Titanium dioxide and zinc oxide nanoparticles induce autophagy associated with toxicity effects in primary human keratinocytes : a dose relevance study

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NANYANG
TECHNOLOGICAL
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**TITANIUM DIOXIDE AND ZINC OXIDE NANOPARTICLES
INDUCE AUTOPHAGY ASSOCIATED WITH TOXICITY
EFFECTS IN PRIMARY HUMAN KERATINOCYTES: A DOSE
RELEVANCE STUDY**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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RELEVANCE STUDY**

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in partial fulfilment of the requirement for the degree of
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To my father.

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Abstract

The application of nanomaterials in cosmetic, sunscreens, and topical products is growing rapidly, even as health concerns arise about the potential hazards to their use. This research work is centered on the toxicological effects of titanium dioxide (TiO₂) and zinc oxide (ZnO) nanoparticles, which are commonly used in sunscreens, in the interaction with primary human keratinocytes. Both TiO₂ and ZnO nanoparticles are found to cause toxicity *in vitro*, such as cytotoxicity, oxidative stress, genotoxicity, and inflammasome activation in a concentration-dependent manner through conducting a dose relevance study. Furthermore, TiO₂ and ZnO nanoparticles are capable of inducing autophagy within a low subtoxic dose range. This is the first study of autophagy induced in primary human keratinocytes by nano TiO₂ and ZnO without other stimuli. The low triggering dose range of autophagy found in keratinocytes would be useful impetus for the research community to look more into the safety limits of nanomaterials applied to skin products.

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Abbreviations

TiO ₂	titanium dioxide
ZnO	zinc oxide
QDs	quantum dots
UV	ultraviolet
SCC	squamous cell carcinoma
SPF	sun protection factor
ROS	reactive oxygen species
RNS	reactive nitrogen species
DNA	deoxyribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
MEF	mouse embryonic fibroblast
BEAS-2B	human bronchial epithelial cell line
V79	Chinese hamster lung fibroblast
HaCaT	transformed aneuploid immortal keratinocyte cell line
RAW264.7	mouse leukaemic monocyte macrophage cell line
SK Mel-28	human melanoma cell line
HK-2	human kidney 2 cell line
p53	tumor protein 53
p62/SQSTEM-1	sequestosome 1
HPRT	hypoxanthine-guanine phosphoribosyl transferase
LDH	lactate dehydrogenase
BNIP	BCL2/adenovirus E1B 19 kDa protein-interacting protein
PRDX3	peroxiredoxin 3
PRNP	prion protein

TXNRD1	thioredoxin reductase 1
IL-1 β	interleukin 1, beta
CXCL9	chemokine (C-X-C motif) ligand 9
ATG	autophagy-related gene
LC3	microtubule-associated protein light chain 3
γ H2AX	phosphorylated histone H2AX
SEM	scanning electron microscopy
TEM	transmission electron microscopy
DLS	dynamic light scattering
HEKCM	EpiGRO TM Human Epidermal Keratinocyte Complete Media
ICP-MS	inductively coupled plasma-mass spectroscopy
ISDD	<i>in vitro</i> sedimentation, diffusion, and dosimetry
MDC	monodansylcadaverine
AUC	area under curve
TWAD	time-weighted average dose
NF- κ B	nuclear factor κ B
TNF	tumor necrosis factor
TRAF6	receptor associated factor
LIR	LC3-interacting region
Akt	protein kinase B (PKB), also known as Akt
ERK	extracellular signal-regulated kinases
JNK	c-Jun N-terminal kinase
p38	p38 mitogen-activated protein kinase
FCS	fluorescence correlation spectroscopy

Chapter 1

Introduction

1.1 Research Background

The conceptual foundations of nanotechnology were inspired by a talk given by Nobel laureate for Physics, Richard Feynman (1918-1988), at an American Physical Society meeting in 1959 [1]. The term “nano-technology” was first coined by Norio Taniguchi (1912-1999), in a 1974 conference [2]. Today, nanotechnology has been applied to a wide range of fields including industry, engineering, biotechnology, medical healthcare, and life sciences [3-5]. For “nanomaterials”, they are small particles with one of the physical dimensions less than 100 nanometer [6].

However, a growing debate regarding human health safety and risks has arisen associated with the rapid development of nanotechnological products and applications. The study of potential hazards to human health caused by nanomaterials is termed “nanotoxicology”. Nanotoxicology is an important subject to nanotechnology because it explores potential health risks induced by engineered nanoparticles, and its study helps to improve and guide the safe design and application of nanotechnology. Identification of nanomaterial-induced potential hazards to human body/tissue will help to advance the field of nanotechnology as a whole, by providing a more well-founded and well-informed framework to consider the benefits of particular nanotechnological products vis-à-vis the potential dangers. To this end, this study centered on the potential toxicity effects of

titanium dioxide (TiO₂) and zinc oxide (ZnO) nanoparticles induced in one type of skin cells, namely primary human keratinocytes.

In skin care products, micro-TiO₂ and ZnO have been used as ultraviolet (UV) filters [7]. Nanotechnology enables these particles to be reduced to nanoscales, with heralded improvements in their UV filtering, optical, and tactile characteristics. For example, the optical properties of TiO₂ and ZnO nanoparticles allow reflection to be reduced, and these particles have higher refractive indices compared with their traditional microscale counterparts [7]. Thus, new cream formulations using these nanoparticles are comparatively more transparent, more fluid, and also more comfortable for daily use. For instance, ZnO nanoparticles are more easily dissolved in oil-formulations so consumers would probably not be able to feel their existence. With the increase of engineered nanoparticles in skin applications such as sunscreens, there are attendant growing concerns about the potential adverse effects of such nano-sized TiO₂ and ZnO¹.

