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NANYANG
TECHNOLOGICAL
UNIVERSITY

**SYSTEMATIC STUDY ON THE INFLUENCE OF PARTICLE
SIZE ON THE CYTOTOXICITY OF NANOPARTICLES**

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A thesis submitted to the Nanyang Technological University
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List of abbreviations

Materials and assays

BEGM: bronchial epithelial growth medium
BSA: bovine serum albumin
CDMEM: complete cell culture medium
DCM: dichloromethane
DMEM: Dulbecco's Modified Eagle Medium
ELISA: enzyme-linked immunosorbent assay
FBS: fetal bovine serum
HA: hydroxyapatite
HPF: Hydroxyphenyl fluorescein
PEMA: poly (ethylene-alt-maleic anhydride)
PI: propidium iodide
PLA: polylactic acid
PLGA: poly(d,l-lactide-co-glycolide acid)
P60: PLGA nanoparticles of primary particle size 60 nm
P100: PLGA nanoparticles of primary particle size 100 nm
P200: PLGA nanoparticles of primary particle size 200 nm
PVA: polyvinyl alcohol
TiO₂: titanium dioxide
T10: TiO₂ nanoparticles of primary particle size 10 nm
T20: TiO₂ nanoparticles of primary particle size 20 nm
T100: TiO₂ nanoparticles of primary particle size 100 nm
T20-PEMA: PEMA coated T20 nanoparticles
T20-chitosan: chitosan coated T20 nanoparticles
T33: TiO₂ particles of specific surface area 33.3 m²/g
T174: TiO₂ particles of specific surface area 174.5 m²/g
TNF- α : tumor necrosis factor-alpha
ZnO: zinc oxide

Equipments:

BET: brunauer-emmett-teller
DLS: dynamic light scattering
FESEM: field emission scanning electron microscope
TEM: transmission electron microscopy
TGA: thermo gravimetric analyzer

Others

ANOVA: a one-way analysis of variance
DI water: distilled water
ESDS: electrochemical spark discharge spallation
GSH: glutathione
IL-1 β : interleukin-1 β
LC: light control
MW: molecular weight
NC: negative control
PBS: phosphate buffered saline
PT pore: permeability transition pore

ROS: reactive oxygen species
SD: standard deviation
UV: ultraviolet
UV-Vis: ultraviolet-visible

Abstract

The study of size effects on the cytotoxicity of poly(d,l-lactide-co-glycolide acid) (PLGA) and titanium dioxide (TiO₂) nanoparticles was reported in this thesis. The main objective of this study is to investigate how particle size can influence the cytotoxicity of PLGA and TiO₂ nanoparticles, and to uncover the mechanism behind such toxic effects. We investigated various cytotoxicity parameters, including cell viability, generation of reactive oxygen species (ROS), mitochondrial depolarization, plasma membrane leakage, increased intracellular calcium concentration and inflammation response, to comprehensively understand the toxicity of different sized nanoparticles after cells were incubated with these nanoparticles for 24 h. Our current results show that PLGA and TiO₂ nanoparticles could trigger size-dependent cytotoxicity to both RAW264.7 cells and BEAS-2B cells. The increased cytotoxicity observed from smaller nanoparticles with larger specific surface area could be explained from their surface area-dependent interactions with biomolecules. TiO₂ nanoparticles could also trigger size- and surface area- dependent phototoxicity. The higher generation of hydroxyl radicals could be the major reason for the higher phototoxicity of TiO₂ nanoparticles with smaller particle size and/or larger surface area. Surface coating of TiO₂ nanoparticles with poly(ethylene-*alt*-maleic anhydride) (PEMA) and chitosan could reduce phototoxicity of TiO₂ nanoparticles, by hindering adsorption of biomolecules and generation of hydroxyl radical (•OH) during photoactivation. Presence of pre-adsorbed extracellular proteins was also found to decrease toxic effects of TiO₂ particles as compared to conditions without extracellular proteins. These results also suggest that surface area, a size related factor, could be a paradigm to predict the cytotoxicity of PLGA and TiO₂ nanoparticles. However, this study does not overlook the importance of some other of factors such as surface functional group, which worth further exploration in future work.

Chapter 1 Introduction

1. 1 Background

Engineered nanomaterials are materials produced with at least one of the dimensions shorter than 100 nm [1]. Nanomaterials possess unique properties, arising from their minute sizes, large surface areas and high surface reactivity, and have thus been explored for a broad range of applications such as in sporting goods, cosmetics and electronics [2-4]. It also has great potential in biomedical field for the purpose of imaging, diagnosis and drug delivery [4]. It was estimated that there were at least 1,300 commercially available products that contain nanomaterials [5]. Because of this, direct or indirect human contact with nanomaterials becomes inevitable, ultimately raising issues pertaining to their safety [4, 6-8]. Currently, safety of nanoparticles is receiving increasing concerns in the regulatory, academic and industrial communities [8]. The debate on the impact of nanotechnology on the development of society is also receiving more attention [8].

Recently there has been a rise in the number of papers reporting on the toxicity of various nanomaterials including fullerene [9], ZnO, TiO₂, CeO₂ [10]. For example, titanium dioxide (TiO₂) nanoparticles and zinc oxide (ZnO) 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 [11-14]. ZnO nanoparticles have also been shown to induce

genotoxicity, which have been reported to be likely because of the Zn^{2+} ions release [11-12]. Besides these metal oxide particles, more attention should also be given to biomedical nanomaterials. Zhao *et al.* [15] reported that hydroxyapatite (HA) nanoparticles of three different surface areas did not cause significant decrease in cell viability, but higher ROS generation was observed in the cells after incubating with HA nanoparticles of large surface areas. PLGA nanoparticles are also widely studied for biomedical uses and have great potential to be utilized in drug delivery systems.

Studies have also shown that the potential physicochemical properties which can affect the toxicity of nanoparticles include particle chemical composition, size, surface area, crystallinity, surface chemistry, surface charge, shape, aggregation state and concentration [6]. 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.

1.2 Problem statement

Although there are more and more papers discussing nanotoxicity, the potential cytotoxicity induced by PLGA and TiO_2 nanoparticles is still inconclusive. Those nanoparticles which are able to dissolve and shed toxic transition metal ions attracted most of the attentions, such as ZnO , CuO and NiO nanoparticles [16], because these particles exhibit very obvious toxic effect to cells and animals. The toxic mechanism of these nanomaterials has been very clear. However those nanomaterials, which do not release toxic metal ions such as PLGA and TiO_2 nanoparticles, are less toxic and less

investigated. Biocompatibility studies on PLGA mainly focused on the toxicity of bulk, film and their extract. Most researchers in biomedical field believe that PLGA nanoparticles are biocompatible and safe to be used in human body. Although PLGA nanoparticles have been studied for the applications in drug delivery system for a long time, very little is known and understood about the safety of these nanoparticles when used in the human body, including the cytotoxic mechanism. Although more than 1,000 articles, on PLGA nanoparticles as drug delivery systems have been published and indexed in the Web of Science, the number of papers with that reports on its cytotoxicity are fewer than 10. Most researches on blank PLGA nanoparticles stopped at viability and cellular uptake study. The in-depth investigation of sublethal effect of PLGA nanoparticles such as oxidative stress and inflammatory effects has not been reported. TiO₂ nanoparticles are even considered as a “natural” nanomaterial and are positively accepted by the general public [17]. However, some reports have pointed out the potential toxicity of TiO₂ nanoparticles, including their ability to induce oxidative stress [18-21], genotoxicity [22-24], and immunotoxicity [25-26]. Although there are more than 5000 papers are discussion the development of TiO₂ nanoparticles, of which less than 2% discuss the toxicity of such nanomaterials. The insufficient knowledge is making the toxicity investigation of PLGA and TiO₂ nanoparticles into an urgent situation. Only once such investigations will have been undertaken can PLGA and TiO₂ nanoparticles be used in human body.

Moreover, how particle properties such as size could influence the potential cytotoxicity of PLGA and TiO₂ nanoparticles has not been sufficiently investigated and the possible mechanism has not been fully understood. Jang *et al.*[27] observed an inverse correlation between the size of TiO₂ particle and antimicrobial effect after photoactivation, but did

not provide the probable mechanism behind this phenomenon. The size effect of these particles on cell models has even been less documented.

Last but not the least, size influence on phototoxicity of TiO₂ nanoparticles is still not clear. There are papers pointing out that the phototoxicity of TiO₂ nanoparticles could be because of the generation of hydroxyl radical during photoactivation [28], but how the size of TiO₂ nanoparticles can influence the generation of hydroxyl radical and their potential phototoxic effect has not been discussed.

1.3 objectives

The focus of this thesis is to study the size effect on the cytotoxicity of both PLGA and TiO₂ nanoparticles. The main objective is to investigate the potential cytotoxicity of PLGA and TiO₂ nanoparticles, and how particle size can influence this outcome. To fulfil this main objective, this study is divided into three detailed parts.

The first part is to synthesize and characterize PLGA and TiO₂ nanoparticles of different size. PLGA nanoparticles are synthesized in house. Reports show that several synthesis parameters could affect the size of PLGA nanoparticles [29]. Because PLGA we used is different from what the literature reported, the factors that would affect the size of PLGA nanoparticles we use are firstly investigated. After synthesis of nanoparticles, all the particles are thoroughly characterized to understand their physiochemical properties which are closely correlated with their *in vitro* and *in vivo* toxic effect.

The second part is to investigate the effect of size on the cytotoxicity of PLGA and TiO₂ nanoparticles. This will be achieved by a series of *in vitro* cytotoxicity test including cell viability, generation of ROS, mitochondrial depolarization, plasma membrane leakage, increased intracellular calcium concentration and inflammation response. The change in the physiological properties or viability of cells will provide us with the potential toxicity of nanoparticles.

The third part is to understand the mechanism behind the cytotoxicity of different-sized PLGA and TiO₂ nanoparticles. This will be achieved by examination of the hypothesis raised from the cytotoxicity studies. A potential method to decrease such cytotoxic effect will also be proposed.

1.4 Scope

In this thesis, we tested the cytotoxicity of three different-sized PLGA and TiO₂ nanoparticles. The phototoxicity of TiO₂ nanoparticles will also be discussed. The scope of this study is exhibited in **Figure 1.1**. Characterization of all nanoparticles used will be conducted before all the *in vitro* studies, including primary particle size, hydrodynamic size (agglomerated size) in both water and complete cell culture medium, specific surface area and endotoxin level.

RAW264.7 (murine macrophage) cells were chosen to represent the phagocytic cell lines, which can actively interact with foreign particles in *in vivo* systems [10, 15, 30]. BEAS-2B (human bronchial epithelial) cells were selected to represent airway epithelium which is one of the defensive cells against inhaled nanoparticles [15]. These two cell lines

are used in this systematic study primarily as biological model systems to illustrate the toxic mechanism of PLGA and TiO₂ nanoparticles, because they are well understood cell lines for nanotoxicology studies [3, 10, 28, 31-33], which enable us to compare our study with others' reports.

Cytotoxicity parameters tested included cell viability, generation of ROS, mitochondrial depolarization, plasma membrane leakage, increased intracellular calcium concentration and inflammation response after 24 h incubation with different nanoparticles. A potential paradigm to explain and predict the cytotoxicity of different sized nanoparticles is proposed. Finally, methods to decrease such nanotoxicity was also proposed and examined in this study.

Figure 1.1 Schematic scope of this thesis

Chapter 2 Literature Review

2.1 Background

2.1.1 Nanotechnology and nanomaterials

Nobel laureate Richard Feynman raised the hypothesis that ‘there’s plenty of room at the bottom’ in his 1959 lecture, which opened a new era in technology - nanotechnology [2]. In 1996, Richard Smalley won the Nobel Prize in Chemistry because of his discovery of fullerene. His finding pushed nanotechnology into a new summit [34]. Till now there are more than 1,300 products in market based on nanotechnology [5] and it was estimated that nanomaterials would have a \$1 trillion market by 2015 [4]. Nanotechnology and nanomaterials have penetration into every aspect of human life including cosmetics, sports good and even biomedical field [4-5]. In this study, we will focus on two types of nanomaterials, which are biomedically used PLGA nanoparticles and cosmetic used TiO₂ nanoparticles.

(a) PLGA nanoparticles

Nanoparticulate delivery system is a novel and intriguing field for researchers who are interested in drug delivery system, because it has some excellent properties to overcome obstacles in administration of plasmid DNA and RNAi materials, certain drugs and vaccines [35]. Several biocompatible and biodegradable materials, such as PLGA, have

been widely investigated as carrier in nanoparticulate delivery system. The polymer matrix can also prevent drug degradation, diminish side effects, increase drug efficacy, and modulate drug release speed by altering formulation method [35-36]. PLGA nanoparticles has been successfully developed to encapsulate drugs including paclitaxel [37], vincristine [38], cisplatin [39], siRNA [40] *etc.*. The chemical structure of PLGA polymer is shown in **Figure 2.1**.

Figure 2.1 Chemical structure of PLGA RESORMER® RG502H. Adapted from the protocol provided by supplier.

PLGA nanoparticles can be synthesized through different methods, including nanoprecipitation [41], emulsion solvent evaporation [29, 42-43], salting-out [44] and solvent displacement [45] method, of which the first two methods are the two most commonly used methods according to literature. The procedure to synthesize PLGA nanoparticles using (1) emulsion solvent evaporation and (2) nanoprecipitation is shown in **Figure 2.2**. The solvent emulsion evaporation technique includes two steps. First, the organic phase should form stable droplet in continuous water phase by using external forces such as high speed stirring or ultrasonication. The surfactant is normally needed in

this step to stabilize the oil droplets. Gradually, the organic solvent is evaporated and homogeneous PLGA nanoparticles form in the end. As for the nanoprecipitation technique, the procedure is much easier and faster as compared with solvent emulsion evaporation technique. Normally, the PLGA is dissolved in water miscible organic solvent first. When the droplet containing PLGA is dropped in to continuous aqueous phase, the organic solvent will diffuse into continuous phase, the solidified PLGA nanoparticles will form simultaneously [46].

Figure 2.2 o/w single emulsion solvent evaporation method and nanoprecipitation method utilized to synthesize PLGA nanoparticles.

The size of PLGA nanoparticles can be changed through some synthesis parameters. Song *et al.* [29] reported that PLGA particles' size was significantly influenced by PLGA concentration, property (the degrees of hydrolyzation and polymerization) of polyvinyl alcohol (PVA), organic solvent composition. It could also be affected by molecular weight of PLGA, PVA concentration and ultrasonication time for solvent emulsion evaporation method as shown in **Table 2.1**.

Table 2.1 The correlation between different synthetic parameters and the size of PLGA nanoparticles. Adapted from Song *et al.* [29].

