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Author(s)	Xiong, Sijing; Tang, Yuxin; Ng, Huiyun Sheena; Zhao, Xinxin; Jiang, Zhelong; Chen, Zhong; Ng, Kee Woei; Loo, Say Chye Joachim
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Specific Surface Area of Titanium Dioxide (TiO₂) Particles Influences Cyto- and Photo- Toxicity

Sijing XIONG¹, Yuxin TANG¹, Huiyun Sheena NG¹, Xinxin ZHAO¹, Zhelong JIANG¹, Zhong CHEN¹, Kee Woei NG¹ and Say Chye Joachim LOO^{1*}

1. School of Materials Science & Engineering, Nanyang Technological University, Block N4.1,
50 Nanyang Avenue, Singapore.

*Corresponding author: Dr. Joachim Say-Chye Loo, Tel: +65-6790-4603, Fax: +65-6790-9081,

Email: joachimloo@ntu.edu.sg

Abstract:

The aim of this study is to examine how different specific surface areas of similar-sized titanium dioxide (TiO₂) particles could influence both cytotoxicity and phototoxicity. TiO₂ particles of different specific surface areas were compared for their toxic effects on RAW264.7 cells in the absence and presence of UV light. From the results, TiO₂ particles with larger specific surface area were found to induce higher cyto- (UV absent) and photo-toxicity (UV activated) to cells after 24 h incubation. The observed cytotoxicity from TiO₂ particles with larger surface area could be explained from their interactions with biomolecules. Upon photoactivation, a larger number of hydroxyl radicals were detected from TiO₂ particles with larger surface area, again suggesting a surface area dependent phototoxic effect. On the other hand, pre-adsorbing TiO₂ particles with extracellular proteins were found to decrease toxicity effects.

Key words: TiO₂, particles, surface area, cytotoxicity, phototoxicity

1. Introduction

Particles of minute sizes and large surface areas have been used in different applications such as cosmetics and electronics (Maynard *et al.* 2006; Xia *et al.* 2006; Nel *et al.* 2006). With an increasing number of consumer products now containing sub-micron, or even nano-sized materials, direct or indirect human contact with these particles becomes inevitable (McCall 2011; Nel *et al.* 2006). In view of this, there has been greater public awareness on the safety of these materials, and correspondingly, an increase in the number of scientific publications pertaining to the toxicity evaluation of these particles in recent years (Oberdörster *et al.* 2005a; Nel *et al.* 2006; Morris *et al.* 2011; Meng *et al.* 2009).

TiO₂ particles is one class of materials that is produced in large volumes, and widely utilized in consumer products because it is relatively inexpensive and chemically stable (Skocaj *et al.* 2011; Araujo and Nel 2009). Due to its ability to absorb, scatter and reflect ultraviolet (UV) light, it is used extensively in sunscreens and cosmetics. At the same time, TiO₂ particles have also been shown to possess strong antimicrobial properties (Skocaj *et al.* 2011) because of its ability to generate reactive oxygen species (ROS) during photoactivation (Bar-Ilan *et al.* 2011; Hoffmann *et al.* 1995). The role of ROS in pathological conditions caused by photoactivated TiO₂ particles has been well documented under different conditions, including in fish cells (Reeves *et al.* 2008), Chinese hamster cell line (Nakagawa *et al.* 1997) and human cells (Gopalan *et al.* 2009). From the literature, it is clearly evident that TiO₂ particles are able to induce oxidative stress (Gurr *et al.* 2005; Kang *et al.* 2008; Long *et al.* 2006; Park *et al.* 2008), genotoxicity (Petkovic *et al.* 2010; Rahman *et al.* 2002; Wang *et al.* 2007) and immunotoxicity (Sayes *et al.* 2006; Palomaki *et al.* 2009), and these observations have been correlated to its physicochemical properties, such as

crystallinity (Sayes *et al.* 2006), particle size (Gurr *et al.* 2005) and surface area (Oberdörster *et al.* 2005a).

We previously reported an inverse relationship between the size of TiO₂ nanoparticles and its *in vitro* cytotoxicity and phototoxicity (Xiong *et al.* 2012). However, it is known that decreasing particle size would also result in a corresponding increase in its specific surface area; and whether cytotoxicity arises from the true effect of particle size or specific surface area is still debatable. In this paper, it was hypothesized that specific surface area, rather than particle size, is the key reason behind an increase in cytotoxicity observed with smaller-sized TiO₂ particles. A similar conclusion was also drawn from an *in vivo* study conducted by Sager *et al.* (Sager *et al.* 2008). However, in that study, the effect of particle size was not fully eliminated, since cytotoxicity comparisons were made between two different sized TiO₂ particles (i.e. ultrafine and fine). As such, the aim of this paper is to investigate the cytotoxicity of similarly-sized TiO₂ particles, albeit with different specific surface areas, and to further investigate whether this observation is maintained under UV activation.

One key consideration in working with high specific surface particles is their ability to adsorb molecules, and there are reports discussing the protein adsorption ability of TiO₂ particles (Wassell and Embery 1996; Oliva *et al.* 2003; Horie *et al.* 2009). When particles enter cell culture medium or biological fluids such as blood, they will be coated with a layer of proteins – a protein corona (Lundqvist *et al.* 2008). This protein corona undergoes dynamic changes even after particles enter cells (Nel *et al.* 2009), and the association and dissociation of proteins from particle surfaces can determine particle-cell interactions (Nel *et al.* 2009), hence influencing toxicity (Xu *et al.* 2010). Since one of the key effects of large surface area is the ability to adsorb

more proteins, it would therefore be of interest in this paper to also decipher this effect by conducting the cytotoxicity tests under three different conditions; i.e. in the presence and absence of fetal bovine serum (FBS) in culture medium, and particles pre-coated with FBS. Murine macrophage RAW264.7 cells were chosen in this study, as this cell line is intensively investigated and well understood in nanotoxicity studies. In our previous studies, we have successfully used this cell line to investigate the cytotoxicity of biomedically used hydroxyapatite (HA) nanoparticles with different specific surface areas (Zhao *et al.*, 2010). The cell line was also utilized for other similar studies on TiO₂ particles (George *et al.* 2011a; Xia *et al.* 2006; Xia *et al.* 2008). Thus, as a platform for comparison with these studies, we chose to use RAW264.7 cells to evaluate and understand the cytotoxicity of TiO₂ particles with different specific surface areas. Towards the end of the paper, a plausible mechanism on how TiO₂ particles may induce cytotoxicity would be proposed, and through this provide valuable insights on how cytotoxicity can be decreased.

