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# Photocatalytic production of hydroxyl radicals by commercial $TiO_2$ nanoparticles and phototoxic hazard identification

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ABSTRACT

This study identifies the phototoxic potential of commercial titanium dioxide nanoparticles ($TiO_2$ NPs) used in sunscreens and consumer products by employing a tiered testing approach comprising physicochemical, *in vitro* and *ex vivo* tests. Our results revealed that all the test samples of $TiO_2$ NPs, varied in surface coating, crystallinity and primary particle size, produced hydroxyl radicals upon UVA photoexcitation as determined by electron spin resonance (ESR) spectroscopy. Their phototoxic potentials were assessed first by combining the validated 3T3 neutral red uptake phototoxicity test and red blood cell phototoxicity test and subsequently in *ex vivo* models of chick chorioallantoic membrane (CAM) and reconstructed human 3D skin model (H3D). Crystalline structure and particle size of $TiO_2$ NPs were found to exert a major influence on the photocatalytic activity and the associated phototoxic effects. Besides, a medium-sized sample with silica/alumina also exhibited high phototoxic potency with no obvious relevance to the enhanced hydroxyl radicals and lipidperoxidation. This effect might be taken place through the interaction of harmful metal ions released from the oxide coating. However, no phototoxicity was observed on a H3D skin model probably due to the lack of efficient percutaneous absorption of $TiO_2$ NPs. This study demonstrates the efficacy of a tiered testing strategy for identifying phototoxic hazards of $TiO_2$ NPs and suggests the need for a comprehensive assessment that takes account of the effects of different coating materials and potential interactions between multiple mechanisms.

## 1. Introduction

The increasing usage of titanium dioxide nanoparticles ($TiO_2$ NPs) in a wide range of consumer products such as sunscreens, personal care and clothing has raised concerns over the possible risk of induced phototoxicity because of their direct exposure to human body and to sunlight. As a promising engineered photocatalyst, $TiO_2$ NPs can be photoactivated upon ultraviolet irradiation and produce reactive oxygen species (ROS), such as hydroxyl radicals (·OH), superoxide anion radicals ($\cdot O_2^-$) and singlet oxygen ($^1O_2$), at the particle surface in aqueous environments (Friehs et al., 2016; Nosaka and Nosaka, 2016). The ROS generated can rapidly react with biomolecules such as lipids, proteins and nucleic acids and are hypothesized to trigger phototoxic effects in live organisms.

Over the past decades, most knowledge of the photocatalytic activity and consequent phototoxicity of $TiO_2$ NPs towards human were gained on a cellular level. Anatase and Degussa P25 $TiO_2$ were shown to induce cytotoxicity in UVA-irradiated human keratinocytes (HaCaT) and fibroblasts, mediated by increased ROS production, lipid/protein peroxidation and organelle dysfunction (Horie et al., 2016; Sayes et al., 2006; Yin et al., 2012). Furthermore, a majority of studies have correlated the toxic effects of $TiO_2$ NPs to their physicochemical properties, such as particle size, crystallinity, surface area, aggregation/agglomeration state and surface modification (Horie et al., 2016; Prasad et al., 2013; Xiong et al., 2013; Yin et al., 2012). Particles of smaller size and anatase forms were generally recognized as more photocatalytic and toxic than larger particles in rutile forms (Zhang and Nosaka, 2014). To alleviate undesirable ROS effects, commercial $TiO_2$ NPs involved in cosmetic industry are usually deactivated by coating with silica or other metal oxide in order to minimize the photocatalytic activity as well as to enhance the dispersion stability(Oguma et al., 2013). Research has shown that the inert oxide coatings of sunscreen $TiO_2$ NPs resulted in

*Abbreviations:* $TiO_2$ NPs, titanium dioxide nanoparticles; ROS, reactive oxygen species; ESR, electron spin resonance spectroscopy; 3T3 NRU PT, 3T3 neutral red uptake phototoxicity test; PIF, photoirritation factor; MPE, mean photo effect; RBC PT, red blood cell phototoxicity test; MDA, malondialdehyde; CAM, chorioallantoic membrane; H3D PT, reconstructed human 3D skin model phototoxicity test

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less ROS (Lewicka et al., 2013). Doping $TiO_2$ NPs with iron ions was demonstrated to inhibit the ROS-mediated cytotoxicity and genotoxicity in HaCaT cells (Ghiazza et al., 2014). However, there is also evidence that certain $TiO_2$ NPs extracted from commercial sunscreens catalyze *in vitro* oxidative damage via photogeneration of ROS and free radicals (Dunford et al., 1997; Millington et al., 2014). Thus, the phototoxicity of various coated $TiO_2$ NPs used in sunscreens and consumer products has not been fully examined and the impacts on human are largely unknown.