Formulation parameters	Mean diameter
MW of PLGA	+
L:G ratio of PLGA	0
PLGA concentration	+*
HD of PVA	+*
PD of PVA	+*
PVA concentration	—
Initial drugs content	0
A/D volume ratio	—*
Aqueous phase pH	0
NaCl concentration	+*
W/O volume ratio	— +
Sonication time	—
Sonication power	0
Removal rate of organic solvents	—

+ represents positive correlation

— represents negative correlation

0 represents no correlation

* represents significant difference

(b) TiO₂ nanoparticles

TiO₂ nanoparticles are one class of nanomaterials that is produced in large volumes, and widely utilized in across industrial and consumer goods, mainly because they are relatively inexpensive and chemically stable [17, 47-48]. The wide use of TiO₂ nanoparticles in sun block creams is mainly due to their ability to absorb wide spectrum

of ultraviolet (UV) light, including UVA as well as UVB. On the other hand, TiO_2 nanoparticles are also known as photocatalyst [18], which enables TiO_2 nanoparticles to be developed into disinfectant products. This property also provide TiO_2 nanoparticles with great potential to be used in biomedical field, such as photodynamic therapy for cancers [18]. The schematic mechanism of the photo-catalytic activity of TiO_2 nanoparticles upon UV activation is shown in **Figure 2.3**. When TiO_2 nanoparticles are exposed to UV light, the photon energy activates the electrons (e^-) up to conduction band from valence band, which leaves only holes (h^+) in the valence band. This pair of electron and hole is called electron hole pairs [49]. The holes (h^+) can interact with adsorbed H_2O or hydroxide ions (OH^-) to generate hydroxyl radicals ($\bullet\text{OH}$) which are highly reactive [50]. Furthermore, the electrons (e^-) in the conduction band can reduce oxygen (O_2) adsorbed onto the particle surface to produce superoxide ions (O_2^-), which can further interact with water in the environment to form hydrogen peroxide in aqueous medium [28, 51]. These ROS generated during photoactivation is very active and able to damage microbes nearby.

Figure 2.3 Schematic mechanism of the photo-catalytic activity of TiO_2 nanoparticles.

2.1.2 Nanotoxicology

Nanoparticles, with size between single molecule and bulk, exhibit some special properties such as small particles size, big surface area [6] as well as some special toxicity. Their small size allows them to enter some special biological compartment [4, 52], much easier than molecules and bulk. Nanoparticles are reported to have the ability to enter interstitium or cells, which may trigger some undesired response such as inflammation [4, 53-54]. For nanoparticles to move into the clinical arena and human daily lives, it is therefore critical that rigorous and independent research uncovers and understands how different physico-chemical properties of these novel materials can influence biocompatibility or toxicity. As such, it is imperative that the safety of PLGA and TiO₂ nanoparticles is rigorously assessed before they can be considered in clinical arena or publicly used materials.

(a) PLGA nanoparticles

Till date, most biocompatibility studies on PLGA have mainly focused on the bulk, film, and microparticulate form of this material [55-58]. For example, one such study by Yoon *et al.* showed that bulk PLGA implants have the ability to trigger an inflammatory response near to the implant site, which was explained to be due to the decreased pH caused by the degradation products [58]. As for PLGA particles, papers with reports on their cytotoxicity are limited. Thus, it is crucial to understand the potential toxicity and cytotoxic mechanism of these materials. More recently, Sivadas *et al.* [57] tested the compatibility of seven types of polymeric microspheres (including PLGA); of which PLGA microspheres were shown to have the highest ability to trigger an inflammatory

response and cause compromised cell monolayer integrity. Pertaining to nano-sized PLGA, Nafee *et al.* [59] reported that although chitosan-coated PLGA nanoparticles did not show obvious cellular toxicity, its biocompatibility was clearly dependent on concentration and cell type. Similarly, Katsikari *et al.* [60] investigated the cytotoxicity of PLGA nanoparticles using MTS and LDH assays and reported a decrease in cell viability with increasing particle concentration. Semete *et al.* [61] reported a systematic cytotoxicity and bio-distribution study of PLGA nanoparticles. The *in vitro* WST-1 test showed more than 75% cell viability after cells interact with PLGA nanoparticles of several different concentrations. From the literature, it is therefore evident that most papers report cytotoxicity simply based on cell viability obtained from assays that test mitochondrial activity *etc.*. On the other hand, to rigorously investigate the biocompatibility or toxicity of nanoparticles through *in vitro* tests, other tests which determine toxicity at the sub-lethal level have to be considered.

(b) TiO₂ nanoparticles

In nanotoxicology studies, more attention is paid to soluble metal oxide particles, such as ZnO, which release toxic metal ions like Zn²⁺ [11-14]. The potential mechanism of cytotoxicity induced by non-soluble metal oxide such as TiO₂ nanoparticles is still controversial. In contrast to ZnO, TiO₂ was classified as a biologically inert material, as reported in some studies [62-63] and is generally positively accepted by the public [17]. However, some reports have pointed out the potential toxicity of TiO₂ nanoparticles, including their ability to induce oxidative stress [18-21], genotoxicity [22-24], and immunotoxicity [25-26]. On the other hand, TiO₂ (anatase) can be excited by light with wavelength no longer than 385 nm [49]. The photons, with sufficient energy, can excite

the electrons in valence band up to conduction band, resulting in the generating electron-hole pairs. The electron-hole pairs can actively interact with adsorbed water or oxygen to generate toxic ROS [64-65]. 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.* [66] 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.* also pointed out the potential photogenotoxicity of TiO₂ nanoparticles [67] to Chinese hamster cell line. In another study, Gopalan *et al.* [68] reported that TiO₂ tended to introduce dose-dependent photogenotoxic effect to human lymphocytes but not to human sperms. The understanding of size influence on the cytotoxicity and phototoxicity of TiO₂ nanoparticles will provide us with further understanding of the mechanism behind these toxic effects of nanomaterials.

2.2 Factors that may affect the toxicity of nanoparticles

Since the toxicity of nanoparticles is correlated to their physicochemical properties, for example, chemical composition, size, shape, surface area, surface chemistry and surface charge *etc.* [1, 69], understanding how these properties can influence cytotoxicity will provide us with useful insights on the possible mechanisms behind their toxicity. In this study, we only focused on two factors, size and a size-related factor surface area.

Size is one of the most important factors in nanotoxicity study. Normally, particle size can determine the physiological response of cells after interaction with different nanoparticles, and also influence the biodistribution and elimination of these materials

[70]. Watari *et al.* [71] studied the toxicity of several particles with size of 200 nm, 100 μm and 10 μm . The neutrophils released increased ROS, tumor necrosis factor-alpha (TNF- α) and interleukin-1 β (IL-1 β) with decreased particles size. Furthermore, they concluded that the size effect could be non-specific and independent of materials from the size effect study of a wide range of particles including Ti, Fe, TiO₂ and polylactic acid (PLA). However, this hypothesis was refuted by some other studies, which believes that this idea of 'one size fit all' ignored the complexity of nanotoxicology [72]. Gurr *et al.* [18] compared cytotoxicity of TiO₂ nanoparticles of different sizes, 10 nm, 20 nm and 200 nm in the absence of photoactivation. 10 nm and 20 nm particles caused increase of ROS, oxidative stress induced damage of lipid and DNA on BEAS-2B cells, but 200 nm particles do not have these toxicities. However, this paper did not provide any reasonable explanation for this result. Some *in vivo* studies also found the correlation between particle size and their cytotoxicity. Oberdorster *et al.* [73] showed that 20 nm TiO₂ particles induced much higher level of inflammatory response as compared to 250 nm TiO₂ particles after 12-week inhalation study in rats. Moreover, in this study Oberdorster *et al.* also find that such toxic effect was well correlated with surface area of particles. They explained the increased toxicity of smaller particles was mainly due to larger surface area, easier access to interstice and increased retention. Surface area is also a crucial factor, which influences nanotoxicity, because nanoparticles has relatively bigger surface areas per unit mass and more active surface [74], and also most of the interaction between nanoparticles and biomolecules happened at particle surface [70]. With decreasing particle size, specific surface area also increases simultaneously. So some papers suggested that the influence of size on the cytotoxicity of nanoparticles is actually the influence of surface area.

Although 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 unknown. Some reports hypothesized that specific surface area might be the key reason behind an increase in cytotoxicity observed with smaller-sized TiO₂ particles, rather than particle size per se. For example, in an *in vivo* study conducted by Sager *et al.* [75], the pulmonary toxicity of two TiO₂ particles with two sizes (ultrafine and fine particles) was studied. It was concluded that specific surface area might be a more accurate dose metric. However, in that study, the effect of particle size was still not fully eliminated, in view that comparisons were made between ultrafine and fine particles. As such, to understand the true influence of size and surface area will help us to understand the mechanism behind nanotoxicology.

2.3 Assays in evaluating the toxicity of nanoparticles

There are many biological and molecular reactions happening in cells upon stimulation with nanoparticles [4, 6]. Among them, the generation of ROS stimulated by nanoparticles is considered a useful paradigm to predict the toxicity of nanomaterials [3]. Too much ROS can cause oxidative stress inside of cells and further cause the calcium ions influx from extracellular medium and intracellular organelles such as endoplasmic reticulum. The rise of intracellular calcium concentration can stimulate calcium ion uptake into mitochondria. If the buffering action exceeds the threshold, it could trigger the opening of permeability transition pore (PT pore) in large scale, causing the mitochondrial depolarization and apoptosis of cells [3]. Thus, oxidative stress could trigger a range of oxidant injuries including mitochondrial depolarization, intracellular calcium ion influx and even cause the death of cells [12]. This oxidative stress involved

pathway is the most widely accepted pathway to understand the cytotoxicity of different nanomaterials [3, 12]. On the other hand, inflammatory response studies will provide us clues on the systematic and chronic toxicity of nanomaterials. Two abiotic tests, namely nanoparticle protein interaction and generation of hydroxyl radicals, can help the understanding of the mechanism behind the size influence on the cytotoxicity of nanoparticles.

2.3.1 Cell viability

Cell viability test is a common assay to evaluate the overall toxicity of materials. Cell viability can be tested in a broad way, such as DNA synthesis and quantification, ATP test and the activity of dehydrogenase. For *in vitro* cell viability test in this study, MTS assay was utilized to quantify the metabolically active cells through the interaction between MTS and dehydrogenase enzymes in live cells, because MTS can be reduced by dehydrogenase to a red coloured formazan. The red coloured solution can absorb light at 490 nm, which is proportional to the amount of formazan and the amount of dehydrogenase in live cells. This is an indirect way to measure the number of live cells through the metabolic activity.

2.3.2 Production of reactive oxygen species (ROS)

ROS include both oxygen radicals as well as certain non-radicals such as H_2O_2 , which are oxidizing agent and/or easily converted into radicals [76]. ROS plays an important role in nanoparticle-mediated injury [3-4]. ROS can be generated from cells. For example, in

mitochondria, the procedure of oxygen metabolism proceeds through several ROS, which are $O_2 \rightarrow O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow OH^{\cdot} \rightarrow H_2O$. In well balanced aerobic metabolism, the ROS production is maintained at cell required level for ATP synthesis [51]. ROS can also generate from materials. For example, photoactivated TiO_2 nanoparticles have been proved to be capable of generating ROS in abiotic conditions [28, 77]. No matter how the ROS generate, if the balance between the production of antioxidant defence and ROS is damaged, the overwhelming ROS can be detrimental to intracellular components such as proteins, lipids and even DNA [51]. There are three levels of oxidative damage. First, when the ROS slightly increased in cells, the defence systems can be up-regulated to protect the cells. Second, when the ROS is excessive to the cells, the cell injury such as proteins oxidation, lipids oxidation, and DNA damage can be observed. Finally, if the cells cannot recover from the ROS induced damage by replacing or repairing those damaged biomolecules, the damage can trigger the cell death through apoptosis or necrosis [76]. The excessive ROS has also been related to the pathogenesis of cancer, diabetes mellitus, arthritis *etc.* [50]. The generation of superoxide in mitochondria can be detected with MitoSOX™ Red mitochondrial superoxide indicator [10, 12], which can selectively target to the mitochondria and then be oxidized by superoxide generated in mitochondria. The red fluorescence observed under fluorescent microscope will indicate the generation of superoxide in mitochondria.

2.3.3 Intracellular calcium concentration

Calcium is a significant regulator in mitochondria to stimulate ATP synthesis. Thus, the damage of calcium homeostasis in mitochondria may induce disease like cardiac ischemia and neuronal toxicity [51]. Brookes *et al.* [51] reported that Ca^{2+} overload in

mitochondria was also related to the generation of ROS and the cause of apoptosis. The Ca^{2+} was both the cause and the effect of ROS generation. The increased Ca^{2+} could stimulate the metabolism rate in mitochondria and enhanced the output of ROS. On the other hand, the increased ROS can stimulate the open of permeability transition (PT) pore to cause Ca^{2+} overload in mitochondria [51]. Another toxic mechanism is that the Ca^{2+} overload can cause the release of antioxidant GSH in mitochondria, resulting in the accumulation of ROS [51]. As for nanotoxicity studies, Xia *et al.*[3] reported that the ambient ultrafine particles and cationic polystyrene nanospheres introduced oxidative stress could be through a calcium regulated pathway. The Fluo-4 Calcium Indicators could be used for the test of intracellular free calcium. Fluo-4 is a dye to detect intracellular calcium levels, which emits green fluorescence under fluorescence microscope when there is an increase in the intracellular calcium ions.

2.3.4 Mitochondrial depolarization

If the oxidative stress in cells is too high, mitochondrial function could be influenced, resulting in mitochondrial depolarization [8]. Mitochondrial perturbation could cause toxic effect to cells, resulting in necrosis, apoptosis and autophagy [8, 78]. A decrease in mitochondrial membrane potential would be detected using cationic dye JC1, which indicates unhealthy mitochondria [12]. JC1 is normally accumulated in the mitochondria of healthy cells. But during mitochondrial depolarization, the dye is released from the mitochondria to the cytosol. This release will cause the fluorescence colour to shift from red to green, which forms the basis of quantifying cells with decreased mitochondrial potential.

2.3.5 Cellular membrane integrity

Propidium iodide (PI) uptake is a method carried out to measure the mortality of cell, which provide similar information as MTS assays but these two methods are based on different mechanisms. The co-staining of cells with both Hoechst and PI can indicate the percentage of dead cells in the system. Hoechst and PI are two DNA interactive agents. The cell-permeable Hoechst is utilized to bind to DNA of both live and dead cells, allowing us to quantify and locate the total cells in each image. The PI can only penetrate into dead cells through the damaged plasma membrane and give out red fluorescence of the nucleus under fluorescence microscope to indicate the number of dead cells. The loss of cell viability always accompanied with decreased the integrity of cell membrane which result in the uptake of PI to stain the nucleus [12].