2. Materials and Methods

2.1 Synthesis of TiO₂ particles

TiO₂ particles of two different specific surface areas were synthesized via thermal treatment of the titanate particles. Firstly, the titanate particles were prepared using a starting titanium foil via a rapid electrochemical spark discharge spallation (ESDS) method in an electrolyte of 10 M NaOH in aqueous solution with a platinum counter electrode (Tang *et al.* 2012). The anodization was conducted at current density of 0.5 A/cm² at room temperature, with a distance of 3.0 cm between the two electrodes. After completion of the experiment, a grey white precipitate was

collected from the solution, yielding the sodium titanate particles. Then, the hydrogen titanate particles were obtained by soaking the precipitates in HCl solution (0.1 M) for several times, followed by washing in deionized water and absolute ethanol before drying in air. Finally, the as-synthesized samples were annealed at 400 °C or 700 °C for 1 hour with a heating rate of 5.0 °C/min, yielding the TiO₂ particles with different specific surface areas of 174.5 and 33.3 m²/g but with the same size, respectively. These two samples are named as T174 and T33 in the following study.

2.2 Characterization of TiO₂ particles

2.2.1 TEM & FESEM

The field emission scanning electron microscope (FESEM, JEOL JSM 6340F) and transmission electron microscope (TEM, JOEL 2010, Japan) were utilized to visualize the size and morphology of TiO₂ particles. TiO₂ particles were first dispersed in methanol at a low concentration and dropped onto a silicon chip and carbon coated copper grids for FESEM images and TEM images respectively. After air dried, the TiO₂ particles were observed under FESEM with an acceleration voltage of 10 kV. TEM images were taken under at an accelerating voltage of 200 kV.

2.2.2 Hydrodynamic size and surface charge of particles

Dynamic light scattering (DLS) technique was used to test the hydrodynamic size and Zeta-potential of TiO₂ particles in water and Complete Dulbecco's Modified Eagle Medium (CDMEM). TiO₂ particles were prepared into stock suspension at a concentration of 3 mg/ml in either deionized water or CDMEM. Ultrasonication in water bath ultrasonicator was conducted

for 10 min to help re-disperse of particles. The working suspension at a concentration of 100 µg/ml was prepared and ultrasonicated for 10 minutes prior to testing using NanoSizer (Malvern Co., UK).

2.2.3 Brunauer-Emmett-Teller (BET) surface area and ultraviolet–visible (UV-Vis) spectra

Nitrogen adsorption/desorption isotherms were measured at 77 K using ASAP2000 adsorption apparatus from Micromeritics. The samples were degassed at 100 °C for 4 h under vacuum before analysis. Optical diffuse reflectance spectra of the samples in solid powder form were measured with a Shimadzu 2550 UV-vis-NIR spectrometer using BaSO₄ as a reference standard with a wavelength range of 300-450nm.

2.3 *In vitro* cytotoxicity study

2.3.1 Cell culture

Immortalized mouse macrophage cell line RAW264.7 (ATCC, # TIB-71) cells were cultured in CDMEM which contained 88% DMEM and was supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin. The cells were incubated in a humidified atmosphere at 37 °C, 5% CO₂ and were passaged in 1:2 or 1:4 ratios upon reaching 70%-80% confluence. Cells between passage 10 and 20 were used for all the experiments.

2.3.2 Cellular metabolic activity test

Cell counting kit-8 (CCK-8), which uses water-soluble tetrazolium salt (WST- 8), was used to determine cell viability through cellular metabolic activity of live cells. Cells were seeded in 24-well plates at a density of 20,000 cells/cm² and left to incubate at 37 °C, 5% CO₂ for 24 h. TiO₂

particles were dispersed in CDMEM to form stock suspensions of 1 mg/ml. The suspensions were ultrasonicated for 10 min to help dispersion of particles and then diluted into working suspensions of concentrations 30, 100, 300 and 1000 $\mu\text{g}/\text{ml}$ with CDMEM. The working suspensions were ultrasonicated for 10 min prior to addition to cells. Old cell medium was replaced with working suspensions (30–1000 $\mu\text{g}/\text{ml}$). For this group, control cells were cultured in medium containing FBS. Cells were incubated for another 24 h. The medium was then removed and replaced with WST-8 working solution which was prepared by diluting WST-8 stock solution with fresh medium. The plates were incubated at 37 °C for 1 h. The solutions were collected, centrifuged to remove residual TiO_2 particles and transferred to a 96-well flat bottom plate. The amount of formazan dye formed, which corresponds to the level of dehydrogenase metabolic activity of all the live cells, was measured using an Infinite200TM micro-plate reader (Tecan Inc., Maennedorf, Switzerland) to test the absorbance at 450 nm. A reference absorbance at 800 nm was also recorded. The viability is equal to the WST-8 absorbance in test wells divided by that in control wells.

2.4 Protein adsorption study

The adsorption of BSA onto TiO_2 particles was tested using Thermo Gravimetric Analyzer (TGA, 2950, HR, V5.4A). TiO_2 particles at a concentration of 1 mg/ml were dispersed in 2 mg/ml of bovine serum albumin (BSA) in Dulbecco's Modified Eagle Medium (DMEM). The suspension was ultrasonicated for 20 min. The particles were collected by centrifugation at 14,000 rpm for 20 min. The particles were washed thrice with Milli-Q water and freeze-dried for 48 h prior to testing. The weight loss curve of bare TiO_2 particles and BSA adsorbed particles was obtained using TGA at a heating rate of 20 °C/min in a nitrogen-filled environment.

2.5 Depletion of FBS and pre-coat particles with FBS

To study the influence of extracellular proteins, TiO₂ particles were prepared in another two conditions, namely depletion of FBS and pre-coat particles with FBS. To remove the extracellular proteins, TiO₂ particles were dispersed in FBS-free medium (98% DMEM, 1% L-glutamine, 1% penicillin-streptomycin), to form stock suspensions of 1 mg/ml. The suspension was ultrasonicated for 10 minutes to help dispersion of particles and then diluted into working suspensions of concentrations 30, 100, 300 and 1000 µg/ml with FBS-free medium. The working suspensions were ultrasonicated for 10 minutes prior to addition to cells. FBS-free medium was used as control in this group of studies.

Pre-coated TiO₂ particles with FBS were prepared by dispersing particles in CDMEM to allow binding of substances from CDMEM to the particles' surface. The suspension was ultrasonicated for 20 minutes and then centrifuged at 14000 rpm for 20 minutes at 25 °C. The supernatant containing unbound proteins was discarded and the pellet was re-suspended in FBS-free medium to form stock suspension of pre-coated TiO₂ particles (1000 µg/ml). Prior to adding to cells, the stock suspension was diluted to working concentrations with FBS-free medium. FBS-free medium was utilized as control in this group of studies.

For these two groups, control cells were cultured in medium without FBS, which is different from the previous group. All the cell culture and cellular metabolic activity test were the same as procedures described previously.

