₂ (T20-chitosan) nanoparticles were synthesized in house. PEMA and chitosan were purchased from Sigma and Sinopharm Chemical Reagent Co. Ltd, respectively. In brief, 200 mg of TiO₂ nanoparticles (T20) was dispersed in 20 ml of methanol with ultrasonication in an ultrasonic cleaner (MRC laboratory instruments Inc., Holon, Israel) for 30 min. The TiO₂ nanoparticles suspension was added into 18 ml of 0.1 % PEMA or chitosan in water. After magnetically stirring for 24 h to evaporate the methanol and allow the coating of PEMA and chitosan on T20 nanoparticles, the samples were freeze-dried for 48 h.

Characterization of TiO₂ nanoparticles

Transmission electron microscopy (TEM)—The primary size of TiO₂ nanoparticles was characterized with transmission electron microscope (TEM, JOEL 2010, Japan). The TiO₂ nanoparticles were first dispersed in methanol and ultrasonicated for 30 min. The

dispersed nanoparticles were dropped onto a carbon-coated copper grids and observed under TEM at an accelerating voltage of 200 kV.

Hydrodynamic size and surface charge of particles—The hydrodynamic size and surface charge of the particles were characterized using dynamic light scattering (DLS) technique. The nanoparticles in powder form were dispersed into stock suspension (3 mg/ml) in water or cell culture medium. The stock solution was ultrasonicated for 10 min and then further diluted in water and cell culture medium at a concentration of 30 µg/ml. Further ultrasonication was conducted for another 10 min just before carrying out DLS measurement using a ZetaPALS zeta potential analyzer (Brookhaven instruments, USA).

Theoretical surface area and Brunauer–Emmett–Teller (BET) surface area—The theoretical surface area of TiO₂ nanoparticles was calculated through the true density and diameter of the nanoparticles as from Eq. (1) (Jang et al. 2001).

$$S = 6/\rho d \quad (1)$$

Where S represented the theoretical surface area, ρ was the true density of materials, and d was the mean diameter of nanoparticles. The true density of TiO₂ was estimated to be 3.90 g/cm³ (Tanaka and Suganuma 2001) according to the information obtained from the suppliers.

The Brunauer–Emmett–Teller (BET) surface area was tested using micromeritics surface area analyzer (ASAP 2000, USA). The powder of TiO₂ nanoparticles was degassed at 200 °C in flowing nitrogen for 4 h prior to nitrogen adsorption.

In vitro cytotoxicity study

Cell culture—The immortalized mouse macrophage cell line RAW264.7 (ATCC # TIB-71) cells were cultured in cell culture medium that is composed of 88 % Dulbecco's modified Eagle medium (DMEM) (Gibco), 10 % fetal bovine serum (FBS) (Benchmark), 1 % sodium pyruvate (Hyclone) and 1 % penicillin–streptomycin (Hyclone). The cells were incubated in a humidified atmosphere containing 5 % CO₂ at 37 °C and sub-cultured every 2 days until they reached 70–80 % confluence.

Dispersion of TiO₂ nanoparticles—The nanoparticles in powder form were dispersed in cell culture medium into stock suspension (3 mg/ml). This stock suspension was ultrasonicated in water bath for 10 min. The stock suspension was further diluted into a working suspension (100 µg/ml), which was ultrasonicated for another 10 min before adding into cells.

Treatment of nanoparticles with/without photoactivation—RAW264.7 cells at concentration of 20,000 cells/cm² were plated in 384-well black plate with transparent bottom and were allowed to grow for 24 h at 37 °C with 5 % CO₂ in a humidified atmosphere. The working suspension of dispersed nanoparticles were added into each well and incubated with cells for another 24 h. For the test groups with UV–Vis exposure, the

excess nanoparticles that did not enter or attach onto cells were washed away with 40 μ l of PBS using a plate washer, for three times at 21 h. After the wash, cells in each well with 25 μ l of PBS were placed under a 100 W xenon arc lamp (LAX-cute, Asahi Spectra) with a light filter to transmit light in the wavelength between 280 and 450 nm. The cells were then exposed to UV-Vis light for 5 min. Finally, another 25 μ l of cell culture medium was added into each well followed by 3-h incubation in standard cell culture conditions.