2.3.6 Inflammation response study

The immune systems in living organisms have many ways to protect themselves from the attacks by “foreign invaders”, which include infectious bacteria, virus as well as natural and synthetic materials [79]. When immunological cells recognize pathogens or any foreign substances, they will be activated to trigger an inflammatory response. The release of inflammatory cytokines, e.g. TNF- α [80] by activated cells is to build up a response to destroy these foreign bodies [53]. Thus, TNF- α is an indicator of the inflammatory responses. However, TNF- α is also related with inflammation, generalized wasting and septic shock [81], programmed cell death [81], metabolic disturbances of cancer patients [82]. TNF- α induction of cytokine cascades can induce and maintain inflammation. Although the evolutionary advantages of inflammatory response could be

the resistance to infection, the unnecessary and prolonged inflammation may cause damages to human health. RAW264.7 cells, a kind of macrophage cells, can release cytokines such as TNF- α with the stimulation of materials in the earlier phase of inflammation [80]. It has been reported that ambient ultrafine particles [3] have the ability to stimulate macrophages to release TNF- α . Because of its serious acute and chronic toxicity, it is crucial to understand how the size of nanoparticles can influence the TNF- α release from macrophage cells. The Enzyme-Linked Immunosorbent Assay (ELISA) test is a mature technique to quantitatively measure the amount of TNF- α released by macrophage.

2.3.7 Nanoparticle-protein interaction

When particles enter cell culture medium or biological fluid such as blood, they will be coated with a layer of proteins, which is called a protein corona [83]. This protein corona undergoes dynamic changes even after particles enter cells [84]. The association and dissociation of protein from particle surfaces can determine the interaction between particles and cells [84], and subsequently influence its toxicity [85]. The interaction between nanoparticles and proteins has been discussed a lot in nanotoxicology studies, because such interaction could influence the wrapping, uptake and biodistribution of nanoparticles, resulting in biocompatible or bio-adverse results [84]. On the other hand, the adsorption or desorption of biomolecules onto nanomaterials may lead to the dysfunction or damage of these adsorbed biomolecules [86-91]. Some reports have discussed the adsorption ability of TiO₂ particles [92-94]. Smaller TiO₂ nanoparticles with larger surface area tend to adsorb more protein, leading to higher toxic effect [94]. Since size and surface area of nano-sized particles could influence their capability to

adsorb proteins, it would therefore be of interest in this thesis to decipher the influence of size and surface area on the interaction between nanoparticles and biomolecules.

2.3.8 Generation of hydroxyl radicals

As we discussed earlier, the photoactivated TiO₂ nanoparticles could generate hydroxyl radicals, which is the reason to use them in disinfectant products. However, highly active and damaging hydroxyl radicals also have potential to cause damaging to cells, because hydroxyl radicals have been proved to be able to oxidize and damage cellular biomolecules such proteins, lipids and even DNA, causing the dysfunction of biomolecules [49, 77, 95-97]. In this study, generation of hydroxyl radicals will be assayed using HPF assay in abiotic conditions to understand how size of TiO₂ particles can influence the generation of hydroxyl radical under photoactivation. This study may also help to understand the mechanism behind the size effect on the phototoxicity of TiO₂ particles.

Table 2.2 Assays and their functions

Marker	Probe	Utility
Protein	Pierce 660	Quantification of proteins on nanoparticles
Hydroxyl radical	HPF	Quantification of hydroxyl radical
Live cell	MTS	Metabolic activity of live cells
Inflammation	ELISA	TNF-α released from cells
Cell nucleus	Hoechst	Localization of cells
Plasma membrane damage	*PI	Damaged plasma membrane integrity
Mitochondrial superoxide	MitoSOX	Generation of mitochondrial superoxide
Mitochondrial membrane potential	JC-1	Mitochondrial depolarization
Intracellular calcium	Fluo-4	Increase of intracellular calcium level

* PI: propidium iodide

2.4 Summary

There are numerous journal papers discussing the fantastic application of PLGA and TiO₂ nanoparticles, but few discussing how the effects of physiochemical properties, such as size of these two materials, could influence their toxic effect. Therefore, the size influence on the cytotoxicity of nano-sized PLGA and TiO₂ particles will be the focus of this study. Reports have shown that generation of ROS, mitochondrial depolarization, plasma membrane leakage, increased intracellular calcium concentration and inflammation response could explain and predict the cytotoxicity of different nanomaterials. All methods are summarized in **Table 2.2**. Thus, in this *in vitro* test, the cytotoxicity of nanoparticles should be thoroughly evaluated through various assays to comprehensively understand how the size of PLGA and TiO₂ nanoparticles could influence their cytotoxicity.

Chapter 3 Materials and Methods

3.1 Investigation of the factors affecting the mean diameter of PLGA particles

The objective of this investigation is to understand how the size of PLGA nanoparticles can be affected by the synthesis factors and to prepare PLGA nanoparticles of different sizes for cytotoxicity studies. In this study, the effect of three processing factors was investigated to understand their influence on size of PLGA nanoparticles. The factors included concentration of PLGA polymer in the organic phase, concentration of PVA in the continuous water phase and components of organic solvent.

3.1.1 PLGA concentration

6 ml solvent mixture was used to dissolve 50 mg, 100 mg, 200 mg and 400 mg of PLGA powder first. The organic solvent mixture was composed of dichloromethane (DCM) and acetone (2/1, v/v). 1.5 ml of fully dissolved PLGA solution was slowly added into 4.5 ml 1% PVA solution (dissolved in water, w/v). Ultrasonication at the oil/water interface for 3 min was conducted to emulsify the organic phase into continuous water phase by utilizing a microtip probe ultrasonicator (Sonics & materials Inc., USA) with ice water bath. The final colloid was collected after magnetically stirring for 24 h. The size and surface charge of nano-sized particles was tested by utilizing DLS technique.

3.1.2 PVA concentration

1.5 ml solvent mixture was used to dissolve 50 mg of PLGA powder first. The organic solvent mixture was composed of DCM and acetone (2/1, v/v). Fully dissolved PLGA polymer solution was added into 4.5 ml of PVA solution of different concentrations slowly. The concentrations of PVA include 0.25%, 0.5%, 1% and 2% (dissolved in water, w/v). Ultrasonication at the oil/water interface for 3 min was conducted to emulsify the organic phase into continuous water phase by utilizing a microtip probe ultrasonicator (Sonics & materials Inc., USA) with ice water bath. The final colloid was collected after magnetically stirring for 24 h. The size and surface charge of nano-sized particles was tested by utilizing DLS technique.

3.1.3 PVA concentration and PLGA concentration

1.5 ml solvent mixture was used to dissolve PLGA powder first. The organic solvent mixture was composed of DCM and acetone (2/1, v/v). Fully dissolved PLGA polymer solution was added into 4.5 ml of PVA water solution (w/v) slowly with the same PLGA and PVA ratio. The concentration of PLGA and PVA is listed in **Table 3.1**. Ultrasonication at the oil/water interface for 3 min was conducted to emulsify the organic phase into continuous water phase by utilizing a microtip probe ultrasonicator (Sonics & materials Inc., USA) with ice water bath. The final colloid was collected after magnetically stirring for 24 h. The size and surface charge of nano-sized particles was tested by utilizing DLS technique.

Table 3.1 PLGA and PVA concentrations

	Set 1	Set 2	Set 3	Set 4
PLGA (mg/1.5 ml)	50	100	200	400
PVA (%w/v)	0.25	0.5	1	2

3.1.4 Organic solvent

1.5 ml solvent mixture was used to dissolve 50 mg of PLGA powder first. The organic solvent mixture was composed of (a) DCM and acetone (1/1, v/v), (b) DCM and acetone (2/1, v/v), (c) DCM and acetone (4/1, v/v) and (d) pure DCM (e) pure chloroform. Fully dissolved PLGA polymer solution was added into 4.5 ml of 1% PVA water solution (w/v) slowly. Ultrasonication at the oil/water interface for 3 min was conducted to emulsify the organic phase into continuous water phase by utilizing a microtip probe ultrasonicator (Sonics & materials Inc., USA) with ice water bath. The final colloid was collected after magnetically stirring for 24 h. The size and surface charge of nano-sized particles was tested by utilizing DLS technique.

3.2 Synthesis of nanomaterials

3.2.1 Synthesis of PLGA nanoparticles of different mean diameters

Nano-sized PLGA particles of different mean diameters were synthesized utilizing different organic solvent or solvent mixture through both nanoprecipitation method and o/w single emulsion solvent evaporation method. Modified nanoprecipitation method [46] was utilized to synthesize the smallest PLGA nanoparticles (60 nm, P60). In brief, 6 ml organic solvent mixture was utilized to dissolve 25 mg PLGA powder. The organic solvent mixture was composed of acetone and dichloromethane (DCM) (39:1, v/v). Fully dissolved PLGA polymer solution was dropped into 18 ml of 0.125% PVA water solution (w/v) slowly under magnetic stirring (990 rpm). The final colloid was collected after 24 h magnetically stirring and then freeze dried for 48 h for future studies.

Modified o/w single emulsion solvent evaporation process [29] was utilized to synthesize 100 nm and 200 nm PLGA nanoparticles (P100 and P200). In brief, 1.5 ml organic solvent or solvent mixture was used to dissolve 50 mg PLGA powder first. For P100, the organic solvent mixture mixed of DCM and acetone (2/1, v/v). For P200, the organic solvent was composed of chloroform only. Fully dissolved PLGA polymer solution was added into 4.5 ml of 1% PVA water solution (w/v) slowly. Ultrasonication at the oil/water interface for 3 min was conducted to emulsify the organic phase into continuous water phase by utilizing a microtip probe ultrasonicator (Sonics & materials Inc., USA) with ice water bath. The final colloid was collected after magnetically stirring for 24 h, after which the nanoparticles were freeze dried for 48 h for future studies.

3.2.2 Surface coating of TiO₂ nanoparticles

Poly (ethylene-*alt*-maleic anhydride) (PEMA) (Sigma Aldrich, Singapore) and chitosan (Sinopharm Chemical Reagent Co. Ltd, China) were utilized to coat TiO₂ nanoparticles. Both PEMA-coated T20 nanoparticles (T20-PEMA) and chitosan-coated T20 nanoparticles (T20-chitosan) nanoparticles were synthesized in house. The surface coating of T20 with PEMA is a new protocol developed in our research group and the surface coating of T20 with chitosan is based on Shi *et.al.*'s report [98]. In brief, 200 mg T20 nanoparticles were dispersed in 20 ml of methanol. Ultrasonication for 30 min was conducted to help re-dispersion of nanoparticles. The dispersed T20 nanoparticles suspension was added into 18 ml of 0.1% PEMA or chitosan solution in water. After magnetically stirring for 24 h to allow the coating of PEMA and chitosan onto T20 nanoparticles, the samples were freeze dried for 48 h.

3.2.3 Synthesis TiO₂ nanoparticles of same size but different surface area

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 [99]. 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 was obtained by soaking the precipitates in HCl solution (0.1 M) for several times, followed by washing in

deionised water and absolute ethanol before drying in air. Finally, the as-synthesized samples were annealed at 400 °C or 700 °C for 1 h at a heating rate of 5.0 °C/min.

3.3 Particles characterization

3.3.1 Field emission scanning electron microscopy (FESEM)

Field emission scanning electron microscope (FESEM, Jeol JSM 6340F) was utilized to take images of PLGA nanoparticles. The nano-sized PLGA particles were firstly dispersed in Milli-Q water and dropped onto a silica chip. After air dried, the PLGA nanoparticles on silica chip were coated with platinum for 70 seconds under vacuum at 20 mA. The acceleration voltage was 2 kV when the images were taken. The primary sizes of PLGA nanoparticles were measured through SEM images of PLGA nanoparticle.

3.3.2 Transmission electron microscopy (TEM)

Nano-sized TiO₂ particles 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. The primary sizes of TiO₂ particles were characterized by TEM (JOEL 2010, Japan). The TiO₂ particles were firstly redispersed in methanol. Ultrasonication for 30 min was conducted to help re-dispersion of nanoparticles. The re-dispersed TiO₂ nanoparticles were dropped onto a carbon coated copper grids. TEM

images were observed and taken under at 200 kV of accelerating voltage. The primary sizes of TiO₂ nanoparticles were measured through TEM images.

3.3.3 Hydrodynamic size and surface charge of particles

Dynamic light scattering (DLS) technique was utilized to characterize the hydrodynamic size and surface charge of nanoparticles. Particles were dispersed in water or cell culture medium into stock suspension at 3 mg/ml first. Ultrasonication for 10 min was conducted to help re-dispersion of nanoparticles. The working solution (30 µg/ml) was ultrasonicated for another 10 min right before DLS tests using a ZetaPALS zeta potential analyzer (Brookhaven instruments, USA).

3.3.4 Brunauer-Emmett-Teller (BET) surface area and theoretical surface area

Micromeritics surface area analyzer (ASAP 2000, USA) was used to characterize the Brunauer-Emmett-Teller (BET) specific surface area of TiO₂ nanoparticles. TiO₂ nanoparticles in powder form were degassed at 200 °C in flowing nitrogen for 4 h before nitrogen adsorption measurement.

The theoretical surface area of PLGA and TiO₂ nanoparticles was calculated through **Equation (1)** [27].

$$S=6/\rho d$$

Equation (1)

S represented the theoretical surface area. ρ was the true density of materials, which is 3.90 g/cm³ for TiO₂ nanoparticles [100] and 1.357 g/cm³ for PLGA nanoparticles [101] according to suppliers' information. d was the mean diameter of nanoparticles.

3.3.5 Endotoxin

Endotoxin attached on nanomaterials may trigger inflammation response of cells directly. To exclude this possibility, PYROGENT[®] Plus single-test with CSE 0.06 EU/ml sensitivity (Lonza) was used to test the endotoxin level at particles concentration 300 µg/ml. The test was conducted according to the protocol provided by supplier.

3.4 *In vitro* cytotoxicity studies

3.4.1 Cell culture

Immortalized mouse macrophage cell line RAW264.7 cells (ATCC, # TIB-71) and human bronchial epithelial BEAS-2B (ATCC, # CRL-9609) cells were chosen to investigate in this study. RAW264.7 cells represent the phagocytic cell lines, which are able to actively interact with nanoparticles in *in vivo* systems [10, 30, 32]. BEAS-2B cells represent airway epithelium which is defensive cells against inhaled nanoparticles [32]. RAW264.7 cells were grown in complete cell culture medium (CDMEM), which 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). BEAS-2B cells were grown in bronchial epithelial growth medium (BEGM) (Lonza). Cells were incubated in a humidified atmosphere at 37 °C, which contained 5% CO₂. Cells were sub-cultured every two days when they reached about 70-80% confluence.

3.4.2 Dispersion of PLGA and TiO₂ nanoparticles

The nanoparticles in powder form were re-dispersed with complete cell culture medium into stock suspension (3 mg/ml), which was ultrasonicated in water bath ultrasonicator for 10 min to help re-dispersion. The stock suspension was further diluted with respective complete cell culture medium into working suspension at different concentrations. Ultrasonication in water bath ultrasonicator was conducted for another 10 min before adding into cells.

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. These 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 particle surface. The suspension was ultrasonicated for 20 minutes and then centrifuged at 14000 rpm and 25 °C for 20 minutes.

The supernatant containing unbound proteins was discarded. 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.