2.6 Treatment of particles with photoactivation

Cells were seeded in 24-well plates at a density of 20,000 cells/cm² and left to incubate at 37 °C, 5% CO₂ for 24 h. Old cell medium was replaced with working suspensions containing 100 µg/ml TiO₂ particles and cells were incubated for 21 hours. The medium with TiO₂ particles were replaced with PBS to prevent interference of phenol red in the medium. Plates were exposed to UVA radiation (VL-215 LC, Vilber Lourmat) at 365 nm with an intensity of 7mW/cm² for 20 min. After which, fresh medium was added to the wells and the cells were incubated for another 3 h. Cellular metabolic activity test were conducted with same procedure described previously.

2.7 Abiotic hydroxyl radical generation test

The ability of TiO₂ particles to generate hydroxyl radicals in an abiotic environment was determined by the HPF assay (3'- (p-hydroxyphenyl) fluorescein assay). TiO₂ particles were dispersed in Milli-Q water to prepare TiO₂ suspension at a concentration of 100 µg/ml. The suspension was ultrasonicated for 20 minutes to help re-dispersion. 90 µl of particle suspensions were added to a 96-well flat plate with transparent bottom. Milli-Q water without particles was used as control. 10 µl HPF working solution, containing 100 µM HPF in Milli-Q water, was added to each well. The plate was exposed to UVA radiation (365 nm) for 0 min, 5 min, 10 min, 15 min, 20 min, 25 min or 30 min. The fluorescent intensity was immediately measured at excitation and emission wavelength of 490 nm /515 nm using the micro-plate reader.

2.8 Statistics

All quantitative data are shown as means ± standard deviation (SD). Statistical analyses were performed using an analysis of variance (ANOVA), followed by Tukey's pairwise comparisons

using SPSS software, version 11.5 (SPSS Inc., Chicago, IL, USA). Significant difference was considered when the *p*-value was lower than 0.05. All the tests repeated four times.

3. Results

3.1 Characterization of TiO₂ particles

TiO₂ particles of different specific surface areas, namely T33 (33.3 m²/g) and T174 (174.5 m²/g), were synthesized and investigated for their respective cytotoxicity in this study (Table 1).

Both particles were found to have similar shapes of flower-like structural morphologies as shown from TEM (Fig. 1a and b) and FESEM (Fig. 1c and d) images. The sizes of the T33 and T174 are similar with a main particle size of $0.8 \pm 0.1 \mu\text{g}$ according to our observation (Tang et al., 2010). As reflected from Table 1, T174 possessed a large specific surface area of $\sim 174.5 \text{ m}^2/\text{g}$, while T33 had a much smaller specific surface area of $\sim 33.3 \text{ m}^2/\text{g}$. The typical nitrogen adsorption–desorption isotherms of the TiO₂ samples are shown in Fig. 2. The isotherm of T174 presents a typical hysteresis loop of type H2 which is attributed to the difference in the adsorption and desorption mechanisms occurring in the “ink-bottle” pores, which possess a high specific surface area around $174.5 \text{ m}^2/\text{g}$ with a pore volume of $0.70 \text{ cm}^3/\text{g}$ (average pore size is 16.1 nm). Upon further increment of the calcination temperature, the hysteresis loop shifts to relatively high pressure and the loop shape changes from the type H2 to type H3, reflecting the appearance of the macropore structures with pore volume of $0.39 \text{ cm}^3/\text{g}$ (average pore size is 47.1 nm). Both these particles were in anatase form, which was confirmed by XRD spectrum (Supporting information, Fig. S1). Diffuse reflectance UV–vis absorption spectra of T33 and T174 (Fig. S2), showed similar UV light absorption profiles, which originated from the wide

band gap of TiO₂. DLS measurements were conducted to examine the hydrodynamic size, polydispersity index (PDI) and surface charge of the particles in both DI water and cell culture medium at a concentration of 100 µg/ml to avoid multiple scattering effect at higher concentration (Table 1). The hydrodynamic sizes of T33 and T174 were 1.209 µg and 0.973 µg, respectively, in DI water, while their hydrodynamic sizes increased slightly to 1.355 µg (T33) and 1.440 µg (T174) in complete cell culture medium. The increase in hydrodynamic size in cell culture medium could be due to the surface adsorption of proteins from CDMEM (Walkey and Chan, 2012; Xia et al., 2006). Both particles exhibited similar positive and negative charge values in water and CDMEM, respectively. The change from positive to negative Zeta potentials in CDMEM could be again due to the surface binding of proteins (Xia et al., 2006).

3.2 Cytotoxicity of TiO₂ particles

Cellular metabolic activity was measured using WST-8 assay, which indirectly indicated the viability of RAW264.7 cells. From Figure 3, the metabolic activity of TiO₂-treated cells were found to decrease at concentrations of 300 µg/ml and above, when compared against the control. There was significant difference between the viability of the cells treated with T174 and T33 at 300 µg/ml and 1000 µg/ml.

3.3 Protein adsorption on TiO₂ particles and its corresponding effects on cytotoxicity

BSA was chosen as a model protein to understand the protein adsorption ability of TiO₂ particles, with different specific surface areas. The weight loss of BSA-adsorbed particles, as measured from TGA, is shown in Figure 4. The decomposition temperature of pristine BSA starts from 188 °C, and the largest mass loss was observed within this range of up to 485 °C. Control bare

T33 and T174 particles without BSA exhibited insignificant changes in mass at this temperature range. Not surprisingly, T174-BSA exhibited the largest mass loss, verifying that more BSA was adsorbed. In summary, TiO₂ particles exhibited strong adsorption to BSA protein, in which a trend of increasing adsorption with increasing specific surface area was observed.

From the trend observed, it is thereby hypothesized that the surface area-dependent cytotoxicity of TiO₂ particles could be related to their surface area-dependent ability to adsorb proteins. In the present study, it is suggested that the cytotoxicity of TiO₂ particles could occur through two possibilities. One possibility is that extracellular proteins adsorbed onto TiO₂ particles can cause a depletion of proteins in the cell culture medium. This possibility was supported by Horie *et al.*'s study (Horie et al. 2009), which demonstrated that toxicity of TiO₂ nanoparticles was due to the depletion of serum in cell culture medium. However, based on our preliminary tests, even at the highest TiO₂ concentration (1000 µg/ml), there was no significant change in protein level in the medium (data not shown), suggesting that cytotoxicity of surface area-dependent TiO₂ particles was less likely due to extracellular nutrient depletion. However, we cannot exclude the possibility that TiO₂ particles may reduce the level of some specific essential factors in cell culture medium, which may ultimately influence the viability of cells. Another more plausible possibility could be that TiO₂ particles may interact with intracellular biomolecules. This interaction may induce depletion or damage of intracellular biomolecules, and if true, decreasing the interactions between TiO₂ particles and intracellular biomolecules could decrease cytotoxicity.