Cellular response characterization—Cytotoxicity parameters including plasma membrane damage, mitochondrial superoxide generation and mitochondrial depolarization were recorded utilizing high-throughput screening method (George et al. 2009). Propidium iodide (PI) nucleic acid stain (Invitrogen, P3566) was utilized to probe the loss of cell viability. The loss of cell viability decreased the integrity of cell membrane, which results in the uptake of PI to stain the cell nucleus. In order to understand the role of intracellular ROS and oxidative stress in causing cell death, MitoSOX (Invitrogen, M36008) was used to detect the generation of superoxide in mitochondria. We also assayed mitochondrial depolarization using JC-1 (Invitrogen, T3168). Hoechst (Invitrogen, H3570) was a membrane-permeable dye that could bind on the nucleic acid to indicate the location and total number of both live and dead cells. The applicability of our HTS assay platform for studying the cytotoxicity of nanoparticles according to hierarchical oxidative stress paradigm has been reported earlier (George et al. 2011a; Xia et al. 2006, 2008). In brief, the cells were washed twice with PBS before adding in 25 μ l dye cocktail (Table 1) to incubate for 30 min in the absence of light in standard 37 °C incubator. The fluorescent images were taken by an automated epifluorescence microscope, Image-Xpress^{micro} (Molecular Devices, Sunnyvale, USA) under 10 $\times$ magnifications. The percentage of cells showing positive signals was automatically calculated using Meta-Xpress software.

Abiotic hydroxyl radical generation test

Hydroxyphenyl fluorescein (HPF, H36004) was utilized in this study to probe the hydroxyl radical generation from the TiO₂ nanoparticles under UV-Vis exposure. HPF working solution (100 μ M, 5 μ l/well) was added into 384-well plate with nanoparticles suspension (100 μ g/ml, 45 μ l/well) in DI water. The fluorescent intensity (F_0) was tested immediately at Excitation/Emission wavelength of 490/515 nm using SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices Corp., USA). After UV-Vis exposure (280–450 nm) for 5 min, the fluorescent intensity (F_1) in each well was tested again. The rate increase in fluorescent intensity per minute was calculated through Eq. (2).

$$\text{Rate increase of fluorescent intensity (} / \text{ min)} = (F_1 - F_0) / 5 \text{ min} \quad (2)$$

Protein adsorption study

TiO₂ nanoparticles were dispersed in DMEM solution with bovine serum albumin (BSA) at a concentration of 2 mg/ml. T20-PEMA and T20-chitosan were washed three times with DI water beforehand to remove unbound PEMA and chitosan. The particles (1 mg/ml) were ultrasonicated for 20 min in BSA-DMEM solution. All of the particles were collected by centrifugation at 14,000 rpm for 20 min at 25 °C and washed with DI water for 3 time before

freeze-drying for 48 h. Thermo Gravimetric Analyzer (TGA, 2950, HR, V5.4A) was used to test the BSA attached on different particles at a heating rate of 20 °C/min in an nitrogen atmosphere.

Statistics

All quantitative data are shown as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) and Tukey's pairwise comparisons were utilized for multiple comparisons. Significant difference was considered when $p < 0.05$. All tests were carried out four times.

Results

Characterization of nanoparticles

TiO₂ nanoparticles of three different sizes were found to be near-spherical in shape as shown from TEM images (Fig. 1). The primary particle sizes were 10 nm (T10), 20 nm (T20) and 100 nm (T100). The hydrodynamic size, polydispersity index (PDI) and zeta potential of the nanoparticles were tested through DLS in both DI water and cell culture medium. The results were summarized in Table 2. All the particles showed size in DI water and cell culture medium in the range from 200 to 700 nm. Although different particles exhibited different surface charge in DI water, the zeta potentials in cell culture medium were similar and lie in the range from -5 to -10 mV. This could be due to the surface binding of proteins from cell culture medium, rendering them to have similar zeta potential values. All of the three particles are mainly composed of anatase except T20 particles, which contain 19 % rutile. The BET surface area measurements showed that the smaller particles had larger specific surface area. The BET surface areas were found to be similar to the theoretically calculated surface areas [from Eq. (1)] which were calculated based on the size of different particles and the true density of the materials. This result confirmed the primary size of TiO₂ nanoparticles measured from TEM analysis.