3.4.3 Metabolic activity test

BEAS-2B and RAW264.7 cells were plated in (20,000 cells/cm²). Cells were allowed to attach and grow for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. The cells were incubated with working suspension of dispersed nanoparticles for another 24 h. After interaction with nanoparticles, the old medium was changed with MTS or WST-8 working solution (10 % in cell culture medium). The cells interacted with MTS or WST-8 assay for about 1 h at standard cell culture condition. To spin down nanoparticles in solution which might interfere with the absorbance of assay, the whole 96-well plate was centrifuged for 10 min at a speed of 250 g. The supernatant was transferred to another 96-well plate. The absorbance of supernatant at 490 nm was quantified using SpectraMax M5e (Molecular Devices Corporation, USA). The absorbance at 690 nm was also tested as a reference.

3.4.4 Photoactivation treatment of nanoparticles with cells

RAW264.7 cells were plated in 384-well plate (20,000 cells/cm²). Cells were allowed to attach and grow for 24 h at 37 °C in a humidified atmosphere with 5% CO₂. The cells were incubated with 100 µg/ml of dispersed TiO₂ nanoparticles in CDMEM for 21 h.

Then, 40 μ l of phosphate buffered saline (PBS) was used to wash the cells by using a plate washer thrice to remove excess nanoparticles that did not enter or attach onto cells. 25 μ l of PBS was remained in each well after wash. After wash, cells were exposed to 280 nm – 450 nm ultraviolet-visible (UV-Vis) light for 5 min in PBS. Finally, another 25 μ l of cell culture medium was mixed into each well. Cells were then cultured in standard cell culture conditions for 3 h and multiparametric assay was conducted to assay the phototoxicity of TiO₂ nanoparticles.

As for the photoactivation treatment used in surface area studies, the light source and exposure time was changed because studies were conducted in different labs. In brief, cells were plated in 24-well plates (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 μ g/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 for 20 min. After which, fresh medium was added to the wells. Cells were placed in incubator for another 3 h. Cellular metabolic activity test were conducted with same procedure described previously.

3.4.5 Co-staining cells with Hoechst and propidium iodide (PI)

RAW264.7 cells were seeded in 35 mm petri-dish (20,000 cells/cm²) and incubate in 37 °C humidified atmosphere with 5% CO₂ for 24 hours prior to TiO₂ particles treatment. For treatment of TiO₂ particles only (without photoactivation), cells were interacted with TiO₂ particles (100 μ g/ml) for 24 h before imaging. To treat TiO₂ particles with

photoactivation, cells were incubated with 100 µg/ml TiO₂ particles of three different conditions for 21 h before changing old medium into PBS. The cells in PBS were then exposed to UVA light at 365nm for 20 minutes. Fresh medium was added into dishes. The cells were incubated for another 3 h before testing. PBS with 1 µM Hoechst (blue) and 5 µM PI (red) was interacted with cells for 5 min. Cells were viewed under 10x objective lens with 80ieclipse upright microscope (Nikon) with magnification of 100 times, and images were taken with NIS-Elements software.

3.4.6 Multiparametric assay

Cytotoxic parameters including leakage of plasma membrane, generation of mitochondrial superoxide, mitochondrial depolarization and increased concentration of intracellular calcium ion were tested utilizing high throughput screening method established by George *et. al.* [12]. The dyes utilized in this thesis included Hoechst (Invitrogen, H3570), PI nucleic acid stain (Invitrogen, P3566), MitoSOX (Invitrogen, M36008), JC-1 (Invitrogen, T3168) and Fluo-4 (Invitrogen, F14202). The usage of the dyes was listed in **Table 3.2** below. Both Hoechst and PI are two DNA interactive agents. The difference is Hoechst is cell-permeable which can bind to DNA of both live and dead cells, allowing us to quantify and locate the total number of cells in each image. However, PI can only penetrate into cells with damaged plasma membrane.

Table 3.2 Fluorescent probes used in this study

Probe	Ex/Em wavelength (nm) (colour)	Utility
Hoechst	355/465 (Blue)	Localization and total number of cells
PI	540/620 (Red)	Damaged integrity of plasma membrane
MitoSOX	510/580 (Red)	Generation of mitochondrial superoxide
JC-1	480/530-590 (Green/Red)	Mitochondrial depolarization
Fluo-4	480/510 (Green)	Increase of intracellular Ca ²⁺ concentration

Wavelength-compatible fluorescent probes were mixed at working concentrations into PBS. Three sets of probe cocktails were prepared. The first cocktail was 1 μ M Hoechst with 5 μ M MitoSOX. The second cocktail was composed of 1 μ M Hoechst and 1 μ M JC-1. The third cocktail included 1 μ M Hoechst, 5 μ M PI and 5 μ M Fluo-4. Cells were washed three times by utilizing PBS to remove serum in medium, because serum in medium may interfere with dyes. 25 μ l dye cocktail per well was added in and then allowed interaction with cells for 30 min in a standard 37 °C incubator in the absence of light. Image-Xpress^{micro} (Molecular Devices, Sunnyvale, USA), an automated epifluorescence microscope, was utilized to take fluorescent images at different wavelength under 10 $\times$ magnifications. The number of cells showing positive signals (No_{positive}) as well as the total cell number (No_{total}) was automatically quantified by using Meta-Xpress software, and the percentage of cells which showed positive signals (percentage of positive cells = $100 \times \text{No}_{\text{positive}} / \text{No}_{\text{total}}$) was calculated by the same software automatically.

3.4.7 Inflammation response study

RAW264.7 cells were plated in a 24-well plate (20,000 cells/cm²). Cells were grown at 37 °C for 24 h in a humidified incubator with CO₂ (5%). Working suspension of dispersed nanoparticles was added into cells. After incubation of cells with nanoparticles for 24 h, the suspension was collected and the TNF- α released into the medium was quantified by Mouse TNF- α ELISA kit (Biolegend) following the protocol provided by supplier.

3. 5 Abiotic particle-protein interaction study

Nanoparticles (1 mg/ml) were dispersed in bovine serum albumin (BSA, 2 mg/ml) in DMEM solution and ultrasonicated for 20 min. All the particles were centrifuged at 14,000 rpm and 25 °C for 20 min to be collected. Milli-Q water was used to wash free BSA for three times. The protein attached on PLGA nanoparticles was quantified using Pierce 660 nm Protein Assay following the manufacture protocol. The adsorption of protein on TiO₂ nanoparticles was tested using Thermo Gravimetric Analyzer (TGA, 2950, HR, V5.4A). The collected TiO₂ nanoparticles were freeze-dried for 48 h before the TGA test. The curve of weight loss for BSA and BSA adsorbed TiO₂ nanoparticles was obtained at a heating rate of 20 °C/min in an atmosphere filled with nitrogen.

3.6 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. 100 μ M HPF working solution (5 μ l/well) was added into 384-well plate with nanoparticles suspension (100 μ g/ml, 45 μ l/well) in distilled water (DI water). The fluorescent intensity (F_0) was tested immediately at 490 nm / 515 nm for Excitation / Emission wavelength by utilizing SpectraMax M5e Multi-Mode Microplate Reader (Molecular Devices Corp., USA). After UV-Vis exposure (280 nm-450 nm) for 5 min, the fluorescent intensity (F_1) in each well was tested again. The rate increase of fluorescent intensity per minute was calculated through **Equation (2)**.

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

Equation (2)

3.7 Statistics

All quantitative data are shown as the means $\pm$ standard deviation (SD). Analysis of statistics was conducted by utilizing Tukey's pair-wise comparisons in a one-way analysis of variance (ANOVA). The significant difference was considered when $p < 0.05$. * represented that the data was significantly different ($p < 0.05$) as compared to the control group. # represented that the data was significantly different ($p < 0.05$) as compared to other particles at same concentration.

Chapter 4 Results and Discussion

4.1 Factors affecting the mean diameter of PLGA nanoparticles

The mean diameter of PLGA nanoparticles can be changed through some synthesis parameters. Song *et al.* [29] reported that size of PLGA nanoparticles was significantly influenced by PLGA concentration, property (the degrees of hydrolyzation and polymerization) of PVA and organic solvent composition. It could also be affected by the PLGA molecular weight (MW), PVA concentration and ultrasonication time for solvent emulsion evaporation method. The aim of this study is to synthesize nano-sized PLGA particles of different sizes for cytotoxicity studies, so we have kept some key parameters such as molecular weight of PLGA to be the same. As such, the component of final particles is the same and the only difference between particles is the size. In this study, we only focus on the influence of three parameters: PLGA concentration, PVA concentration and organic solvent component.

4.1.1 PLGA concentration influences the size of PLGA nanoparticles

PLGA nanoparticles were synthesized by changing the PLGA concentrations in organic phase only. The hydrodynamic size and surface charge of particles were tested using DLS techniques. **Figure 4.1** showed increase of PLGA concentration in organic phase caused the increase in hydrodynamic size of PLGA nanoparticles. The reason could be that the increase of PLGA polymer concentration increased the viscosity of organic phase, which

diminished the net shear stress and formed bigger particles [29]. Another possibility is that the decrease of PLGA polymer concentration increased the elimination of organic solvent, which allowed more coalescence of droplets before solidification [29]. The zeta potential of PLGA particles did not significantly altered according to the change of PLGA concentrations, because the surface charge of these particles could be resulted from the charge of PVA on surface of nanoparticles.

Figure 4.1 Change in PLGA concentration with particle size and surface charge.

Triangle represents particle size. Square represents zeta potential.

4.1.2 PVA concentration influence the size of PLGA nanoparticles

PLGA nanoparticles were synthesized by changing the surfactant PVA concentrations in water phase only. **Figure 4.2** showed the size of PLGA particles diminished with the incensement of PVA concentration in aqueous phase. At low concentration, the PVA may not enough to form small particles, because smaller particles have higher surface area which needs more PVA to cover their surface in order to decrease the interfacial tension

[102] during emulsification process. However, we also observed that the increase of PVA concentration after 1% did not obviously reduce the size of nanoparticles. This could be because with the increase in PVA concentration, the viscosity in aqueous phase also increased, which resulted in decreased net shear stress [42]. So based on our results, below concentration of 1% (w/v) for PVA solution, the effect of surfactant PVA in reduction of interfacial tension was the predominant effect. However, when PVA concentration higher than 1%, the viscosity effect in aqueous phase became predominant, which terminated the decrease of particle size with increased PVA concentration. The zeta potential did not significantly vary with the different sized PLGA nanoparticles.

Figure 4.2 Change in PVA concentration with particle size and surface charge.

Triangle represents particle size. Square represents zeta potential.

4.1.3 PLGA concentration and PVA concentration co-influence the size of PLGA nanoparticles

PLGA nanoparticles were synthesized by changing PLGA concentration and the surfactant PVA concentrations simultaneously, while keeping the ratio of PLGA to PVA the same. **Figure 4.3** showed the mean diameter of PLGA nanoparticles decreased with both increase of PLGA concentration and PVA concentration. The trend is similar to the trend in **Figure 4.2**, which means the PVA concentration influence more on size of PLGA nanoparticles than PLGA concentration. Due to the same surface coating of PVA, the zeta potential of different sized PLGA nanoparticles did not show statistic difference.

Figure 4.3 Change in PLGA concentration and PVA concentration simultaneously with particle size and surface charge. Triangle represents particle size. Square represents zeta potential.

4.1.4 Component of organic solvent influences the size of PLGA nanoparticles

PLGA nanoparticles were synthesized by changing components of organic solvent. **Figure 4.4** showed the mean diameter of PLGA nanoparticles dramatically increase with the increased ratio of DCM in organic phase. Acetone is a water miscible solvent. The fast diffusion of acetone from organic phase to aqueous phase could significantly decrease the interfacial tension between water and oil phase, and thus decreased the particle size as compared with DCM alone [29, 103]. So the increase of water miscible solvent can more effectively decrease the particles size. Due to the same surface coating of PVA, the surface charge of different sized PLGA nanoparticles did not show statistic difference.

Figure 4.4 Change in organic solvent with particle size and surface charge. Triangle represents particle size. Square represents zeta potential.

In summary, the influence of these three factors is in the following sequences: component of organic solvent > PVA concentration > PLGA concentration. Thus, we proposed to use different organic solvent to synthesize PLGA nanoparticles of different sizes while

keeping all the other parameters the same. Based on **Figure 4.4**, using solvent composed of DCM/acetone (2/1, v/v), we could obtain particles with DLS size around 150 nm. We further hypothesized that if we use pure acetone, which is water miscible, the particles size will largely decrease. While if we use pure chloroform, which is more viscous than DCM, the particle size will dramatically increase.

4.1.5 Synthesis of PLGA nanoparticles of different particle sizes

PLGA nanoparticles of three different sizes were successfully synthesized by using different organic solvent. The characterization data for the particles tested are listed in **Table 4.1**. Their SEM images and schematic structure are shown **Figure 4.5**, which reflects the primary sizes and morphology of PLGA nanoparticles. The PLGA nanoparticles of three different sizes were spherical and measured with a mean diameter of 61 nm for P60 (A & D), 94 nm for P100 (B & E) and 205 nm for P200 (C & F), using the SEM images. DLS technique was used to measure the hydrodynamic size and zeta potential of the nanoparticles in both water and complete cell culture medium CDMEM and BEGM (**Table 4.1**). The hydrodynamic size was bigger than their primary size. Different particles showed similar surface charge in water within the range from -17 mV to -23 mV. However, the zeta potentials of nanoparticles in CDMEM and BEGM were less negative and showed similar charge within the range of -2 mV to -10 mV. This could be due to the adsorption of the proteins and ions in the cell culture medium onto the particle surface, resulting in the neutralization of surface charge [3]. The smaller particles are relatively easier to agglomerate as compared with larger particles. The obvious size increase of smaller particles (especially for P60) in water and cell culture medium is mainly due to the agglomeration of the nanoparticles. As for the cases in cell culture

medium, not only the protein corona increased the size of particles, but also the high salt concentration in medium amplified the agglomeration of particles. The BET method was not applicable for PLGA nanoparticles due to the low melting point of PLGA polymer. Theoretical surface areas were calculated through the size and true density of the materials (**Equation (1)**), which indicated that smaller particles had larger surface areas. The endotoxin test was conducted to exclude the possibility that the inflammation response of the cells was due to the endotoxin attached on the materials. From **Table 4.1**, the endotoxin in the materials was lower than 0.06 EU/ml at the highest particle concentration tested (300 µg/ml). This result suggested that the inflammation response of the cells could be more likely due to the particles themselves instead of the endotoxin in the samples.

Table 4.1 By using different organic solvent, PLGA nanoparticles with three different sizes were synthesized and characterized.