To investigate this, the approach would be to pre-coat TiO₂ particles with extracellular proteins, so as to reduce exposed particle surface, and hence interaction with intracellular biomolecules. To investigate this, cells were exposed to TiO₂ particles in another two conditions, namely

particles dispersed in medium without FBS (Figure 5a) and FBS-coated particles dispersed in FBS-free medium (Figure 5b). All results were normalized to their corresponding control. In the absence of FBS (Figure 5a), cells exhibited significantly lower viability than the control in a dose-dependent manner when treated with 300 and 1000 $\mu\text{g/ml}$ TiO_2 particles. At both concentrations, treatment with T174 resulted in a more significant decrease in cellular metabolic activity when compared to T33 treated cells. For the second group (Figure 5b), cells treated with FBS-coated particles did not show significant difference in metabolic activity for TiO_2 concentrations of 300 $\mu\text{g/ml}$ and below. At 1000 $\mu\text{g/ml}$, however, both T33- and T174- treated cells showed metabolic activity significantly lower than the control ($p<0.01$). When comparing these two conditions, particles pre-adsorbed with FBS were shown to have the less significant cytotoxic effects, indicating that pre-adsorption of proteins onto TiO_2 particles did have an effect on cytotoxicity.

3.4 Phototoxicity of TiO_2 particles

To further investigate the effect of specific surface area on phototoxicity, TiO_2 particles of concentration of 100 $\mu\text{g/ml}$ were used. From our preliminary tests, cell viability was not compromised when treated with 100 $\mu\text{g/ml}$ of TiO_2 particles (both T33 and T174) in the absence of UV irradiation in all three conditions investigated. Pre-tests also showed that UV light exposure up to 20 minutes without TiO_2 particles did not induce cell death (Supporting Information, Figure S3). Figure 6 shows cellular metabolic activity of RAW264.7 cells after being treated with TiO_2 particles with and without UV light activation. In all three conditions, the photoactivated T33 and T174 particles caused significant decrease in cell viability as compared to the control ($p<0.05$). A larger decrease in cell viability was observed in the absence

of FBS (Figure 6b). It was also observed that T174 tend to trigger higher phototoxicity compared to T33. Similar trend was also observed when co-staining cells with Hoechst and PI (Supporting Information, Figure S4 to S9).

3.5 Generation of hydroxyl radical under UVA exposure

HPF was utilized to quantify the amount of hydroxyl radicals generated by TiO₂ particles during photoactivation. HPF is a ROS indicator which emits green fluorescence when oxidized by hydroxyl radicals, and fluorescence intensity is proportional to the amount of hydroxyl radicals generated. Figure 7 shows the fluorescent intensity of HPF assay at different interaction times, for control (water), 100 µg/ml T33 and 100 µg/ml T174 particles in the absence and presence of UV. Controls displayed no hydroxyl radical generation in both conditions. In the absence of UV irradiation, it was observed that both T33 and T174 particles did not generate any hydroxyl radicals. With photoactivation, however, T33 and T174 were able to elicit hydroxyl radical generation in a time-dependent manner. T174 was also observed to induce more hydroxyl radical generation as compared to T33 at all the time points studied.

4. Discussion

Particle surface area has always been regarded as an important parameter in particle toxicity studies (Oberdörster *et al.* 2005b; Oberdörster *et al.* 2005a; Nel *et al.* 2006). The aim of the current study was to establish a link between TiO₂ particle surface area and its influence on both cytotoxicity and phototoxicity. Results of the current study demonstrated that TiO₂ particles with larger surface area were able to elicit more cytotoxic and phototoxic effects than particles with

smaller surface area, although both particle samples had similar sizes. This observation indicated the importance of particle surface area as a factor in influencing the toxicity of TiO₂ particles. As mentioned earlier, the ability of TiO₂ particles to elicit cytotoxic effects in the absence of UV irradiation is still very much under debate. Contrary to studies which reported that non-irradiated TiO₂ particles are biologically inert (Lindenschmidt *et al.* 1990; Ophus *et al.* 1979; Skocaj *et al.* 2011), the current study observed that at high concentrations (1000 µg/ml), TiO₂ particles with large surface area were able to induce significant cytotoxic effects in the absence of photoactivation. Cytotoxicity was clearly dose-dependent. The reason why we chose concentrations as high as 1000 µg/ml is because studies with unrealistic high doses can be useful as proof-of-principle studies or hypothesis-forming studies. It can reflect dose of repeated exposures and identify hazard. The high concentration tested in our study can also resemble ‘hot spots’ of deposition in respiratory tract.

Although hydroxyl radical generation, leading to oxidative stress, is considered by many to be the primary mechanism responsible for TiO₂ phototoxicity (George *et al.* 2011a; Bar-Ilan *et al.* 2011), the mechanism underlying the induction of cytotoxic effects by TiO₂ particles without photoactivation is still not well understood. It was observed in the current study that without UV irradiation, TiO₂ particles, regardless of their surface area, did not produce hydroxyl radicals. Thus, the cytotoxic effects observed from TiO₂ particles with large surface areas in the current study were probably due to a mechanism other than hydroxyl radical generation.

TiO₂ particles have been reported to cause oxidative stress (Gurr *et al.*, 2005; Kang *et al.*, 2008; Long *et al.*, 2006; Park *et al.*, 2008), immunotoxicity (Palomaki *et al.*, 2009; Sayes *et al.*, 2006) and even genotoxicity (Petkovic *et al.*, 2010; Rahman *et al.*, 2002; Wang *et al.*, 2007) in the absence of photoactivation. Particle-biomolecule interaction could be one possible explanation

for this (Horie et al., 2009). Macrophages are able to uptake particles with sizes in the range of 250 nm to 3 μg (Hillaireau, 2009) and numerous studies have proven that TiO_2 particles can be internalized by cells and enter different cellular organelles such as lysosomes (Jin et al., 2008) and mitochondria (Long et al., 2006). Based on literature and our current knowledge, particles could interact with biomolecules after being taken up into cells with the following possibilities. First, particles may cause the depletion of some key biomolecules. For example, the depletion of intracellular protein glutathione (GSH) in human embryonic kidney (HEK293) cells was reported by Wang et al. (2009) after the cells were incubated with SiO_2 nanoparticles of different sizes. The cytotoxicity of these SiO_2 nanoparticles was observed to be sized-dependent. The authors explained that smaller SiO_2 nanoparticles of larger surface areas could trigger higher level of GSH depletion, resulting in increased generation of ROS. Second, the particles may cause denaturation and/or erroneous folding of intracellular biomolecules. For example, studies have found that TiO_2 particles could interrupt cytoplasmic proteins such as microtubule protein in *Arabidopsis thaliana* (Wang et al., 2011). This phenomenon has been discussed earlier by Gheshlaghi et al. (2008), who showed that TiO_2 particles can bind tubulin heterodimers leading to the conformation change of protein. Others have reported some pathological changes of proteins such as aggregation or unfolding of proteins after interaction with particles (Lynch and Dawson, 2008; Norde and Giacomelli, 2000; Sabatino et al., 2007; Shen et al., 2007). TiO_2 nanoparticles were reported to have strong adsorption effect on trypsin and can inhibit the activity of trypsin by changing α -helix and β -sheet ratio of the protein structure (Wang et al., 2010). Similar damage in the secondary structure of proteins due to particle–protein interaction between CdS quantum dots and human hemoglobin was also observed (Shen et al., 2007). The secondary structure of BSA was irreversibly changed after interaction with polystyrene (Norde

and Giacomelli, 2000) or asbestos (Sabatino et al., 2007). Based on the current study, we therefore inferred that TiO₂ particles with larger surface area would be able to adsorb more proteins, which could cause depletion or impairment of key biomolecules in cells and influence normal cell functions. As such, with extracellular protein in cell culture medium or pre-coating TiO₂ particles with extracellular proteins would potentially avoid such phenomenon, and was thus found to decrease cytotoxicity. The potential mechanism is illustrated in Fig. 8.