The phototoxicity of TiO₂ nanoparticles was size- and surface area-dependent

RAW 264.7 cell line is used in this investigation primarily as a biological model system to illustrate the mechanism of action TiO₂ nanoparticles under light activation. It is a well-understood cell line model for nanotoxicology studies (George et al. 2011a; Xia et al. 2006, 2008; Zhao et al. 2010, 2012; Xiong et al. 2011). Moreover, we have shown the utility of RAW 264.7 cells to illustrate the role of bandgap energy of TiO₂ nanoparticles in determining their phototoxicity under near-visible light activation (George et al. 2011a). The negative control (NC) received neither particles nor UV-Vis light, while light control (LC) received UV-Vis light only. From Fig. 2, LC showed some damage to cells when compared with NC, but is not statistically significant ($p > 0.05$). This meant that the RAW264.7 cells exposed to UV-Vis light (280–450 nm) for 5 min did not cause any significant cytotoxicity. For the cells treated with non-photoactivated nanoparticles only, the cytotoxicity of the nanoparticles alone is not significant, which is less than 1 % for the percentage of positive cells in PI uptake and MitoSOX tests. A slight increase in JC-1 signals (2.5 %) was observed in the T10-treated (smallest size) group. However, after photoactivation, all particle-treated cells exhibited obvious increase in PI uptake, which is indicative of cell death. The mortality of T10-treated RAW264.7 cells increased to ~30 % after photoactivation, which was

significantly higher than the mortality of cells treated with larger particles T20 (9.5 %) and T100 (9.0 %) with photoactivation. A similar trend in mitochondrial superoxide was also observed to increase, revealing the possible underlying mechanism of cell death—due to generation of reactive oxygen species (ROS) and subsequent induction of oxidative stress in cells. From Fig. 2, it is evident that T10-treated cells, with photoactivation, showed a higher generation of mitochondrial superoxide when compared to the larger particles of T20 and T100. The JC-1 test revealed a slight decrease in mitochondrial membrane potential compared with non-activated groups. This meant that mitochondrial depolarization could be one of the damages caused by the phototoxicity of TiO₂ nanoparticles but it may not be the main reason responsible for the death of cells.

An inverse relationship between phototoxicity and the size of TiO₂ nanoparticles was observed. This was consistent with the fact that smaller particles had larger surface area per unit mass compared to larger particles. Thus, we hypothesized that the higher cytotoxicity induced by smaller particles is related to their higher surface area and thus a larger number of surface-exposed TiO₂ molecules. Particle concentration was then converted from weight/ml into surface area/ml, which was 166.0, 50.4 and 17.2 cm²/ml for T10, T20 and T100, respectively. From Fig. 3, the cytotoxicity results showed excellent correlation with the surface area, because the R^2 for PI uptake, MitoSOX and JC-1 results were 0.967, 0.917 and 0.966, respectively. These observations indicated that cell membrane damage, ROS generation and mitochondrial depolarization caused by photoactivated TiO₂ nanoparticles were proportional to the surface area of nanoparticles.

Surface coating of TiO₂ nanoparticles with PEMA or chitosan decreased phototoxicity

Since it was shown that phototoxicity was correlated with exposed surface area, it was further hypothesized that the toxic effects would be minimized via coating the surface of TiO₂ nanoparticles. To eliminate the influence of surface charge, we chose two differently charged materials to coat the TiO₂ nanoparticles (T20), which are negatively charged PEMA and positively charged chitosan. After surface coating with PEMA or chitosan, the Zeta potential of T20 nanoparticles changed from 32.3 to -50.4 mV and 51.4 mV respectively (Table 2), showing the successful coating of PEMA or chitosan onto the surface of T20 nanoparticles. From PI uptake results shown in Fig. 4a, the mortality of photoactivated coated T20 nanoparticles decreased significantly ($p < 0.05$). Similarly, MitoSOX staining results showed that cells with mitochondrial peroxide generation also decreased in both T20-PEMA-and T20-chitosan-treated groups compared with T20 nanoparticles treated groups (Fig. 4b). Decreased mitochondrial damage was also observed in JC-1 results (Fig. 4c). In summary, the surface coating of TiO₂ nanoparticles, regardless of the charge of materials, could decrease the cytotoxicity of TiO₂ nanoparticles during photoactivation.

Hydroxyl radical generation was responsible for the phototoxicity of TiO₂ nanoparticles

Based on Fig. 5, smaller particles tend to have a higher rate of hydroxyl radical generation under photoactivation in abiotic conditions. This could be due to two reasons. First, for smaller particles, more photoactivated electrons and holes could reach the TiO₂ surfaces. Second, more molecules such as H₂O and O₂ are adsorbed onto TiO₂ surfaces to interact with these photoactivated electrons and holes to generate more hydroxyl radicals (Zhang et

al. 2010). Surface coating of T20 nanoparticles with PEMA or chitosan was shown to significantly decrease the generation of hydroxyl radicals ($p < 0.05$).