		P60	P100	P200
Primary Particle Size (nm)		61	94	205
water	Size (nm)	101	149	207
	PDI	0.046	0.097	0.071
	Zeta Potential (mV)	-17.0	-21.4	-22.9
CDMEM	Size (nm)	192	120	196
	PDI	0.379	0.246	0.212
	Zeta Potential (mV)	-2.7	-6.7	-5.0
BEGM	Size (nm)	536	158	233
	PDI	0.343	0.131	0.110
	Zeta Potential (mV)	-6.4	-9.6	-3.6
Theoretical Surface Area (m²/g)		72	46	22
BET Surface Area (m²/g)		/	/	/
Endotoxin (EU/ml)		<0.06	<0.06	<0.06

Figure 4.5 (A to C) Scanning electron microscopic pictures of PLGA nanoparticles: (A) PLGA 60 nm, (B) PLGA 100 nm, (C) PLGA 200 nm. (D to F) Schematic structure of PLGA nanoparticles. Spherical core represented hydrophobic PLGA presented inside of the particles. Curled line represented the hydrophilic surfactant exhibited on the surface of PLGA nanoparticles.

In summary, PLGA concentration, PVA concentration and organic solvent component could influence the size of PLGA nanoparticles in synthesis, but the influence of organic solvent component was most obvious. By using different organic solvent, we successfully synthesized PLGA nanoparticles with three different sizes (60 nm, 100 nm and 200 nm).

4.2 Size influences the cytotoxicity of PLGA nanoparticles

4.2.1 Limited size effect of PLGA nanoparticles on cell viability

To test the cytotoxicity of PLGA nanoparticles, the particles were incubated with RAW264.7 and BEAS-2B cells for 24 h. The concentrations are from 10 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$. For *in vitro* cell viability test, MTS assay was utilized to quantify the metabolically active cells through the interaction between MTS and dehydrogenase enzymes in live cells. **Figure 4.6** exhibited the metabolic activity of cells after interaction with PLGA nanoparticles. The graph indirectly indicates the viability of live cells in each group as compared to the negative control (0 $\mu\text{g/ml}$). **Figure 4.6** showed no apparent cytotoxicity for PLGA nanoparticles regardless of the size. This result is consistent with other reports that PLGA nanoparticles do not show obvious acute lethal toxicity to cells within 24 h of incubation [104-105].

Figure 4.6 The metabolic activity of live cells after cells were incubated with PLGA nanoparticles for 24 h. The metabolic activity of cells was quantified by MTS assay and normalized to negative control (0 $\mu\text{g/ml}$). (A) Viability of RAW264.7 cells after incubation with PLGA nanoparticles of three different sizes (B) Viability of

BEAS-2B cells after incubation with PLGA nanoparticles of three different sizes. The data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.2.2 Cells displayed different responses to different sized PLGA nanoparticles in the multiparametric assay

The multiparametric assay was conducted after RAW264.7 and BEAS-2B cells were exposed to different sized PLGA nanoparticles for 24 h. The potential cytotoxic effect of PLGA nanoparticles were shown in **Figure 4.7**. The results indicated the percentage of positive cells for each parameter whose fluorescence intensity was above a certain threshold using MetaXpress software. Limited damaging response was observed in both of RAW264.7 and BEAS-2B cells after incubation with PLGA nanoparticles (positive cells < 10%). However, the smallest PLGA nanoparticles (P60) triggered similar increase in the intracellular calcium flux in both RAW264.7 and BEAS-2B cells. Calcium is an important regulator of mitochondrial function. The disturbance of cytosolic calcium homeostasis may trigger profound influence on cell functions [51]. High concentration of Ca^{2+} , especially in pathology, has negative effect on the function of mitochondria through ROS generation [51]. But based on our current results, the slight increase in the calcium concentration in P60 treated cells did not cause obvious cell death or damage of organelles.

Figure 4.7 Multiparametric assays to detect the *in vitro* cytotoxicity of different sized PLGA nanoparticles after 24 h incubation with (A to D) RAW264.7 and (E to H) BEAS-2B cells. The concentration ranges from 10 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$. The cells were stained for 30 min with the dye cocktails to assay for (A and E) MitoSOX staining, (B and F) JC-1 staining, (C and G) PI uptake and (D and H) Fluo-4 staining. The

percentage of cells showed positive fluorescence above a certain threshold was recorded by using MetaXpress software. The data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.2.3 PLGA nanoparticles triggered size and concentration dependent inflammation response.

Nanoparticles have different ways of entering human body and activating the immune system. PLGA nanoparticles, proposed to be used in biomedical field, were designed to deliver drugs through intravenous administration. So the inflammatory response of cells after interaction with PLGA nanoparticles is a key parameter to evaluate the biocompatibility of PLGA nanoparticles. In this study, we tested the TNF- α released from RAW264.7 cells after 24 h incubation with PLGA nanoparticles (**Figure 4.8**). The graph showed an obvious trend that smaller particles tend to trigger more release of cytokine TNF- α from RAW264.7 cells. P60 and P100 nanoparticles started to show statistical difference when compared to control from 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, respectively. The P200 nanoparticles did not trigger significant release of TNF- α up to 300 $\mu\text{g/ml}$. This result indicated that PLGA nanoparticles with size larger than 100 nm would be safer for biomedical applications.

Figure 4.8 The TNF- α released from RAW264.7 cells after the cells were stimulated by PLGA nanoparticles for 24 h. The TNF- α released from macrophages into medium was measured by ELISA and normalized to negative control (0 $\mu\text{g/ml}$). The data represents means $\pm$ SD, $n=4$. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.2.4 The protein adsorption on PLGA nanoparticles was size-dependent

The cytotoxicity studies showed that the cytotoxicity of PLGA nanoparticles was size-dependent. Unlike other nanomaterials such as ZnO, PLGA nanoparticles do not release toxic ions like Zn^{2+} . Thus, the potential mechanism behind this cytotoxicity could be due to the size-dependent interaction between nanoparticles and intracellular biomolecules adsorbed onto nanoparticles. Smaller nanoparticles have larger surface area

and higher percentage of molecules exposed on particle surface to interact with surrounding biomolecules. To investigate this hypothesis, we studied the adsorption of BSA (a model biomolecule) to different sized PLGA nanoparticles. Pierce 660 test were chosen to test the protein adsorption onto PLGA nanoparticles. **Figure 4.9** shows the protein quantification of BSA attached on PLGA nanoparticles through Pierce 660 assay. The theoretical surface area of PLGA nanoparticles (per 100 mg) and the amount of BSA adsorbed onto 100 mg PLGA nanoparticles for P60, P100 and P200 were listed in the table of **Figure 4.9**. The protein adsorption correlated well with the surface area of the PLGA nanoparticles ($R^2 > 0.9$).

Nanoparticles	P60	P100	P200
BSA(mg) adsorped on 100 mg particles	0.662	0.441	0.106
Surface area per 100 mg (m ²)	7.2	4.6	2.2

Figure 4.9 Protein quantification of BSA attached on PLGA nanoparticles through Pierce 660 assay. The data represents mean $\pm$ SD, n=4.

The data therefore suggests that the size dependent cytotoxicity of PLGA nanoparticles could be related to different surface area of different-sized nanoparticles. Smaller particles have bigger surface area and more molecules exposed on particle surface to interact with intracellular biomolecules nearby.

4.3 Size influences the cytotoxicity of TiO₂ nanoparticles

4.3.1 Physical characterization of TiO₂ nanoparticles

The characterization data for TiO₂ nanoparticles tested are listed in **Table 4.2**. Their TEM images and schematic structures are shown **Figure 4.10**, which reflects the primary size and morphology of TiO₂ nanoparticles. The TiO₂ nanoparticles of three different sizes were near-spherical particles with primary size 10 nm for T10 (A), 20 nm for T20 (B) and 100 nm (C) for T100 as shown in **Figure 4.10**. DLS technique was used to measure the hydrodynamic size and zeta potential of the nanoparticles in both water and complete cell culture medium CDMEM and BEGM (**Table 4.2**). The hydrodynamic size was bigger than their primary size. Although different particles showed different surface charge in water, the zeta potentials in CDMEM and BEGM were similar within the range of -5 mV to -13 mV. This could be due to the adsorption of the proteins and ions in the cell culture medium onto the particle surface, resulting in the neutralization of surface charge [3]. The BET method was utilized to quantify the specific surface area of TiO₂ nanoparticles. From the results shown in **Table 4.2**, the smallest TiO₂ nanoparticles (T10) had the highest BET surface area (166.0 m²/g). The experimental BET surface area was close to the theoretical surface areas calculated through the size and true density of the materials (**Equation (1)**), which confirmed the size gotten from TEM images. These results all indicated that smaller particles had larger surface areas. The endotoxin test was conducted to exclude the possibility that the inflammation response of the cells was due to the endotoxin attached on the materials. From **Table 4.2**, the endotoxin in the materials was lower than 0.06 EU/ml at the highest particle concentration of 300 µg/ml. This result

suggested that the inflammation response of the cells could be more likely due to the particles themselves instead of the endotoxin in the samples.

Table 4.2 TiO₂ nanoparticles with three different sizes were characterized

		T10	T20	T100
Primary Particle Size (nm)		10	20	100
water	Size (nm)	669	307	349
	PDI	0.186	0.263	0.224
	Zeta Potential (mV)	-16.9	32.3	19.1
CDMEM	Size (nm)	262	338	444
	PDI	0.005	0.180	0.248
	Zeta Potential (mV)	-5.7	-7.7	-9.3
BEGM	Size (nm)	1457	707	1336
	PDI	0.298	0.289	0.255
	Zeta Potential (mV)	-11.0	-12.1	-9.9
Theoretical Surface Area (m²/g)		154	73	15
BET Surface Area (m²/g)		166.0	50.4	17.2
Endotoxin (EU/ml)		<0.06	<0.06	<0.06

Figure 4.10 (A to C) TEM images of TiO₂ nanoparticles of 3 different sizes (A) T10, TiO₂ nanoparticles of size 10 nm, scale bar 10 nm; (B) T20, TiO₂ nanoparticles of size 20 nm, scale bar 20 nm; (C) T100, TiO₂ nanoparticles of size 100 nm, scale bar 100 nm. (D to F) Schematic structure of TiO₂ nanoparticles. Sphere represented TiO₂ nanoparticles of different sizes.

4.3.2 Size effect of TiO₂ nanoparticles on cell viability

To test the cytotoxicity of TiO₂ nanoparticles, the particles of concentrations ranging from 10 µg/ml to 300 µg/ml were incubated with RAW264.7 and BEAS-2B cells for 24 h. For *in vitro* cell viability test, MTS assay was utilized to quantify the metabolically active cells through the interaction between MTS and dehydrogenase enzymes in live cells. **Figure 4.11** exhibits the metabolic activity of cells after interaction with TiO₂ nanoparticles. The graph indirectly indicates the viability of live cells in each group as compared to the negative control (0 µg/ml). **Figure 4.11** shows that cell viability was generally uncompromised (> 80%) after 24 h incubation with TiO₂ nanoparticles.

However, viability of BEAS-2B cells was statistically lower after they were exposed to 300 mg/ml of T10 and T20 nanoparticles as compared to the negative control (Figure 4.11 B).

Figure 4.11 The metabolic activity of live cells after cells were incubated with TiO₂ nanoparticles for 24 h. The metabolic activity of cells was quantified by MTS assay and normalized to negative control (0 µg/ml). (A) Viability of RAW264.7 cells after incubation with TiO₂ nanoparticles of three different sizes (B) Viability of BEAS-2B cells after incubation with TiO₂ nanoparticles of three different sizes. The data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p < 0.05$) as compared to the control group. # represented that the data was significantly different ($p < 0.05$) as compared to other particles at same concentration.

4.3.3 Cells displayed different responses to different sized TiO₂ nanoparticles in the multiparametric assay

The multiparametric assay was conducted after RAW264.7 and BEAS-2B cells were exposed to different sized TiO₂ nanoparticles for 24 h. The results shown in **Figure 4.12** indicated the percentage of positive cells for each parameter whose fluorescence intensity was above a certain threshold using MetaXpress software. The TiO₂ nanoparticles exhibited obvious size and concentration dependent cytotoxicity to both RAW264.7 and BEAS-2B cells (**Figure 4.12**). The PI uptake results confirmed the results in MTS tests that only T10 and T20 triggered ~10% cell death at the highest concentration of 300 µg/ml. Size-dependent generation of mitochondrial superoxide was observed in both RAW264.7 and BEAS-2B cells. ROS generation after the interaction with TiO₂ nanoparticles in the *in vitro* studies was also observed in BEAS-2B cells by other groups [18, 24]. Only T10 triggered statistically increased signal in intracellular calcium flux parameter in both RAW264.7 and BEAS-2B cells at the concentration higher than 100 µg/ml. In summary, smaller particles tend to trigger higher level of damage to both RAW264.7 and BEAS-2B cells in this *in vitro* investigation.

Figure 4.12 Multiparametric assays to detect the *in vitro* cytotoxicity of different sized TiO_2 nanoparticles after 24 h incubation with (A to D) RAW264.7 and (E to H) BEAS-2B cells. The concentration ranges from 10 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$. The cells were stained for 30 min with the dye cocktails to assay for (A and E) MitoSOX staining, (B and F) JC-1 staining, (C and G) PI uptake and (D and H) Fluo-4 staining. The

percentage of cells showed positive fluorescence above a certain threshold was recorded by using MetaXpress software. The data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.3.4 TiO₂ nanoparticles triggered size and concentration dependent inflammation response

Nanoparticles have different ways of entering the human body and activating the immune system. Although TiO₂ nanoparticles are more commonly used in sunscreens, they still have great potential to enter human body through lung and gastrointestinal tract [17]. The stimulation of immune system is possible after the particles entering human body. In this study, we tested the TNF- α released from RAW264.7 cells after 24 h incubation with TiO₂ nanoparticles (**Figure 4.13**). The graph shows an obvious trend that smaller particles tend to trigger more release of cytokine TNF- α from RAW264.7 cells. T10 triggered the highest level of cytokine release starting from 10 μ g/ml. Both T20 and T100 triggered similar amount of TNF- α release, which shows statistical difference from the control at 100 μ g/ml and 300 μ g/ml. The TNF- α release stimulated by TiO₂ nanoparticles was also observed by other studies [26, 106]. TiO₂ could not only trigger the expression of TNF- α but also stimulate the release of other cytokines including IL-6 and MIP-1 α in macrophages [26]. The increase in maturation of macrophage and antigen presentation triggered by TiO₂ nanoparticles was also observed in Palomaki *et al.*'s study [26]. Similar trend was also observed by Oberdorster *et al.* from their *in vivo* study [107] whereby after intratracheal instillation of different sized TiO₂ nanoparticles, obvious acute inflammatory

response was indicated by the lung lavage parameters. This pulmonary inflammatory response correlated well with surface area of particles instead of particle mass, volume, or numbers, so similarly smaller particles with higher surface area could also trigger higher level of inflammatory response.