In addition, phototoxicity tests of the current study revealed that TiO₂ particles, when irradiated with UV, elicited lower cell viability as compared to non-irradiated particles at the same concentration. Again, particles with larger surface area also tended to trigger higher phototoxicity as compared with particles with smaller surface area, suggesting the possible role that particle surface area may play in influencing phototoxic effects of TiO₂. Unlike in the absence of UV light, the generation of hydroxyl radicals was chiefly responsible for the phototoxicity of TiO₂ particles, which again was specific surface area dependent, and its mechanism is illustrated in Fig. 8. Photons with enough energy can excite the electrons (e⁻) in the valence band of TiO₂ particles to the conduction band during photoactivation, leaving holes (h⁺) in valence band, which is called electron hole pairs (Maness et al., 1999). In conduction band, the electrons (e⁻) can react with oxygen (O₂) nearby and generate superoxide ions (O₂⁻). On the other hand, the holes (h⁺) in valence band can interact with adsorbed water or hydroxide ions (OH⁻) and generate hydroxyl radicals (•OH) (Brookes et al., 2004; George et al., 2011a). Highly active and damaging hydroxyl radicals can oxidize and damage cellular biomolecules such proteins, lipids and even DNA, causing the dysfunction of these biomolecules (Almquist and Biswas, 2002; Circu and Aw, 2010; Finkel and Holbrook, 2000; George et al., 2011b; Maness et al., 1999). In this study, generation of hydroxyl radicals by photoactivated particles, as

indicated by HPF assay, was time- and surface area-dependent (shown in Fig. 7). Throughout the whole study, at all-time points, photoactivated particles with larger surface area were shown to produce more hydroxyl radicals. Moreover, the highly unstable hydroxyl radicals can only non-specifically attack biomolecules in a diffusion-controlled reaction (Vidosava, 2004). Thus, the biomolecules adsorbed onto TiO₂ particles are highly susceptible to the damage of hydroxyl radicals during photoactivation. Lipid peroxidation occurring in cellular membrane, will decrease the fluidity of membrane and eventually increase permeability for ions (Vidosava, 2004). It was also reported that oxidized protein would be more susceptible to enzymic proteolysis (Dukan et al., 2000). Obviously, if some key biomolecules such as enzymes, lipids and DNA were attached on TiO₂ particles and oxidized by hydroxyl radicals or some other ROS such as superoxide ions, the viability of cells will be threatened (Vidosava, 2004). In summary, the current study suggested that the increased phototoxic effects observed in particles with larger surface area may be due to an increase in hydroxyl radical generation, and the hydroxyl radicals generated on particles of larger surface area could attack more intracellular biomolecules adsorbed onto TiO₂ particles.

As discussed previously, both cytotoxicity and phototoxicity of TiO₂ particles could be related to the biomolecule adsorption on TiO₂ particles. The interaction between the surface of TiO₂ particles and intracellular biomolecules such as some key enzymes will be the focus of our future work. To uncover the morphology and functional change of biomolecules exposed to TiO₂ particles will help to understand the mechanism behind the toxicity of TiO₂ particles and also help to establish an abiotic system to predict the toxicity of different particles.

5. Conclusion

The current study demonstrated that TiO₂ particles with larger surface area were able to elicit more cytotoxic and phototoxic effects in the absence and presence of UV irradiation respectively. Adsorption of more biomolecules could be the reason for increased cytotoxicity of TiO₂ particles with larger surface area. Hydroxyl radical generation together with biomolecules adsorption could be responsible for the surface area related phototoxic effect of TiO₂ particles. These results indicated that particle surface area is an important factor in influencing cytotoxicity and phototoxicity of TiO₂ particles. Furthermore, the presence of extracellular proteins was also found to be able to decrease toxic effects of TiO₂ particles in this study.

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Conflict of interest

The authors declared that there are no conflicts of interest.

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Table 1 Physical characterization of TiO₂ particles with different surface areas

	T33	T174
Main particle size (μm)	0.8 ± 0.1	0.8 ± 0.1
Crystal structure	Anatase	Anatase
In water		
Hydrodynamic size (μm)	1.209 ± 0.153	0.973 ± 0.087
PDI	0.316 ± 0.086	0.545 ± 0.114
Zeta potential (mV)	9.3 ± 0.8	10.6 ± 0.9
In CDMEM		
Hydrodynamic size (μm)	1.355 ± 0.027	1.440 ± 0.239
PDI	0.562 ± 0.171	0.537 ± 0.066
Zeta potential (mV)	-8.2 ± 0.6	-8.9 ± 0.4
BET surface area (m ² /g)	33.3	174.5