On the other side, a number of *in vivo* studies on human volunteers or animal models revealed that sunscreen $TiO_2$ NPs, coated or not, retain mainly in the outer layers of skin (stratum corneum) (Filon et al., 2015; Schilling et al., 2010). This lack of significant penetration of $TiO_2$ NPs into viable skin layers would apparently limit its toxicity *in vivo*. Park found no phototoxic effect by nano-$TiO_2$ exposure on guinea pig skin and a reconstructed human skin model (Park et al., 2011). However, this study was limited to only one test sample of $TiO_2$. On the contrary, there is robust evidence that sunscreen $TiO_2$ NPs induced phototoxicity by increasing ROS generation in aquatic animals such as *Daphnia magna* and zebrafish (Bar-Ilan et al., 2012; Ma et al., 2012; Wyrwoll et al., 2016). The conflict of current research might be ascribed to the 1) large variety of physicochemical properties of $TiO_2$ NPs that may influence the result interpretation; 2) heterogeneity of experimental systems and model sensitivities; and 3) interference of NPs with assay components (*e.g.* $TiO_2$ with LDH assay components) (Friehs et al., 2016). Therefore, although several *in vitro* methods have been developed and validated for assessing phototoxicity, there remains the need to fully characterize their performance and prediction capability in the case of $TiO_2$ NPs.

The objective of the present study was to develop a means for identifying the potential phototoxic hazard of commercial $TiO_2$ NPs used in sunscreens and consumer products. Six different types of $TiO_2$ NPs were investigated in this study and their physicochemical properties *i.e.* crystal phase, primary particle size, morphology, aggregation size and specific surface area were characterized. The photocatalytic ability of $TiO_2$ NPs was evaluated by photoformation of ·OH radicals upon UVA irradiation by using electron spin resonance (ESR) spectroscopy. The phototoxicity was assessed by a tiered testing approach combing several *in vitro* and *ex vivo* methods, including the validated 3T3 neutral red uptake phototoxicity test, a red blood cell phototoxicity test (RBC PT) supplemented with malondialdehyde (MDA) measurement for membrane integrity, a photo-CAM assay using chorioallantoic membrane (CAM) and a human 3D skin model phototoxicity test (H3D PT).

## 2. Materials and methods

### 2.1. Nano-$TiO_2$ samples and characterization

Of the $TiO_2$ NPs chosen for this study, three samples coated with silica and/or alumina were obtained from cosmetic ingredient manufacturers: R21 (catalog # Ti-Si 2000, rutile, Uni-Powder, China), AR52 (catalog # SIMP-301A, anatase/rutile, Shanghai SIMP Biotechnology, China) and AR55 (catalog # anatase/rutile, Jiangsu Hehai nanometer science and Technology, China). Two samples of anatase $TiO_2$ NPs coated with silane were purchased from Aladdin: A23 and A108 (catalog # T104940 and T104946, anatase, Aladdin, China). P25 $TiO_2$ (catalog # Aeroxide® P25, anatase/rutile, Acros Organics, US) was also evaluated as an uncoated standard material. In this study, the $TiO_2$ NPs were designated by their crystalline structure and mean particle size as independently characterized.

For all the nano-$TiO_2$ samples, the X-ray diffraction (XRD) patterns were determined by a diffractometer (Ultima IV, Rigaku, Japan) operated at 40 kV and 40 mA with a Cu-Kα wavelength X-ray source. The morphology, primary particle size and elemental analysis of $TiO_2$ NPs were conducted using a field emission scanning electron microscope (FESEM, Hitachi S-4800, Hitachi High-Technologies, Japan) equipped with an X-ray energy dispersive spectroscopy (EDS) detector. Before FESEM measurements, samples were sputter-coated with Au by an ion sputter (Hitachi E-1045, Japan) under 15 mA for 120 s. The Brunauer–Emmett–Teller (BET) surface areas of $TiO_2$ NPs were measured using a micromeritics analyser (ASAP, Autosorb-IQ-MP, Quantachrome Instruments, US).