Figure 4.13 The TNF- α released from RAW264.7 cells after the cells were stimulated by TiO₂ nanoparticles for 24 h. The TNF- α released from macrophages into medium was measured by ELISA and normalized to negative control (0 µg/ml). The data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.3.5 The protein adsorption on TiO₂ nanoparticles was size-dependent

All the cytotoxicity studies showed that the cytotoxicity of TiO₂ nanoparticles was size-dependent. Unlike other nanomaterials such as ZnO, TiO₂ nanoparticles do not release toxic ions like Zn²⁺. Thus, the potential mechanism behind this cytotoxicity could be due to the size-dependent interaction between nanoparticles and intracellular biomolecules adsorbed onto nanoparticles. Smaller nanoparticles have larger surface area and higher percentage of molecules exposed on particle surface to interact with surrounding biomolecules. To investigate this hypothesis, we studied the adsorption of BSA (a model biomolecule) to different sized TiO₂ nanoparticles. The TGA result of BSA adsorption onto TiO₂ nanoparticles is shown in **Figure 4.14**. The weight decreased more for T10-BSA and T20-BSA when compared with T100-BSA in the temperature range from 188 °C to 485 °C, which was the major weight loss temperature range of BSA protein. This indicated more BSA adsorbed onto T10 and T20 nanoparticles than on relatively larger T100 nanoparticles. By comparing the results in **Figure 4.9** and **Figure 4.14**, 100 mg of P100 nanoparticles could only adsorb 0.441 mg of BSA, but 100 mg of T100 nanoparticles could adsorb more than 2 mg of BSA. This could be the reason for the higher cytotoxicity of 100 nm TiO₂ nanoparticles as compared with 100 nm PLGA nanoparticles. BSA remained attached on these nanoparticles after extensive washing, which indicated a high-affinity interaction between BSA and nanoparticles especially for TiO₂ nanoparticles.

Figure 4.14 TGA analysis of BSA adsorption on TiO₂ nanoparticles. T10-BSA, T20-BSA and T100-BSA represent the weight decrease of particles after interaction with protein BSA. This indicated more BSA adsorbed onto T10 and T20 nanoparticles than on relatively larger T100 nanoparticles.

Although it has been found that reaction between nanoparticles and proteins is related to some pathological changes such as protein aggregation or unfolding, the mechanism of these damages is still poorly investigated and understood [108]. Some studies have

reported that the interaction between nanoparticles and biomolecules could lead to dysfunction or damage of biomolecules [108-111]. Wang *et al.* [109] investigated the interaction between TiO₂ nanoparticles and trypsin. They observed strong physical adsorption effect of TiO₂ nanoparticles on enzyme trypsin and found inhibitory effect of TiO₂ nanoparticles on trypsin activity by changing the α -helix and β -sheet ratio of the protein structure. Deng *et al.* [108] found the interaction between poly (acrylic acid) coated gold nanoparticles and fibrinogens would induce the unfolding of protein fibrinogen, which resulted in the activation of Mac-1 reception and inflammation response. Smaller particles (5 nm) tend to bind more fibrinogen than bigger particles (20 nm), and the adsorption is proportional to the surface area of nanoparticles. Wang *et al.* [112] reported a sized dependent toxicity of silica nanoparticles on cultured human embryonic kidney (HEK293) cells. 20 nm SiO₂ nanoparticles were more toxic than 50 nm SiO₂ nanoparticles. The possible reason could be related to the depletion of glutathione (GSH, a tripeptide responsible for the homeostasis of free radicals in cells) and the increase of ROS, and smaller particles with higher surface area tend to trigger higher level of GSH decrease in cells. Horie *et al.* [94] showed similar results that smaller TiO₂ nanoparticles tend to adsorb more proteins and cause severe toxic effect to human keratinocyte HaCaT cells and human lung carcinoma A549 cells. However their explanation was that the cytotoxicity of TiO₂ nanoparticles was due to the depletion of proteins in the cell culture medium. But based on our data, even 300 μ g/ml of TiO₂ nanoparticles did not cause obvious decrease of proteins in the cell culture medium (data not shown here). So the sized dependent cytotoxicity of TiO₂ nanoparticles is less likely due to nutrient depletion. Instead, the adsorption, denaturalization or even the damage of biomolecules could be a more reasonable explanation.

4.3.6 Summary

Figure 4.15 Schematic of the possible mechanism behind the size dependent cytotoxicity of PLGA and TiO₂ nanoparticles

The data therefore suggests that the size dependent cytotoxicity of PLGA and TiO₂ nanoparticles should be related to different surface area of different-sized nanoparticles. Smaller particles have bigger surface area and more molecules exposed on particle surface to interact with intracellular biomolecules nearby. The potential mechanism of such size-dependent cytotoxicity was exhibited in **Figure 4.15**. When nanoparticles come in to contact with intracellular biomolecules, these biomolecules may adsorb onto the surface of the particles and interact with these “intruder” nanoparticles. These adsorption and interactions may then trigger a series of responses in cells including generation of ROS, mitochondrial depolarization, plasma membrane leakage, intracellular calcium

influx and cytokine release. Thus, our studies demonstrated that the particles size can influence the cytotoxicity of nanoparticles. Smaller particles tend to have higher cytotoxicity, as smaller particles have more molecules exposed on the surface of particles to interact with surrounding biomolecules such as proteins. The protein adsorption could be an important paradigm to predict the *in vitro* cytotoxicity of nanoparticles, especially for low toxic nanoparticles such as PLGA and TiO₂ nanoparticles.

4.4 Size influences the phototoxicity of TiO₂ nanoparticles

4.4.1 The phototoxicity of TiO₂ nanoparticles was size dependent

In this study, RAW264.7 macrophages were used as a model biological system for testing of the phototoxicity of TiO₂ nanoparticles. The negative control (NC) received neither particles nor UV-Vis light, while light control (LC) received UV-Vis light only. From **Figure 4.16**, LC showed some damage to cells when compared with NC, but was not statistically significant ($p > 0.05$). This meant that the UV-Vis exposure (280 nm – 450 nm) for 5 min to RAW264.7 cells did not cause any significant cytotoxicity. For the cells treated with non-photoactivated nanoparticles only, the cytotoxicity of the nanoparticles alone was not significant, which was less than 1% for the percentage of positive cells in PI uptake and MitoSOX tests. A slight increase of 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 ROS and subsequent induction of oxidative stress in cells. From **Figure 4.16**, 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. With all the particles and cell treatment

conditions, the intracellular calcium concentration tested by Fluo-4 did not show obvious change as compared to their corresponding control. This indicated the phototoxicity of TiO₂ nanoparticles may not related to the change of intracellular calcium concentration.

Figure 4.16 Potential damage to RAW264.7 cells after being treated with or without UV light (280 nm - 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 µg/ml. Cells were stained with (A) MitoSOX, (B) JC-1, (C) PI uptake and (D) Fluo-4 to probe cytoplasm mitochondrial superoxide generation, mitochondrial depolarization, membrane integrity and intracellular calcium influx, respectively. Smaller particles triggered higher levels of phototoxicity to cells in all 3 assays. Data represents means $\pm$ SD, n=4. * represented that the data

was significantly different ($p<0.05$) as compared to the control group (NC is control for non UV exposed group; LC is control for UV exposed group.). # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.4.2 The phototoxicity of TiO₂ nanoparticles was surface area dependent

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 was related to their higher surface area and thus a larger number of surface-exposed TiO₂ molecules. Particle concentration was then converted from weight/volume into surface area/volume, which was 166.0 cm²/ml, 50.4 cm²/ml and 17.2 cm²/ml for T10, T20 and T100, respectively. From **Figure 4.17**, the cytotoxicity results showed excellent correlation with the surface area, because the R² 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.

Figure 4.17 Toxicity of photoactivated TiO₂ nanoparticles was correlated with surface area. The surface areas normalized to volume were 166.0 cm²/ml, 50.4 cm²/ml and 17.2 cm²/ml for T10, T20 and T100, respectively. Cytotoxicity outcome showed excellent correlation with the surface area for (A) MitoSOX, (B) JC-1 and (C) PI uptake, but was irrelevant with (D) Fluo-4 results. Data represents means $\pm$ SD, n=4.

4.4.3 Hydroxyl radical generation was responsible for the phototoxicity of TiO₂ nanoparticles

Based on **Figure 4.18**, 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 [113].

Figure 4.18 Hydroxyl radical generation during photoactivation of TiO₂ nanoparticles tested by HPF assay. Smaller particles generated more hydroxyl radicals during photoactivation of TiO₂ nanoparticles. Data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

The hypothesis of this study was that phototoxicity of TiO₂ nanoparticles was size dependent. 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 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 **Figure 4.17**, the mitochondrial superoxide production, mitochondrial depolarization and loss of cell plasma membrane integrity correlated well with the surface area of the different-sized nanoparticles ($R^2 > 0.9$). 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.

Figure 4.19 Schematic of the possible mechanism behind the size dependent phototoxicity of TiO₂ nanoparticles

With our results, we present here a model to describe the potential mechanism of TiO₂ nanoparticles induced phototoxicity, as shown in **Figure 4.19**. 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 [49]. The holes (h^+) can interact with adsorbed H_2O or hydroxide ions (OH^-) to generate hydroxyl radicals ($\bullet OH$) which are highly reactive and toxic to cells [50], as hydroxyl radicals ($\bullet OH$) can inflict detrimental toxicity on cellular proteins, lipids and even DNA, resulting in the oxidation and dysfunction of biomolecules [49, 77, 95-97]. Furthermore, the electrons (e^-) in the conduction band can reduce oxygen (O_2) adsorbed onto the particle surface to produce superoxide ions (O_2^-), which can further react with water in the environment to form hydrogen peroxide in cells [28, 51]. ROS generation is widely considered as a molecular paradigm for the toxicity of nanoparticles [3]. Excessive ROS generation would cause oxidative stress in cells [114-115]. If the oxidative stress exceeds the threshold of the cellular antioxidant defences, additional mitochondrial perturbation such as mitochondrial depolarization would occur [4, 51, 116]. 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 TiO_2 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 [3]. HPF results indicated that ROS generation may result directly from hydroxyl radicals generated by photoactivated TiO_2 nanoparticles.

Furthermore, we found size-dependent biomolecule adsorption could also be related to this size- and surface area- dependent phototoxicity. Smaller particles with higher percentage of molecules on their surface [6] can generate more ROS such as $\bullet OH$. The highly unstable hydroxyl radicals can non-specifically attack biomolecules such as DNA, proteins and lipids in a diffusion-controlled reaction [117]. Intuitively, if smaller particles could adsorb more biomolecules onto their surface, this would increase the possibility

that these adsorbed biomolecules could be damaged by photo-activated hydroxyl radicals. 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 [97, 118]. 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 [117]. For example, if the lipid peroxidation happened in cellular membrane, the fluidity of membrane will decrease, resulting in increased permeability for ions [117]. The oxidized protein would increase the susceptibility to enzymic proteolysis [119]. 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 (**Figure 4.14**), similarly shown by Horie *et al.* [94]. Thus, more biomolecules tend to be damaged by smaller TiO₂ nanoparticles.

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 [120-122].

4.4.4 Summary

In this study, we demonstrated that the TiO₂ nanoparticles exhibited increased cytotoxicity upon UV-Vis activation also 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. These studies provided clues of the mechanism behind the phototoxicity of TiO₂ nanoparticles.

4.5 Surface coating decreased the phototoxicity of TiO₂ nanoparticles

4.5.1 Physical characterization of TiO₂ nanoparticles and nanoparticles with surface coating

Since it was shown that phototoxicity was correlated to 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. The SEM images were shown in **Figure 4.20**. The hydrodynamic size, PDI and zeta potential of the nanoparticles were tested through DLS technique in both DI water and cell culture medium. The results were summarized in **Table 4.3**. All the particles showed size in DI water and cell culture medium in the range from 300 to 800 nm. After surface coating with PEMA or chitosan, the zeta potential of T20 nanoparticles in DI water changed from 32.3 mV to -50.4 mV and 51.4 mV respectively, showing the successful coating of PEMA or chitosan onto the surface of T20 nanoparticles. 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 -7 mV 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 [3].

Figure 4.20 (A to C) SEM pictures of bare and coated T20 nanoparticles: (A) bare T20 nanoparticles, (B) PEMA coated T20 nanoparticles (T20-PEMA), (C) chitosan coated T20 nanoparticles (T20-chitosan). (D to F) Schematic structure of bare and coated T20 nanoparticles. (D) bare T20 nanoparticles. Spherical particles represents bare T20 nanoparticles. (E) T20-PEMA and chemical structure of PEMA. Spherical core represents T20 nanoparticles. Green curled line represents PEMA. (F) T20-chitosan and chemical structure of chitosan. Spherical core represents T20 nanoparticles. Red curled line represents chitosan.

Table 4.3 Characterization of TiO₂ nanoparticles and particles with surface coating

		T20	T20-PEMA	T20-chitosan
Primary Particle Size (nm)		20	20	20
water	Size (nm)	307	643	408
	PDI	0.263	0.349	0.276
	Zeta Potential (mV)	32.3	-50.4	51.4
CDMEM	Size (nm)	338	633	742
	PDI	0.180	0.330	0.306
	Zeta Potential (mV)	-7.7	-9.6	-7.2

4.5.2 The phototoxicity of TiO₂ nanoparticles was decreased after surface coating with PEMA and chitosan

Multiparametric assays were conducted to test the cytotoxicity of TiO₂ nanoparticles and the same particles after surface coating. The negative control (NC) received neither particles nor UV-Vis light, while light control (LC) received UV-Vis light only. From **Figure 4.21**, LC did not show some statistically significant damage to cells when compared with NC ($p > 0.05$). This meant that the RAW264.7 cells exposed to UV-Vis light (280-450 nm) alone for 5 min did not cause any significant cytotoxicity. The cells treated with non-photoactivated nanoparticles only did not show statistically difference as compared to control across all the particles tested. However, after photoactivation, T20-treated cells exhibited obvious increase in PI uptake and MitoSOX, which is indicative of cell death and mitochondrial superoxide respectively. From PI uptake results, 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. Decreased mitochondrial damage was also observed in JC-1 results. Same as results in **Figure 4.16**, the phototoxicity of TiO₂ nanoparticles did not show any relationship with the change of intracellular calcium concentration. In summary, the surface coating of TiO₂ nanoparticles, regardless of the charge of materials, could decrease the cytotoxicity of TiO₂ nanoparticles during photoactivation.

Figure 4.21 Potential damage to RAW264.7 cells after being treated with or without UV light exposure (280 nm - 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 (T20-PEMA, T20 and T20-chitosan), the cells were treated with different nanoparticles at a concentration of 100 $\mu\text{g/ml}$, and assayed for (A) MitoSOX staining, (B) JC-1 staining, (C) PI uptake and (D) Fluo-4 staining. After surface coating with PEMA or chitosan, the phototoxicity of T20 nanoparticles decreased. Data represents means $\pm$ SD, $n=4$. * represented that the data was significantly different ($p<0.05$) as compared to the control group (NC is control for non UV exposed group; LC is control for UV exposed group). # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.5.3 Hydroxyl radical generation was decreased after surface coating of T20 nanoparticles

Based on **Figure 4.22**, T20 nanoparticles could trigger high rate of hydroxyl radical generation under photoactivation in abiotic conditions. Surface coating of T20 nanoparticles with PEMA or chitosan was shown to significantly decreased the generation of hydroxyl radicals ($p<0.05$), especially for PEMA coated T20 particles.