Protein adsorption was size dependent and decreased after surface coating of TiO₂ nanoparticles

It was shown that smaller particles tended to generate more hydroxyl radicals during photoactivation. The hydroxyl radicals are viciously reactive, which attacks whatever nearby and reacts at the site of formation (Vidosava 2004; Halliwell 1996). Intuitively, if smaller particles could adsorb more biomolecules onto their surface, this would increase the possibility that these biomolecules could be damaged by photoactivated hydroxyl radicals. BSA was therefore chosen as a model to understand the adsorption ability of biomolecules onto different-sized nanoparticles. BSA is a type of biomolecules which will gradually decompose with the increase in temperature; however, TiO₂ nanoparticles are relatively stable with temperature below 600 °C. So TGA analysis technique can be utilized to semi-quantify the amount of BSA attached onto TiO₂ nanoparticles (Simi and Abraham 2009). Figure 6 shows the TGA analysis of pure BSA, nanoparticles and nanoparticles-BSA. From Fig. 6a, a major weight loss of BSA protein was observed from 188 to 485 °C, corresponding to its decomposition temperature. Based on Fig. 6b, T10-BSA, T20-BSA and T100-BSA nanoparticles exhibited a much larger mass loss within this temperature range compared to their uncoated counterparts T10, T20 and T100 nanoparticles, corresponding to mass loss due to the adsorbed BSA. It was also shown that smaller particles (T10 and T20) adsorbed more BSA than larger particles (T100). From Fig. 6c, the T20-PEMA and T20-chitosan particles also showed a mass loss, which proved the successful surface coating of PEMA and chitosan onto T20 nanoparticles. Although there are mass loss observed for T20-PEMA and T20-chitosan nanoparticles, T20-PEMA, T20 and T20-chitosan nanoparticles still exhibited lower drop in mass at the decomposition temperature range of BSA as compared to counterpart T20-PEMA-BSA, T20-BSA and T20-chitosan-BSA nanoparticles. The difference in weight loss between BSA-adsorbed nanoparticles and corresponding nanoparticles indicated the amount of BSA attached on the particles, which was in the following sequences T20 > T20-chitosan > T20-PEMA. This result confirms that surface coating of T20 nanoparticles with PEMA or chitosan could decrease the BSA adsorption on T20 nanoparticles.

Discussion

The hypothesis of this study was that phototoxicity of TiO₂ nanoparticles was size dependent. We chose TiO₂ nanoparticles of different sizes to conduct this study. T20 is the most widely used and studied Degussa TiO₂ P25 nanoparticles, which composed of 81 % anatase and 19 % rutile (Ji et al. 2010). To understand the mechanism behind the phototoxicity of this particle can provide valuable information in the application of Degussa P25 nanoparticles. To study the size effect on the phototoxicity of TiO₂ nanoparticles, we chose another two particles T10 and T100, which composed of anatase. X-ray powder diffraction (XRD) patterns of T10, T20 and T100 nanoparticles were shown in supporting information fig. S1, which indicated the crystal structure of these three TiO₂ nanoparticles. RAW264.7 cells were treated with TiO₂ nanoparticles of different sizes with and without

UV–Vis excitation. The PI staining test exhibited that the lethal toxicity of TiO₂ nanoparticles after UV–Vis activation was size dependent. Smaller particles, with correspondingly larger surface area, tend to cause higher cytotoxicity as compared to larger particles (smaller surface area) of the same weight/ml concentration. To uncover the mechanism behind the phototoxic effect of TiO₂ nanoparticles, a multi-parametric cytotoxicity analysis was carried out. The intracellular perturbations included mitochondrial superoxide generation and mitochondrial depolarization. Based on Fig. 3, the mitochondrial superoxide production, mitochondrial depolarization and loss of cell plasma membrane integrity correlated well with the specific surface area of the particles ($R^2 > 0.9$). To understand the cause of oxidative stress in cells, we tested the generation of hydroxyl radicals during photoactivation of TiO₂ nanoparticles in an abiotic condition. The results of HPF test point out that oxidative stress in cells could be due to the ROS generated by photoactivated TiO₂ nanoparticles directly.