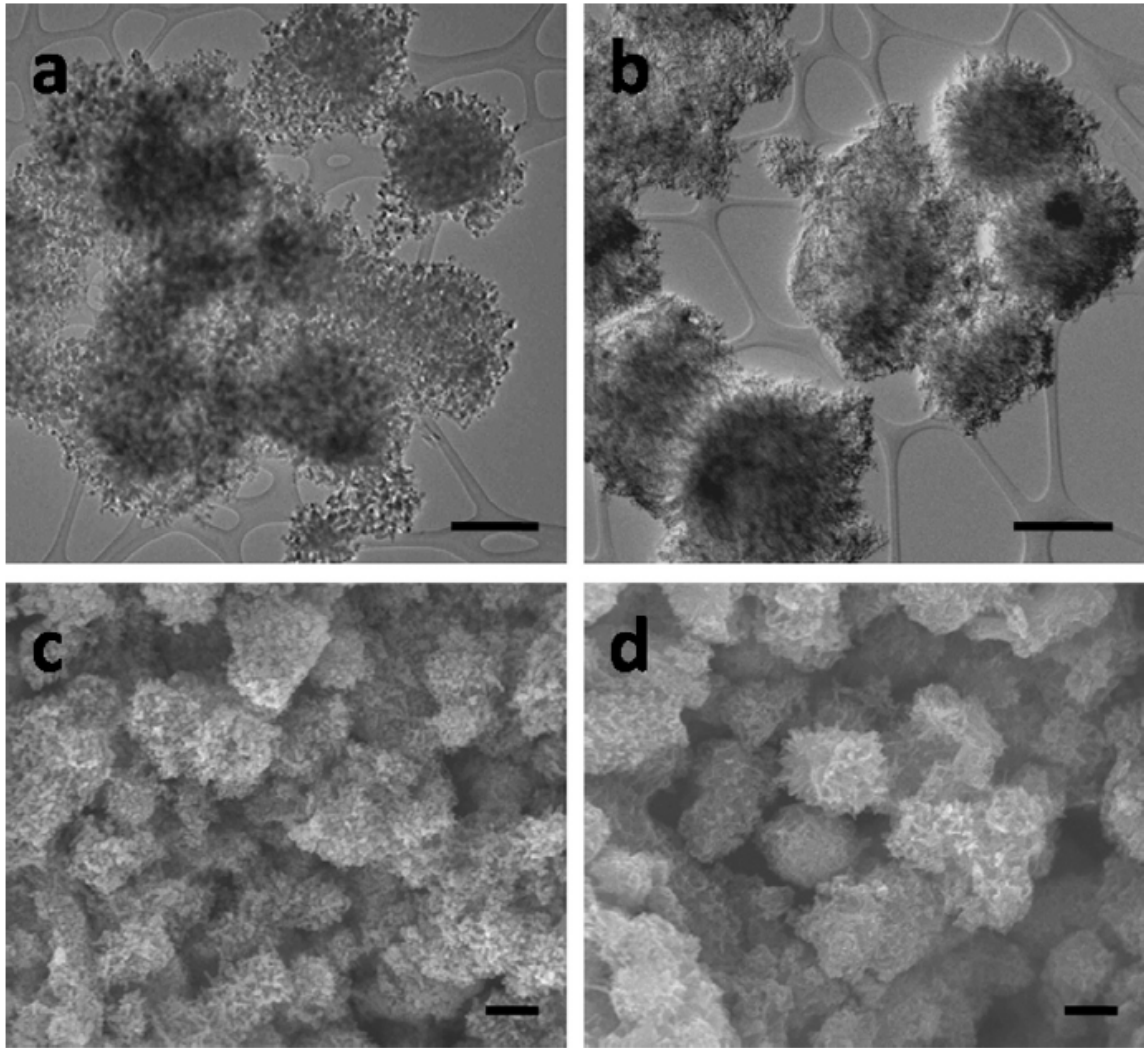


Fig. 1 (a, b) TEM micrographs of TiO_2 particle samples with different surface areas (a) T33 (b) T174; (c, d) FESEM micrographs of TiO_2 particle samples with different surface areas (c) T33 (d) T174. All scale bars represent 500 nm.

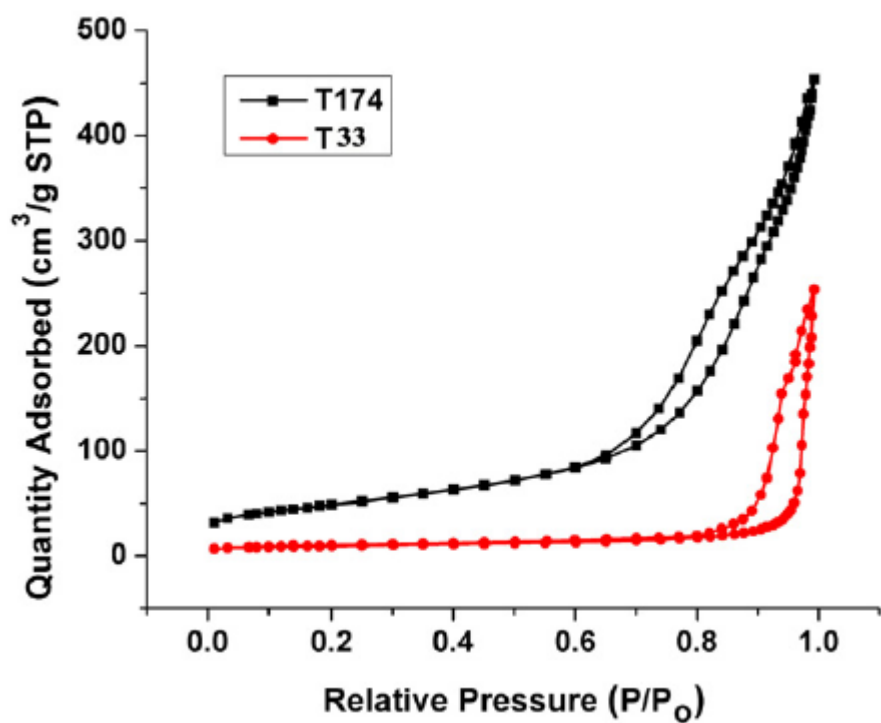


Fig. 2 Nitrogen adsorption-desorption isotherms of T33 and T174. The surface areas of the T33 and T174 samples are around 33.3 m²/g and 174.5 m²/g respectively.

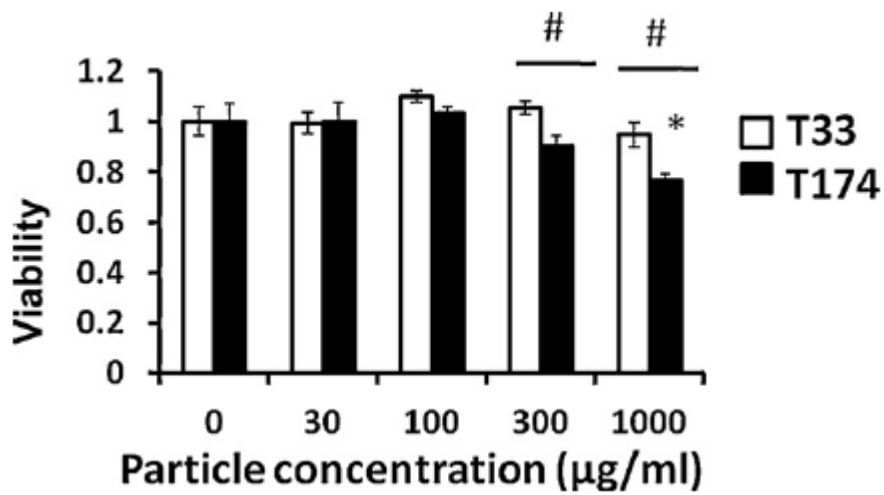


Fig.3 The metabolic activity of live cells quantified by WST-8 assay after RAW264.7 cells were treated with TiO₂ particles of two different surface areas dispersed in CDMEM for 24 h. Metabolic activity was normalized to negative control (0 µg/ml). Data represent means ± SD, n=4. * $p < 0.05$ compared with corresponding negative control (0 µg/ml). # $p < 0.05$ between two particles at the same concentration.