Figure 4.22 Hydroxyl radical generation during photoactivation of TiO_2 nanoparticles tested by HPF assay. The surface coating of T20 nanoparticles with PEMA or chitosan significantly decreased the hydroxyl radicals generated by T20 nanoparticles. Data represents means $\pm$ SD, $n=4$. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.5.4 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 [117, 123]. Intuitively, if smaller particles could adsorb more biomolecules onto their surface, this would increase the possibility that these biomolecules could be damaged by photo-activated hydroxyl radicals. BSA was therefore chosen as a model to understand the adsorption ability of biomolecules onto different sized nanoparticles. **Figure 4.23** shows the TGA analysis of pure BSA, nanoparticles and nanoparticles-BSA. From **Figure 4.23A**, a major weight loss of BSA protein was observed from 188 °C to 485 °C, corresponding to its decomposition temperature. Based on **Figure 4.23B**, 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.

Figure 4.23 TGA analysis of BSA adsorption onto TiO₂ nanoparticles. (A) BSA, (B) T20, T20-PEMA, T20-chitosan and their corresponding BSA adsorbed nanoparticles. The surface coating of T20 nanoparticles with PEMA or chitosan decreased the BSA adsorption onto T20 nanoparticles.

Based on cytotoxicity of different sized TiO₂ nanoparticles, we found the reason behind the size-dependent phototoxicity of TiO₂ nanoparticles was the change of surface area. In this study, we 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 ($\bullet\text{OH}$) generation by T20 during the photoactivation process. The potential mechanism is exhibited in **Figure 4.24**. The decreased cytotoxicity could be due to four possibilities. First, the surface coating decreased the effective surface area for interaction between surface TiO_2 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 [77]. Second, the PEMA or chitosan attached on the surface of TiO_2 nanoparticles quenched the activity of photo-activated TiO_2 surface and resulted in non-harmful oxidized PEMA or chitosan, which prevent further disturbance inside cells. Third, the surface coating of TiO_2 nanoparticles may decrease light intensity reached at the particles. Based on the UV-Vis test (**Figure 4.25**), 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 **Figure 4.22**, PEMA or chitosan on the surface of TiO_2 nanoparticles might be able to block the attachment of biomolecules on the surface of TiO_2 nanoparticles. The decreased adsorption of biomolecules decreased the possibility of biomolecules to be damaged by ROS, and thus cytotoxicity.

Figure 4.24 Schematic of the possible mechanism behind decrease phototoxicity of TiO_2 nanoparticles after surface coating

Figure 4.25 UV–Vis absorption spectra of T20, T20-chitosan and T20-PEMA nanoparticles. The nanoparticles were dispersed in water at a concentration of 50 $\mu\text{g/ml}$. The UV-Vis absorption spectra were tested by using UV Spectrometer. Both T20-chitosan and T20-PEMA nanoparticles showed decreased UV-Vis absorbance as compared to bare T20 nanoparticles. The T20-PEMA nanoparticles showed more obvious decrease in UV absorbance than T20-chitosan nanoparticles. This could be because more PEMA was attached onto T20 nanoparticles as compared to chitosan, which was proven in TGA studies.

4.5.5 Summary

In this study, we demonstrated that the TiO₂ nanoparticles exhibited increased cytotoxicity upon UV-Vis activation. After surface coating of TiO₂ nanoparticles with PEMA or chitosan, the phototoxicity of TiO₂ nanoparticles decreased, which proved by PI-uptake and MitoSOX studies. This decreased phototoxicity after surface coating of TiO₂ nanoparticles might be due to the hindrance of biomolecule adsorption and hydroxyl radicals ($\bullet$ OH) production in the photo-activation process. Higher ROS generation from bare particles was detected under both biotic and abiotic conditions. TGA analysis revealed that less protein could be adsorbed onto surface coated TiO₂ nanoparticles. This study provided clues on how to alleviate such toxicity of TiO₂ nanoparticles.

4.6 Surface area, a size related factor, influenced the cytotoxicity and phototoxicity of TiO₂ particles

We previously reported an inverse relationship between the size of TiO₂ nanoparticles and its *in vitro* cytotoxicity. 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 unknown. In this study, it was hypothesized that specific surface area might be the key reason behind an increase in cytotoxicity observed with smaller-sized TiO₂ particles, rather than particle size per se. Similar observations were also reported from an *in vivo* study conducted by Sager *et al.* [75]. The pulmonary toxicity of two TiO₂ particles with different sizes (ultrafine and fine particles) was studied, and it was concluded that specific surface area might be a more accurate dose metric. However, in that study, the effect of particle size was still not fully eliminated, in view that comparisons were made between ultrafine and fine particles. As such, the aim of this study is to investigate the cytotoxicity of similar-sized TiO₂ particles, with different specific surface areas, and to further investigate whether this observation is maintained under UV activation. A plausible mechanism on how TiO₂ particles may induce cytotoxicity was also proposed, which may provide valuable insights on how the cytotoxicity of TiO₂ particles can be decreased.

4.6.1 Characterization of TiO₂ particles

TiO₂ particles of different 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 4.4**). With the same distribution of particles size, we achieve the different surface area by varying the inner pore size and pore volume of porous TiO₂ particles. Both particles were found to have flower-like structural morphologies and were of similar size –diameters ~0.8 µm as shown from TEM (**Figure 4.26 A, B**) and FESEM (**Figure 4.26 C, D**) images. As reflected from **Table 4.4**, the T174 sample possessed a high specific surface area which was around 174.5 m²/g with a pore volume of 0.70 cm³/g (average pore size of 16.1 nm), while the T33 sample had a low specific surface area around 33.3 m²/g with pore volume of 0.39 cm³/g (average pore size is 47.1 nm). The typical nitrogen adsorption-desorption isotherms of the TiO₂ samples are shown in **Figure 4.27**. Both particles were in anatase form. DLS measurements were conducted to examine the hydrodynamic size, 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 (**Table 4.4**). The hydrodynamic size of T33 and T174 was 1.209 µm and 0.973 µm respectively in DI water, while their hydrodynamic sizes increased slightly to 1.323 µm (T33) and 1.164 µm (T174) in complete cell culture medium. The small hydrodynamic size increase in cell culture medium could be due to the surface adsorption of proteins from CDMEM [3, 124]. Both particles exhibited similar positive and negative charge values in water and CDMEM respectively. The change from positive to negative Zeta potentials in CDMEM is again due to the surface binding of proteins.

Table 4.4 Physical characterization of TiO₂ particles with different surface area

		T33	T174
Primary Particle Size (μm)		0.8	0.8
Cristal structure		Anatase	Anatase
water	Size (μm)	1.209	0.973
	PDI	0.316	0.545
	Zeta Potential (mV)	9.3	10.6
CDMEM	Size (μm)	1.323	1.164
	PDI	0.465	0.518
	Zeta Potential (mV)	-8.7	-9.0
BET Surface Area (m ² /g)		33.3	174.5

Figure 4.26 (A, B) TEM micrographs of TiO₂ particle samples with different surface areas (A) T33 (B) T174; (C, D) FESEM micrographs of TiO₂ particle samples with different surface areas (C) T33 (D) T174. All scale bars represent 500 nm.

Figure 4.27 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.

4.6.2 Cytotoxicity of TiO₂ particles

Cellular metabolic activity was measured using WST-8 assay, which indirectly indicated the viability of RAW264.7 cells. From **Figure 4.28**, 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.

Figure 4.28 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. The metabolic activity of cells was normalized to negative control (0 µg/ml). Data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.6.3 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 4.29**. 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.

Figure 4.29 TGA analysis of BSA adsorption onto TiO₂ particles. (A) BSA, (B) T33, T174, T33-BSA and T174-BSA.

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. The first possibility is that extracellular proteins adsorbed onto TiO₂ particles causing a depletion of proteins in the cell culture medium. This possibility was supported by Horie *et al.*'s study [94], which demonstrated that the toxicity of TiO₂ nanoparticles was due to the depletion of serum in cell culture medium. However, based on our preliminary tests, even in the presence of highest TiO₂ concentration (1000 µg/ml) we used, there was no significant change in protein level in the medium (data not shown here), indicating that the cytotoxicity of surface area-dependent TiO₂ particles is less likely due to the nutrient depletion. As such, we proposed another possibility that TiO₂ particles may interact with intracellular biomolecules. The interaction may induce depletion or damage of intracellular biomolecules. If the second possibility is true, decreasing the interactions between TiO₂ particles and intracellular biomolecules could decrease the cytotoxicity of TiO₂ particles.

In this case, by pre-coating TiO₂ particles with extracellular proteins, there would be lesser particle surface exposed to interact with intracellular biomolecules upon cell entry, leading to decreased cytotoxicity. To investigate this, cells were exposed to TiO₂ particles in another two conditions, namely particles dispersed in medium without FBS (**Figure 4.30A**) and FBS-coated particles dispersed in FBS-free medium (**Figure 4.30B**). In the absence of FBS (**Figure 4.30A**), cells exhibited significantly lower viability than the control in a dose-dependent manner when treated with 300 and 1000 µg/ml TiO₂ 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 4.30B**), cells treated with FBS-coated particles did not show significant

difference in metabolic activity for TiO₂ concentrations of 300 µg/ml and below. At 1000µg/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 TiO₂ particles did have an effect on cytotoxicity.

Figure 4.30 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 interact with (A) particles dispersed in FBS free medium; (B) particles pre-coated with FBS and then dispersed in FBS free medium. Data represents means $\pm$ SD, n=4. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

4.6.4 Phototoxicity of TiO₂ particles

To further investigate the effect of specific surface area on phototoxicity, TiO₂ particles of concentration of 100 µg/ml was used. From our preliminary tests, cell viability was not compromised when treated with 100 µg/ml of TiO₂ 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 TiO₂ particles did not induce cell death (**Figure 4.31**). **Figure 4.32** shows cellular metabolic activity of RAW264.7 cells after being treated with TiO₂ 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 4.32 B**). 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 (**Figure 4.33 to Figure 4.38**).

Figure 4.31 The metabolic activity of live cells quantified by WST-8 assay after RAW264.7 cells were treated with or without UV light. Data represents means $\pm$ SD, $n=4$.

Figure 4.32 The metabolic activity of live cells quantified by WST-8 assay after RAW264.7 cells were treated with control or 100 $\mu\text{g/ml}$ of two TiO_2 particles for 24 h. The metabolic activity of cells was normalized to negative control (0 $\mu\text{g/ml}$). Cells were interact with (A) particles dispersed in medium with FBS (B) particles dispersed in FBS free medium; (C) particles pre-coated with FBS and then dispersed in FBS free medium. Data represents means $\pm$ SD, $n=4$. * represented that the data was significantly different ($p<0.05$) as compared to the control group. # represented that the data was significantly different ($p<0.05$) as compared to other particles at same concentration.

Figure 4.33 RAW264.7 cells were treated with medium with FBS or 100 $\mu\text{g/ml}$ particles (T33 and T174) dispersed in medium with FBS for 24 h prior to co-staining with 1 μM Hoechst 33342 (blue) and 5 μM PI (red). All cells were stained with Hoechst in blue color. Only dead cells showed red nuclei in PI column and purple in merged images.

Figure 4.34 RAW264.7 cells were treated with medium with FBS or 100 $\mu\text{g/ml}$ particles (T33 and T174) dispersed in medium with FBS as well as UV light exposure for 20 min prior to co-staining with 1 μM Hoechst 33342 (blue) and 5 μM PI (red). All cells were stained with Hoechst in blue color. Only dead cells showed red nuclei in PI column and purple in merged images.

Figure 4.35 RAW264.7 cells were treated with FBS free medium or 100 $\mu\text{g/ml}$ particles (T33 and T174) dispersed in FBS free medium for 24 h prior to co-staining with 1 μM Hoechst 33342 (blue) and 5 μM PI (red). All cells were stained with Hoechst in blue color. Only dead cells showed red nuclei in PI column and purple in merged images.

Figure 4.36 RAW264.7 cells were treated with FBS free medium or 100 $\mu\text{g/ml}$ particles (T33 and T174) dispersed in FBS free medium as well as UV light exposure for 20 min prior to co-staining with 1 μM Hoechst 33342 (blue) and 5 μM PI (red). All cells were stained with Hoechst in blue color. Only dead cells showed red nuclei in PI column and purple in merged images.

Figure 4.37 RAW264.7 cells were treated with FBS free medium or 100 $\mu\text{g/ml}$ particles (T33 and T174) pre-coated with FBS but re-dispersed in FBS free medium for 24 h prior to co-staining with 1 μM Hoechst 33342 (blue) and 5 μM PI (red). All cells were stained with Hoechst in blue color. Only dead cells showed red nuclei in PI column and purple in merged images.

Figure 4.38 RAW264.7 cells were treated with FBS free medium or 100 $\mu\text{g/ml}$ particles (T33 and T174) pre-coated with FBS but re-dispersed in FBS free medium as well as UV light exposure for 20 min prior to co-staining with 1 μM Hoechst 33342 (blue) and 5 μM PI (red). All cells were stained with Hoechst in blue color. Only dead cells showed red nuclei in PI column and purple in merged images.

4.6.5 Generation of hydroxyl radical under UVA exposure

HPF was utilized to quantify the amount of hydroxyl radicals generated by TiO_2 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 4.39** 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.

Figure 4.39 HPF assay to test the generation of hydroxyl radicals during photoactivation of TiO₂ particles.

Particle surface area has always been regarded as an important parameter in particle toxicity studies [1, 4, 6]. 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. There was indeed a direct correlation between particle surface area and the ability of TiO₂ to elicit both cytotoxic and phototoxic effects, which is similar to that observed by Sager *et al.* [75].

As mentioned earlier, the ability of TiO₂ particles to elicit cytotoxic effects in the absence of UV irradiation is still pretty much under debate. Contrary to studies which reported that non-irradiated TiO₂ particles were biologically inert [17, 62-63], 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 therefore clearly dose-dependent. Although hydroxyl radical generation, leading to oxidative stress, is considered by many to be the primary mechanism responsible for TiO₂ phototoxicity [28, 64], 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 some other mechanisms instead of hydroxyl radical generation.