With our results, we present here a model to describe the potential mechanism of TiO₂ nanoparticles induced phototoxicity, as shown in Fig. 7. When TiO₂ nanoparticles are exposed to UV light, the photon energy excites the electrons (e^-) in the valence band to the conduction band and leave holes (h^+) in the valence band, giving rise to electron hole pairs (Maness et al. 1999). The holes (h^+) can interact with adsorbed H₂O or hydroxide ions (OH⁻) to generate hydroxyl radicals ($\cdot$ OH) which are highly reactive and damaging to cells (Dröge 2002), as hydroxyl radicals ($\cdot$ OH) can inflict detrimental damage on cellular proteins, lipids and even DNA, resulting in the oxidation and dysfunction of biomolecules (Maness et al. 1999; Almquist and Biswas 2002; George et al. 2011b; Circu and Aw 2010; Finkel and Holbrook 2000). Furthermore, the electrons (e^-) in the conduction band can reduce oxygen (O₂) adsorbed onto the particle surface to produce superoxide ions (O₂⁻), which can further react with water in the environment to form hydrogen peroxide in cells (Brookes et al. 2004; George et al. 2011a). ROS generation is widely considered as a molecular paradigm for the toxicity of nanoparticles (Xia et al. 2006). Excessive ROS generation would cause oxidative stress in cells (Perraud et al. 2004; Tan et al. 1998). If the oxidative stress exceeds the threshold of the cellular antioxidant defenses, additional mitochondrial perturbation such as mitochondrial depolarization would occur (Nel et al. 2006; Lin and Beal 2006; Brookes et al. 2004). These disturbances might further cause apoptosis or necrosis of cells followed by an increase in cell membrane permeability. From the current results, we found that the phototoxicity of TiO₂ nanoparticles to RAW264.7 cells could be mainly due to the ROS generation. ROS could be spontaneously generated by the materials or during the interaction between particles and cellular components (Xia et al. 2006). HPF results indicated that ROS generation may result directly from hydroxyl radicals generated by photoactivated TiO₂ nanoparticles.

Furthermore, we found the size-dependent cytotoxicity of TiO₂ nanoparticles after photoactivation could be due to two reasons, size-dependent ROS generation and size-dependent biomolecule adsorption. Smaller particles with higher percentage of molecules on their surface (Oberdörster et al. 2005) can generate more ROS such as $\cdot$ OH, which was proved by HPF test. The highly unstable hydroxyl radicals can non-specifically attack biomolecules such as DNA, proteins and lipids in a diffusion-controlled reaction (Vidosava 2004). The damage of proteins induced by oxidative stress has been widely studied. The

formation of carbonyl derivatives is considered as one of the possible modifications caused by oxidative stress, which is through oxidation-induced peptide cleavage or direct oxidation of certain amino acid side chains (Finkel and Holbrook 2000; Stadtman 1992). Biomolecules such as proteins attached onto the surface of TiO₂ nanoparticles enhanced the likelihood to be damaged or denatured by the hydroxyl radicals on the surface TiO₂ nanoparticles. Obviously, if this reaction happens to some key biomolecules such as DNA, proteins and lipids, the state of the cells will be affected (Vidosava 2004). For example, if the lipid peroxidation happened in cellular membrane, the fluidity of membrane will decrease, resulting in increased permeability for ions (Vidosava 2004). The oxidized protein would increase the susceptibility to enzymic proteolysis (Dukan et al. 2000). Smaller particles with more TiO₂ molecules exposed on the surface of TiO₂ nanoparticles can adsorb more biomolecules such as proteins on their surfaces. The protein adsorption study proved that smaller particles with higher surface area could adsorb more proteins, similarly shown by Horie et al. (2009). Thus, more biomolecules tend to be damaged by smaller TiO₂ nanoparticles.