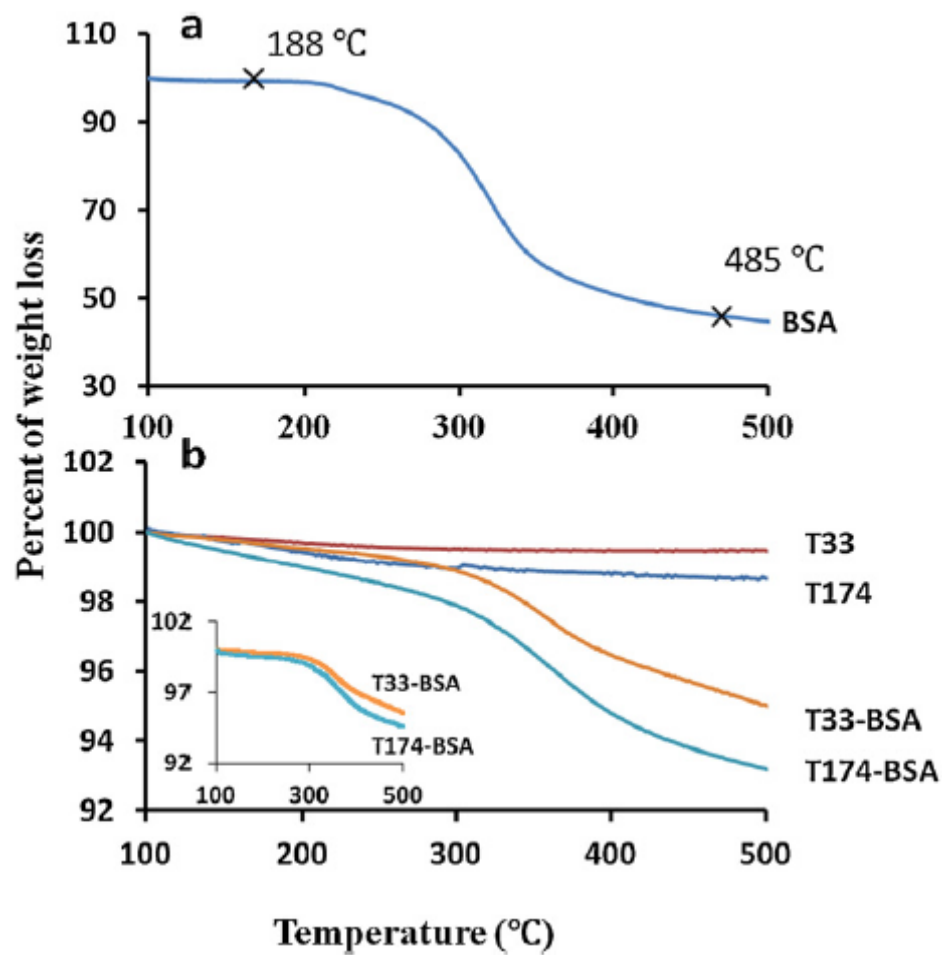


Fig. 4 TGA analysis of BSA adsorption onto TiO₂ particles. (a) BSA, (b) T33, T174, T33-BSA and T174-BSA.

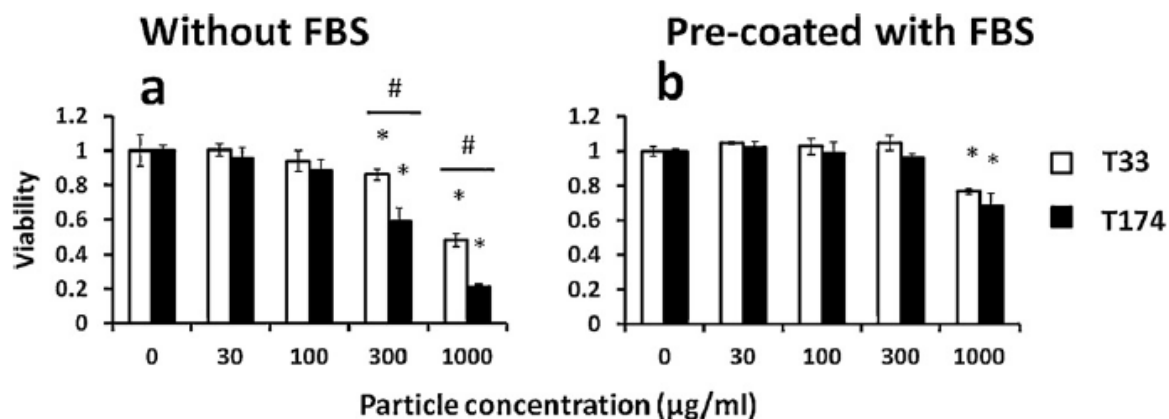


Fig. 5 The metabolic activity of live cells quantified by WST-8 assay after RAW264.7 cells were treated with TiO₂ particles of two different surface areas for 24 h. The metabolic activity of cells was normalized to negative control (0 µg/ml). Cells were exposed to (a) particles dispersed in FBS free medium; (b) particles pre-coated with FBS and then dispersed in FBS free medium. Data represent means ± SD, n=4. * $p < 0.05$ compared with corresponding negative control (0 µg/ml). # $p < 0.05$ between two particles at the same concentration.