Unlike ZnO particles, TiO₂ particles are relatively stable and do not release toxic metal ions in cell culture medium [125]. However TiO₂ particles were reported to be able to cause oxidative stress [18-21], immunotoxicity [25-26] and even genotoxicity [22-24] in the absence of photoactivation. Particle-biomolecule interaction could therefore be one possible mechanism to explain the cytotoxicity of TiO₂ particles [94]. Horie *et al.* [94]

reported that, among the particles examined, namely NiO, ZnO, TiO₂, CeO₂, SiO₂, and Fe₂O₃ particles, TiO₂ particles showed the strongest protein adsorption abilities. A size-dependent protein adsorption profile onto TiO₂ particles was also observed in Horie's studies, whereby smaller particles tend to adsorb more proteins than larger particles. This observation was subsequently correlated to an increase in cytotoxicity, in which the explanation given was due to the depletion of proteins in the cell culture medium. However, based on our preliminary tests, a complete depletion of proteins would occur at particle concentrations higher than 100 mg/ml (data not shown here). With this, the cytotoxicity of TiO₂ particles observed in our study is therefore less likely due to their interaction with extracellular proteins such as nutrient depletion, but could instead be due to the interaction with intracellular biomolecules. On the other hand, numerous studies have proved that TiO₂ particles can be taken by cells and enter different cellular organelles such as lysosome [126] and mitochondria [20]. Based on literature and our current knowledge, the particles could influence the 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 glutathione (GSH) in human embryonic kidney (HEK293) cells was reported by Wang *et al.* [112] after the cells were incubated with SiO₂ nanoparticles of different sizes. The cytotoxicity of these SiO₂ nanoparticles was observed to be sized-dependent. The author explained that smaller SiO₂ nanoparticles of larger surface area could trigger higher level of GSH depletion, resulting in increased generation of ROS. Second, the particles may cause denaturation and/or even damage of intracellular biomolecules. For example, studies have found that TiO₂ particles could interrupt cytoplasmic proteins such as microtubule protein in *Arabidopsis thaliana* [86]. This phenomenon has been discussed earlier by Gheshlaghi *et al.* [87], who showed that TiO₂ particles can bind tubulin heterodimers leading to the conformation change of protein. Others have reported that some pathological changes of proteins such as

aggregation or unfolding of proteins after interaction with particles [88-91]. TiO₂ 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 [109]. Similar damage in the secondary structure of proteins due to particle-protein interaction between CdS quantum dots and human hemoglobin was also observed [91]. The secondary structure of BSA was irreversibly changed after interaction with polystyrene [89] or asbestos [90]. However, the potential mechanism behind the correlation between protein adsorption and cytotoxicity of particles is still poorly investigated and not fully understood [108]. Based on this study we can only suggest that TiO₂ particles with larger surface area should be able to adsorb more biomolecules. The presence of extracellular proteins can preoccupy the surface of particles resulting in decreased adsorption of intracellular proteins and decreased cytotoxicity (**Figure 4.40**). However, the molecular mechanism that underlies the surface area dependent cytotoxicity of TiO₂ particles is still unknown. Our current understanding can only allow us to hypothesize that, upon adsorption of biomolecules onto TiO₂ particles, depletion or impairment of some key biomolecules in cells may influence normal cell functions, which may eventually lead to built-up of intracellular stress due to accumulation of impaired biomolecules, causing cell damage and cell death.

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 that possible role of particle surface area may play in influencing phototoxic effects of TiO₂. Unlike in the absence of UV light, the generation of hydroxyl radicals could be responsible for the phototoxicity of TiO₂ particles, which again is also specific surface

area dependent. The formation of hydroxyl radicals from photoactivated TiO₂ particles has been discussed in Section 4.4. In brief, photoactivation of TiO₂ particles can trigger the formation of electron hole pairs [49] on TiO₂ particles, which actively interact with adsorbed oxygen, water or hydroxide ions to generate hydroxyl radicals (•OH) [28, 51]. Highly active and damaging hydroxyl radicals can oxidize and damage cellular biomolecules such proteins, lipids and even DNA, causing the dysfunction of biomolecules [49, 77, 95-97]. In this study, generation of hydroxyl radicals by photoactivated particles, as indicated by HPF assay, was time- and surface area-dependent (Shown in **Figure 4.39**). 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 nearby in a diffusion-controlled reaction [117]. Thus, the biomolecules adsorbed onto TiO₂ particles are highly susceptible to the hydroxyl radicals generated by TiO₂ particles during photoactivation. 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 B, 2004). Lipid peroxidation occurring in cellular membrane will decrease the fluidity of membrane and eventually increase permeability for ions [117]. It was also reported that oxidized protein would be more susceptible to enzymic proteolysis [119]. 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 radical 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. Thus, we hypothesized that the

presence or absence of extracellular proteins should influence the interaction between particles and intracellular biomolecules and further influence the toxic effects of TiO₂ particles. In order to prove this hypothesis, the toxicity of TiO₂ particles in three different conditions, which were TiO₂ particles dispersed in medium with FBS, particles dispersed in medium without FBS and FBS-coated particles dispersed in FBS-free medium, were compared. It was revealed that within the three conditions tested, similar toxicity trend was observed between particles of different surface areas. However, across the conditions, lower toxicity was observed in the presence of FBS or FBS-coated particles which indicated that the presence of extracellular proteins could decrease the cytotoxicity and phototoxicity of TiO₂ particles. The similar toxicity of particles in FBS containing medium and particles pre-coated with FBS indicated that the role of proteins on surface of particles is more important than that of proteins in the cell culture medium. Some other studies have also reported that the presence of serum in culture medium could reduce toxicity of particles [127-130]. 10% serum in the culture medium decreased the percentage of micronuclei in human lung epithelial cells caused by single-walled carbon nanotubes (SWCNTs) [130]. Most of the papers believed that serum or proteins could alleviate particle aggregation, resulting in different cellular uptake [130]. However, based on DLS data, these two types of particles investigated were well dispersed in both water and cell culture medium. The particles did not severely aggregate regardless of the presence or absence of serum. Petri-Fink *et al.*[129] explained the decreased toxicity was because of the presence of serum on the surface of particles, which hindrance the uptake of particles. However, contradicting results was reported by Dutta *et al.*[131], who believed that the reason for increased uptake of SWCNTs when they were suspended in 10–15% serum was that the material adsorbed albumin and changed the protein structure, leading to the particles to be scavenged by macrophages. Our TGA results pointed out that the TiO₂ particles have strong adsorption to proteins, which is consistent with Horie

et al.'s report [94]. Particles with larger surface area could adsorb more proteins and also trigger higher level of cytotoxic effect. Thus, we proposed another possibility that the adsorption of proteins could be the reason for the cytotoxicity and pre-coating of particles with extracellular proteins in serum or cell culture medium could pre-occupy the surface of particles, decrease the interaction between surface of particles and intracellular biomolecules and finally decrease the toxicity of particles. These results also provided an insight to the importance of culture medium condition used during testing, because different test systems can result in contrasting observations [132]. The use of surfactant in toxicity study should also be noted with special concerns because reports have shown that the surfactant can further decrease the toxicity of particles [131], which could be because surfactant could modify the surface property of particles such as increasing hydrophilicity and also influence the interaction between particles and intracellular biomolecules. Dutta *et al.* [131] found that silica nanoparticles pre-coated with Pluronic F127 significantly decreased the adsorption of serum proteins and also decreased toxic effects. 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.

Figure 4.40 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 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.

4.6.6 Summary

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.

Chapter 5 Conclusions

The systematic study of the size effect on the cytotoxicity of PLGA and TiO₂ nanoparticles was reported in this thesis. The first objective of this study is to synthesize and characterize PLGA and TiO₂ nanoparticles of different sizes. Firstly, we investigated synthetic factors that may influence the size of PLGA nanoparticles. We found the size of PLGA nanoparticles could be mostly influenced by PLGA concentration, PVA concentration and organic solvent in the synthesis procedure. The importance of these factors are in this order, organic solvent > PVA concentration > PLGA concentration. Thus, by using different organic solvent and synthesis techniques, we synthesized PLGA nanoparticles of different sizes, which are 60 nm, 100 nm and 200 nm PLGA nanoparticles. The different sized TiO₂ nanoparticles were all from commercial sources, but T20 nanoparticles were coated with PEMA and chitosan. TGA analysis proved the successful coating of PEMA and chitosan onto T20 nanoparticles. Fully characterization study was conducted to understand the properties of all these nanomaterials.

The second objective of this study is to investigate the influence of size on the cytotoxicity of PLGA and TiO₂ nanoparticles. For both PLGA and TiO₂ nanoparticles, smaller nanoparticles with higher surface area showed higher level of cytotoxicity. Smaller TiO₂ particles with higher surface area showed higher level of phototoxicity. After surface coating of TiO₂ nanoparticles, their phototoxicity decreased. As such, we hypothesized that the increased surface area of smaller particles was the reason behind the size dependent cytotoxicity of PLGA and TiO₂ nanoparticles. By comparing two TiO₂ nanoparticles of similar size and shape but different surface area, we found the particles

with higher surface area showed higher level of cytotoxicity and phototoxicity, which confirmed our hypothesis.

Last but not the least, understanding of the mechanism behind the cytotoxicity of different-sized PLGA and TiO₂ nanoparticles is the third object of this study. First, we found smaller nanoparticles with larger surface area were always tending to adsorption of more biomolecules, which could be responsible for the increased cytotoxicity of smaller particles. The phototoxicity of TiO₂ nanoparticles was also related to their particle size, which could be because smaller particles had more TiO₂ molecules exposed on the surface of nanoparticles. Thus, smaller particles could generate more hydroxyl radicals during photoactivation to cause damage to the biomolecules adsorbed on TiO₂ nanoparticles. As such, surface area, a size related factor, could be a paradigm to predict the cytotoxicity and phototoxicity of TiO₂ nanoparticles. The surface coating of TiO₂ nanoparticles and addition of extracellular proteins could decrease such cytotoxicity and phototoxicity, which was also proven in this *in vitro* study.

Chapter 6 Recommendation for Future Work

Based on our observations and understanding about the size effects on the cytotoxicity of PLGA and TiO₂ particles, it will be meaningful to conduct further study to uncover the molecular mechanism behind their cytotoxicity. Due to the lack of understanding of chronic and *in vivo* effect, we propose to conduct genotoxicity and zebrafish studies to further substantiate the conclusion which has been raised in this thesis. These investigations will provide a more comprehensive understanding of the influence of size, surface area and some other factors such as surface functional group on nanotoxicity.

6.1 Develop an abiotic system to predict the toxicity of non-soluble nanoparticles

Although the biomolecular mechanism is still unclear, our current study has suggested that the interaction between particles and biomolecules should be related to these size and surface area dependent cytotoxicity. Thus, to further prove our hypothesis, 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 nanoparticles will help to understand the mechanism behind the size influence on nanotoxicity. The mechanism has great potential to be utilized to predict the toxicity of different particles in an abiotic condition and in a high throughput way.

The proposed method for this study includes three parts. First of all, we need to identify the key proteins attached on nanoparticles using techniques such as 2-D PAGE [133]. Second, after recognized the proteins which has potential to interact with the surface of nanoparticles, we will investigate the change of protein structure and the influence on the function of proteins after the interaction between protein and nanoparticles. Finally, after the understanding of the interaction between particles and proteins, an abiotic system will be developed to predict the toxicity of non-soluble nanoparticles.

6.2 Systematic study on the factors that may influence the genotoxicity of PLGA and TiO₂ nanoparticles

Although there are thousands of studies focused on the acute toxicity of nanoparticles including both *in vitro* and *in vivo* studies, the chronic toxicity of nanomaterials such as genotoxicity and carcinogenicity are not sufficiently discussed and understood. It was found in our previous study that the cytotoxicity of ZnO nanoparticles was related to their ability to activate p53 pathways in BJ cells and also cause the damage of double stranded DNA [134]. This finding suggest that nanomaterials may be able to initiate chronic toxicity such as carcinogenesis, and special concern should be paid to nanomaterials that are frequently used in the daily life of consumers such as components in sunscreens. Based on our current results, we found that PLGA nanoparticles were relatively safe and did not induce obvious genotoxicity in our *in vitro* studies. However, TiO₂ nanoparticles could promote genotoxicity on a skin fibroblast cell line (BJ cells) through DNA damage. But how the factors such as size and surface area could influence their cytotoxicity is still unknown. Thus a systematic study on the factors that may influence the cytotoxicity of

PLGA and TiO₂ nanoparticles will provide use with a more comprehensive understanding about the chronic toxicity of these nanomaterials.

6.3 *In vivo* toxicity of PLGA and TiO₂ using normal and transgenic zebrafish

We propose to use zebrafish to conduct our *in vivo* studies because of two reasons. First zebrafish is a new model discovered to be used in nanotoxicity studies in recent years, and also it can be used in the high throughput screening test [16, 64]. Second, zebrafish can be utilized to predict the influence of nanomaterials to environment.

By using zebrafish in our *in vivo* studies, we can answer lots of unknown questions. Whether PLGA and TiO₂ nanoparticles will cause delayed hatch, malformation or even death of zebrafish? Will their toxic effects be influence by different factors such as particle size? How will the property of TiO₂ nanoparticles influence their phototoxicity to zebrafish? And by using well established transgenic zebrafish we may find the mechanism behind these questions.

Chapter 7 References

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Appendix I: Publications

1. **Xiong, S.**; Tang, Y.; Ng, H.; Zhao, X.; Chen, Z.; Ng, K. and Loo, J., Specific surface area of TiO₂ particles influences cyto- and photo- toxicity. *Toxicology*, accepted.
2. **Xiong, S.**; George, S.; Yu, H.; Damoiseaux, R.; France, B.; Ng, K. and Loo, J., Size effect on the cytotoxicity of Poly-(D, L-lactide-co-glycolide) (PLGA) and titanium dioxide (TiO₂) nanoparticles. *Archives of Toxicology*, in press.
3. **Xiong, S.**; George, S.; Yu, H.; Ji, Z.; Lin, S.; Damoiseaux, R.; France, B.; Ng, K. and Loo, J., Size of TiO₂ nanoparticles influences their phototoxicity: an *in vitro* investigation. *Archives of Toxicology* 2012:1-11.
4. **Xiong, S.**; Zhao, X.; Heng, B.; Ng, K. and Loo, J., Cellular uptake of Poly-(D, L-lactide-co-glycolide) (PLGA) nanoparticles synthesized through solvent emulsion evaporation and nanoprecipitation method. *Biotechnology Journal* 2011, 6, (5), 501-508.
5. Kathawala, M.; **Xiong, S.**; Richards, M.; Ng, K.; George, S. and Loo, J., Emerging *in vitro* models for safety screening of high volume production nanomaterials under environmentally relevant exposure conditions. *Small*, in press.
6. Yu, H.; Lui, Y.; **Xiong, S.**; Leong, W.; Wen, F.; Nurkahfianto, H.; Rana, S.; Leong, D.; Ng, K. and Tan, L., Insights into the role of focal adhesion modulation in myogenic differentiation of human mesenchymal stem cells, *Stem Cells and Development*, in press.
7. Setyawati, M.; Khoo, S.; Eng, B.; **Xiong, S.**; Zhao, X.; Das, G.; Tan, T.; Loo, J.; Leong, D. and Ng, K., Cytotoxic and Genotoxic Characterization of Titanium Dioxide, Gadolinium Oxide and Poly(lactic-co-glycolic acid) Nanoparticles in Human Fibroblasts. *Journal of Biomedical Materials Research: Part A*, accepted.
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11. Ng, K.; Khoo, S.; Heng, B.; Setyawati, M.; Tan, E.; Zhao, X.; **Xiong, S.**; Fang, W.; Leong, D. and Loo, J., The role of the tumor suppressor p53 pathway in the cellular DNA damage response to zinc oxide nanoparticles. *Biomaterials* 2011, 32, 8218-8225.
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