In the experiments, the $TiO_2$ NPs were prepared in stock suspensions at a concentration of 10,000 μg/mL in either ultrapure water or in phosphate-buffered saline solution (PBS, pH7.4). Sonication in water bath was conducted for 10–15 min until the suspension appear homogenous to the naked eye. Other concentrations were prepared by serial dilution prior to testing. The working suspensions of $TiO_2$ NPs (100 μg/mL) used in the photo-CAM assay and H3D PT were assessed for agglomeration and aggregation by dynamic light scattering (DLS, Malvern Zetasizer Nano, UK).

### 2.2. ESR measurement of hydroxyl radicals

The photoformation of hydroxyl radicals (·OH) by UVA-irradiated $TiO_2$ NPs was determined by ESR spectroscopy, using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as the spin trapping agent (Tang et al., 2017). An aliquot of 500 μL $TiO_2$ suspension in ultrapure water (200 μg/mL) were put into a sealed 1 cm × 1 cm quartz cuvette with the addition of 500 μL 400 mM DMPO (Dojindo Laboratories, Japan) solution. The quartz cuvette were exposed to radiation from a high power spot UVA source (UVEC-4, Lanpulike, $\lambda_{max}$ = 365 nm, 100 mW/cm$^2$) for 8 min which gives a UVA dose of 48 J/cm$^2$ as measured by a Spectronics AccuMax XF-1000 radiometer equipped with a XS-365 UVA sensor. After irradiation, about 50 μL aliquot of the DMPO-$TiO_2$ suspensions was immediately transferred to a 0.5 mm diameter glass capillary tube and the spin-trapping ESR spectra were recorded on a Micro-ESR (Active Spectrum, CA) at ambient temperature using the following settings: 1 G field modulation, 100 G scan range, 15 mW microwave power and 40 scan times. A pure DMPO solution in the absence of $TiO_2$ NPs was subjected to the same procedure as control.

### 2.3. 3T3 neutral red uptake phototoxicity test (3T3 NRU PT)

The 3T3 NRU PT was conducted as described in the Organisation for Economic Cooperation and Development (OECD) guideline 432 (OECD, 2010) using 3T3 Balb/c fibroblasts (ATCC CCL-163), passages 65–75. The light source used in the 3T3 NRU PT and other phototoxicity tests of this manuscript was a solar simulator (SOL 500, Dr Hönle, Germany) equipped with a H1 filter to obtain a radiation dose of 5 J/cm$^2$ UVA. To counteract the interference of $TiO_2$ NPs accumulated in cells, the optical density value of a parallel plate control without the addition of neural red (NR) was subtracted from its NR-treated counterpart for each sample, under both irradiation and dark conditions. The phototoxicity was assessed using photoirritancy factor (PIF) and/or mean photo effect (MPE) determined by a Phototox Version 2.0 software (ZEBET, Germany). According to the guideline, a test substance with a PIF $> 2$ and $< 5$ or an MPE $> 0.1$ and $< 0.15$ is predicted as "probably phototoxic" while a PIF $> 5$ or an MPE $> 0.15$ predicts: "phototoxicity".

### 2.4. Red blood cell phototoxicity test (RBC PT) and MDA measurement

The RBC-PT was performed according to the ECVAM DB-ALM Protocol No. 81 (ECVAM, 1994) in which rabbit erythrocytes were employed to detect photoinduced cell membrane damage (hemolysis) and hemoglobin oxidation. The RBC suspensions were prepared from freshly obtained rabbit blood from Beijing Xinglong Experimental Animals Co. Ltd. (Experimental Animal Production License No. SCXK (Beijing) 2016-0003, China), in which the blood samples were withdrawn from the marginal vein of healthy New Zealand white rabbits. The RBC suspension was prepared by washing with PBS (pH7.4) for