Based on the previous results, we further hypothesized that if the surface of TiO₂ nanoparticles was pre-coated with other materials, the phototoxicity of TiO₂ nanoparticles should decrease. We used PEMA and chitosan to coat T20 nanoparticles. Cytotoxic tests showed obvious decrease in phototoxicity after surface coating of T20 nanoparticles based on PI uptake and MitoSOX staining results. The HPF test further proved that the surface coating of T20 nanoparticles could decrease hydroxyl radicals ($\cdot\text{OH}$) generation by T20 during the photoactivation process. The decreased cytotoxicity could be due to four possibilities. First, the surface coating decreased the effective surface area for interaction between surface TiO₂ molecules and other molecules such as water and oxygen. The active electron hole pair may recombine and release the energy in the form of heat (Almquist and Biswas 2002). Second, the PEMA or chitosan attached on the surface of TiO₂ nanoparticles quenched the activity of photoactivated TiO₂ surface and resulted in non-harmful oxidized PEMA or chitosan, which prevent further disturbance inside cells. Third, the surface coating of TiO₂ nanoparticles may decrease light intensity reached at the particles. Based on the UV–Vis test (supporting information, Fig. S2), the absorbance of UV light largely decreased after coating with PEMA, which indicated that these coated particles are less able to absorb UV and thus reduce the possibilities of free radical formation. The fourth reason could be that there is less BSA adsorption for the coated particles. For example, BSA adsorption on T20 nanoparticles decreased after surface coating. From Fig. 7, PEMA or chitosan on the surface of TiO₂ nanoparticles might be able to block the attachment of biomolecules on the surface of TiO₂ nanoparticles. The decreased adsorption of biomolecules decreased the possibility of biomolecules to be damaged by ROS, and thus cytotoxicity.

This study provided a novel clue to understand the mechanism behind the phototoxicity of different-sized TiO₂ nanoparticles. These findings showed that the surface area and more specifically, the number of TiO₂ molecules exposed on the surface of TiO₂ nanoparticles can be used to predict the potential phototoxic effects of TiO₂ nanoparticles. With this understanding, we can find ways to modify the phototoxicity of TiO₂ nanoparticles and design safer nanomaterials. Despite the increased toxicity shown in smaller TiO₂ nanoparticles, they have the potential to be used in biomedical applications. It was reported

that photoactivated TiO₂ nanoparticles could selectively induce toxicity against cancer cells (Cai et al. 1992; Lagopati et al. 2010; Stefanou et al. 2010). On the other hand, the surfaces of these particles can be coated with materials such as PEMA and chitosan to reduce their potential toxic effects, for use in consumer products such as sunscreens. But the current results are not adequate to conclude whether the size or size-related properties such as surface area played a more important role, which will be the focus of future studies. Further studies are also needed to understand the interaction between TiO₂ nanoparticles and biomolecules. These findings have great potential to be used to predict the cytotoxicity and phototoxicity of different TiO₂ nanoparticles in an abiotic condition.

Conclusion

In this study, we demonstrated that the TiO₂ nanoparticles exhibited increased cytotoxicity upon UV–Vis activation in a size-dependent manner. The size-related property, active surface area, could be related to such size-dependent phototoxicity of TiO₂ nanoparticles. ROS generation and biomolecule adsorption could be the two major reasons responsible for the increased cytotoxicity of smaller particles after photoactivation. Higher ROS generation from smaller particles was detected under both biotic and abiotic conditions. TGA analysis revealed that more protein could be adsorbed onto smaller nanoparticles. The surface coating of TiO₂ nanoparticles with PEMA or chitosan could decrease their phototoxicity, which might be due to the hindrance of biomolecule adsorption and hydroxyl radicals (·OH) production in the photoactivation process. This provided the mechanism behind the phototoxicity of TiO₂ nanoparticles and clues on how to alleviate such toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. TEM micrographs of 3 spherical TiO₂ nanoparticle samples with different sizes. **a** T10, 10 nm; *scale bar* 20 nm, **b** T20, 20 nm; *scale bar* 20 nm, **c** T100, 100 nm; *scale bar* 100 nm

Fig. 2.

Potential damage to RAW264.7 cells after being treated with or without UV light (280–450 nm) for 5 min. NC represented negative control which received neither particles nor UV light. LC represented light control which received UV light only. For test groups (T10, T20 and T100), the cells were treated with the different nanoparticles at 100 $\mu\text{g}/\text{ml}$. Cells were stained with a PI, b MitoSOX and c JC-1 to probe cytoplasm membrane integrity, mitochondrial superoxide generation and mitochondrial depolarization, respectively. Smaller particles triggered higher levels of phototoxicity to cells in all 3 assays. Data represent mean $\pm$ SD, $n = 4$. * $p < 0.05$ compared with corresponding control (NC is control for non-UV exposed group; LC is control for UV exposed group). # $p < 0.05$ compared with other two particles treated groups

Fig. 3.