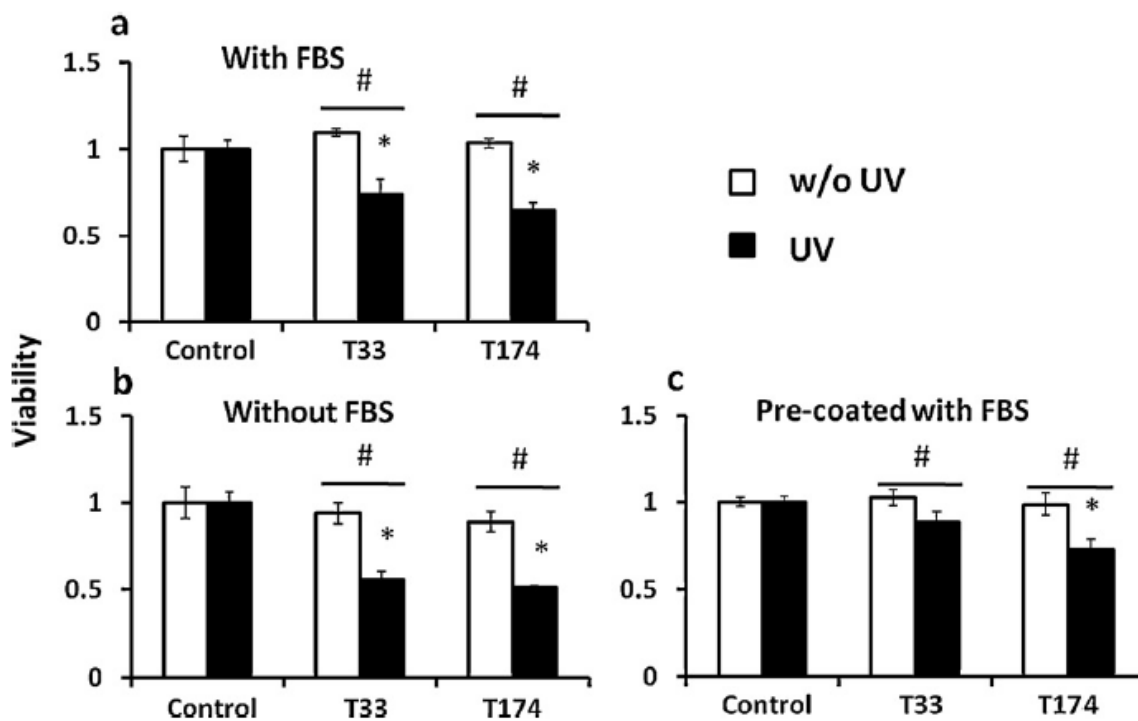


Fig. 6 The metabolic activity of live cells quantified by WST-8 assay after RAW264.7 cells were treated 100 $\mu\text{g}/\text{ml}$ of two TiO_2 particles for 24 h, with or without UV exposure. The metabolic activity of cells was normalized to negative control (0 $\mu\text{g}/\text{ml}$). Cells were exposed to (a) particles dispersed in medium with FBS (b) particles dispersed in FBS free medium and (c) particles pre-coated with FBS and then dispersed in FBS free medium. Data represent means \pm SD, $n=4$. * $p<0.05$ compared with corresponding control (0 $\mu\text{g}/\text{ml}$). # $p<0.05$ between two particles at the same concentration.

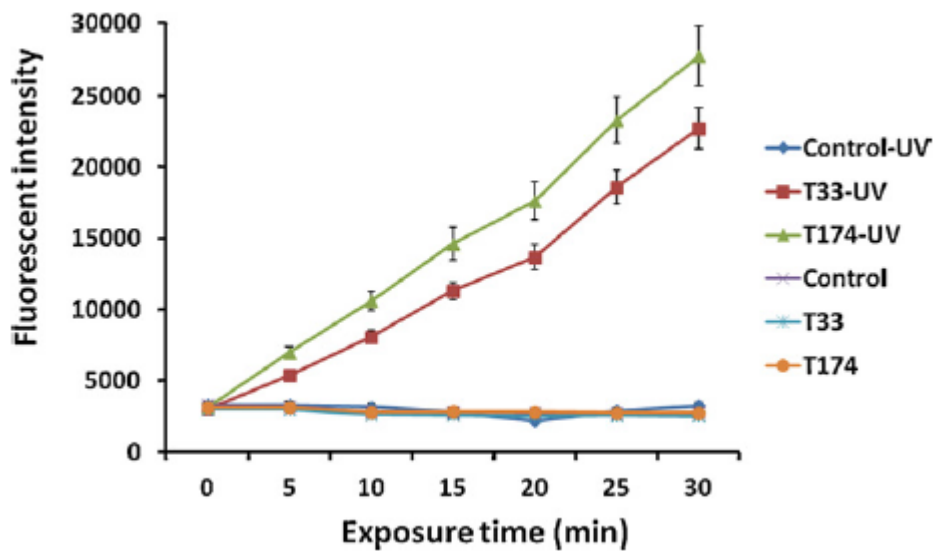


Fig. 7 HPF assay to test the generation of hydroxyl radicals during photoactivation of TiO_2 particles.

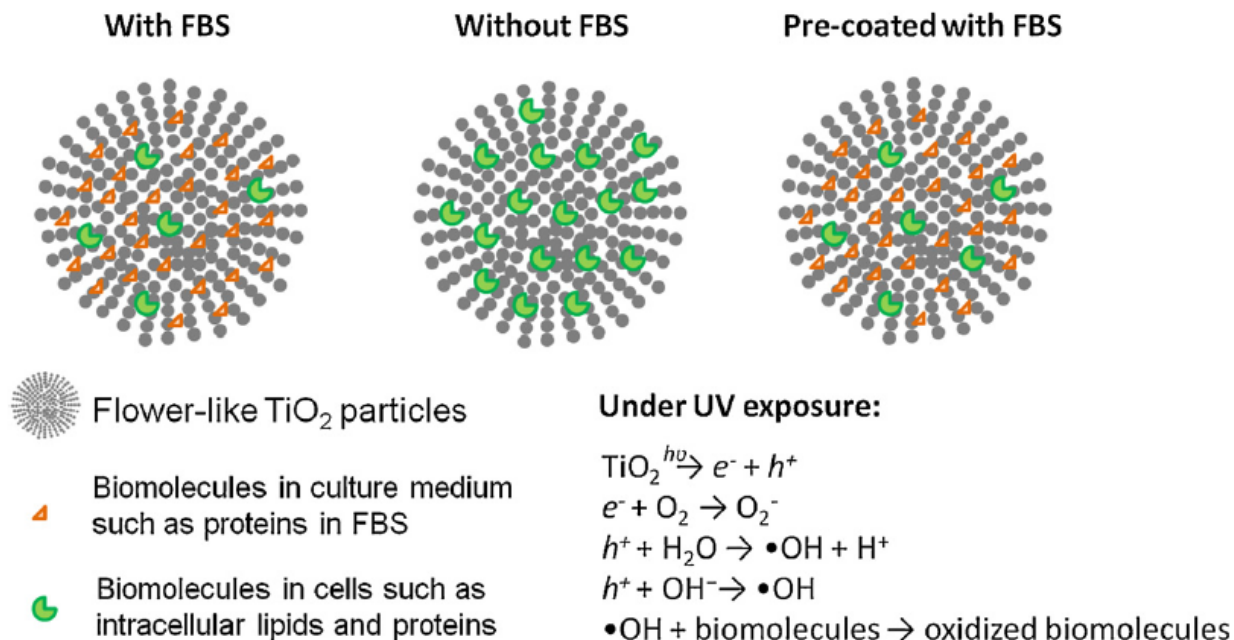


Fig. 8 Possible mechanisms behind the cytotoxicity and phototoxicity of flower-like TiO₂ particles. TiO₂ particles may be able to interact with biomolecules in cells. Particles with larger specific surface area may interact more with surrounding biomolecules, resulting in high cytotoxicity of the particles. However, with extracellular protein in cell culture medium or pre-coating TiO₂ particles with extracellular proteins would potentially decrease the interaction between particles and intracellular biomolecules. On the condition that TiO₂ particles were activated by UV light, the photons with enough energy can excite the electrons (e⁻) in the valence band of TiO₂ particles to the conduction band to generate electron hole pairs. In conduction band, the electrons (e⁻) can react with oxygen (O₂) nearby and generate superoxide ions (O₂⁻). On the other hand, the holes (h⁺) in valence band can interact with adsorbed water or hydroxide ions (OH⁻) and generate hydroxyl radicals (•OH). Highly active and damaging hydroxyl radicals can oxidize and damage cellular biomolecules such proteins, lipids and even DNA, causing the dysfunction of these biomolecules.