Toxicity of photoactivated TiO₂ nanoparticles was correlated with surface area. The surface areas normalized to volume were 166.0, 50.4 and 17.2 cm²/ml for T10, T20 and T100, respectively. Cytotoxicity outcome showed excellent correlation with the surface area for a PI uptake, **b** MitoSOX and **c** JC-1 results ($R^2 > 0.9$). Data represent mean $\pm$ SD, $n = 4$

Fig. 4.

Potential damage to RAW264.7 cells after being treated with or without UV light exposure (280–450 nm) for 5 min. NC represented negative control that received neither particles nor UV light. LC represented light control that received UV light only. For test groups (T20-PEMA, T20 and T20-chitosan), the cells were treated with different nanoparticles at a concentration of 100 $\mu\text{g}/\text{ml}$, and assayed for **a** PI uptake, **b** MitoSOX staining and **c** JC-1 staining. After surface coating with PEMA or chitosan, the phototoxicity of T20 nanoparticles decreased. Data represent mean $\pm$ SD, $n = 4$. * $p < 0.05$ compared with corresponding control (NC is control for non-UV exposed group; LC is control for UV exposed group). # $p < 0.05$ compared with the other particles treated group

Fig. 5.

Hydroxyl radical generation during photoactivation of TiO₂ nanoparticles tested by HPF assay. Smaller particles generated more hydroxyl radicals during photoactivation of TiO₂ nanoparticles. The surface coating of T20 nanoparticles with PEMA or chitosan significantly decreased the hydroxyl radicals generated by T20 nanoparticles. Data represent mean $\pm$ SD, $n = 4$. * $p < 0.05$ between T10, T20 and T100. # $p < 0.05$ between T20-PEMA, T20 and T20-chitosan

Fig. 6. TGA analysis of BSA adsorption onto TiO_2 nanoparticles. **a** BSA, **b** T10, T20, T100 and their corresponding BSA-adsorbed nanoparticles, **c** T20, T20-PEMA, T20-chitosan and their corresponding BSA-adsorbed nanoparticles. Smaller particles absorbed more BSA than bigger particles. The surface coating of T20 nanoparticles with PEMA or chitosan decreased the BSA adsorption onto T20 nanoparticles

Fig. 7.

Schematic of the possible mechanism behind the phototoxicity of TiO₂ nanoparticles. When TiO₂ nanoparticles are exposed to UV light, the photon energy excite the electrons (e^-) in the valence band to the conduction band and leave holes (h^+) in the valence band to form electron hole pairs. The holes (h^+) can interact with adsorbed H₂O or hydroxide ions (OH^-) to generate reactive hydroxyl radicals ($\cdot OH$) which can interact with biomolecules nearby and result in the oxidation and dysfunction of biomolecules. The electrons (e^-) in the conduction band can reduce oxygen (O₂) adsorbed onto the particle surface to produce superoxide ions (O₂⁻), which can further react with water in the environment to form hydrogen peroxide in cells. Surface coating of TiO₂ nanoparticles with PEMA or chitosan can effectively decrease generation of hydroxyl radicals ($\cdot OH$) and adsorption of biomolecules

Table 1

Three groups of probe cocktails used

Fluorescence probe cocktail	Ex/Em wavelength (nm)	Indication
1 μ M Hoechst + 5 μ M PI	355/465 and 540/620	Damaged plasma membrane integrity
1 μ M Hoechst + 5 μ M MitoSOX	355/465 and 510/580	Generation of mitochondrial superoxide
1 μ M Hoechst +1 μ M JC-1	355/465 and 480/530–590	Mitochondrial depolarization

Table 2Characterization of TiO₂ nanoparticles with different size and surface coating

	T10	T20	T100	T20-PEMA	T20-chitosan
Primary particle size (nm)	10	20	100	20	20
Cristal structure	Anatase	Ana*/Rut*81/19	Anatase	/	/
Water					
Size (nm)	669	307	349	643	408
PDI	0.186	0.263	0.224	0.349	0.276
Zeta potential (mV)	-16.9	32.3	19.1	-50.4	51.4
CDMEM					
Size (nm)	262	338	444	633	742
PDI	0.005	0.180	0.248	0.330	0.306
Zeta potential (mV)	-5.7	-7.7	-9.3	-9.6	-7.2
Theoretical surface area (m ² /g)	154	73	15	/	/
BET surface area (m ² /g)	166.0	50.4	17.2	/	/

Ana* anatase, Rut* rutile