Firstly, the precise dose range of these nanoparticles used in the formulation of commercial sunscreens is unknown. There is a lack of information on the concentration and physico-chemical properties of nanoparticles used in cosmetic products as well as on market fractions of nanoparticle-containing products [8, 9]. Little information has been revealed to the public and the dose range of nanoparticles used in sunscreens may remain as trade secrets. Thus, it cannot be assumed that their dosages are necessarily safe. Secondly, there are concerns regarding the skin penetration of TiO₂ and ZnO nanoparticles due to their small size less than 100 nm, as well as potential toxicological effects if they manage to cross the skin barrier and interact with skin cells.

¹ It should be noted that the mere fact of a particular material's longstanding use is not a certain guarantee of its safety and non-toxicity. A classic example is asbestos – a seemingly innocuous fibrous silicate material, recorded to have been first mined 4000 years ago, then proliferated widely in industry from the 19th Century onwards. It was finally recognized for its dangers, and subsequently banned in the U.S. in the 21st Century. (Singapore banned it for certain uses in 1989.)

Human skin is the body's largest defense system that prevents external injuries and hazards. The stratum corneum, the outer layer of human epidermis, plays the main function of skin protection [10]. Physical properties of TiO₂ and ZnO nanoparticles make them highly mobile, which may lead to skin penetration of these small particles through small cuts or wounds, for example. Thus, certain health concerns underlie the motivations of this study to explore the potential toxicological outcomes from the interaction of TiO₂ and ZnO nanoparticles with one predominant cell type of epidermis, primary human keratinocytes in a wide dose regime and to determine what dosage levels would begin to cause toxicity.

1.2 Hypotheses and Objectives

In this context, the hypotheses of this study are to assess the validity of the following claims:

- TiO₂ and/or ZnO nanoparticles are capable of activating autophagy in primary human keratinocytes;
- TiO₂ and ZnO nanoparticles cause a number of toxicity effects in primary human keratinocytes;
- There are differences in the dose range of TiO₂- and ZnO-induced autophagy and toxicity effects in primary human keratinocytes.

The objectives of this study are:

- To determine whether TiO₂ and/or ZnO nanoparticles are autophagy inducers;
- To identify the potential toxicity effects caused by TiO₂ and ZnO nanoparticles in primary human keratinocytes;

-
- To profile TiO₂- and ZnO-induced autophagy (if any) and toxicity in a wide dose relevance study in primary human keratinocytes as well as to identify the effectively influential dosage range (if any).

1.3 Summary of Research Scope

This study achieves these through a detailed examination of TiO₂- and ZnO-induced autophagy and toxicity effects under different dose regimes in keratinocytes. It first characterizes physical properties of TiO₂ and ZnO nanoparticles. Computation of dosimetry simulation is employed to calculate the maximum administered particle mass delivered to cells in any dose range, which has been validated against empirical results over the parameter ranges of interest. Autophagy and cytotoxicity induced by TiO₂ and ZnO nanoparticles are compared in both administered and delivered dose across a wide range, respectively. A number of assessments of potential toxicity effects such as oxidative stress, genotoxicity, programmed cell death, and inflammasome activation are performed on cells under the exposure of TiO₂ and ZnO nanoparticles of different dose regimes, respectively. Bio-transmission electron microscopy is finally used to observe nanoparticle-uptake, autophagy, apoptosis, necrosis, and other ultrastructural changes in keratinocytes exposed to the different dosage ranges of TiO₂ and ZnO nanoparticles. What this research presents is a dose relevance study of exposure of TiO₂ and ZnO nanoparticles to primary human keratinocytes.

Chapter 2

Literature Review

2.1 Outline

This chapter reviews related work in the areas of skin application of TiO₂ and ZnO nanoparticles, nanoparticles physico-chemical features relevant to their toxicity, toxicity of nanoparticles in healthy and compromised skins, systemic toxicity studies of nanoparticles, and possible biological mechanisms of nanotoxicology, which served as motivations and comparisons for this study.

2.2 Nanoparticles in Products for Skin Application

2.2.1 Nanoparticles in Sunscreens: safe or unsafe?

There has been much literature that described the detrimental effects of UV exposure such as sunburn or other sun-related diseases [11]. One method of sunlight protection is the routine use of sunscreens, which has been mostly recognized and accepted by the public. Current evidence available indicates that sunscreens provide protective benefits to reduce the development of squamous cell carcinoma (SCC) [12].

The application of inorganic UV filters (i.e. TiO₂ and ZnO) is due to their stability and low allergic reaction under UV exposure [11]. But the use of TiO₂ and ZnO were compromised by their large particle sizes and poor dispersity in early-generation sunscreen formulations, which produced an opaque and white appearance on the skin accompanied by a grainy feeling.

Nanotechnology has helped to reduce these shortcomings of both UV filters by reducing the particle size into nano-range. Modern formulations of sunscreens contain nano-sized components, that is, 1-100 nm in diameter. TiO₂, remains to be one of the primary nanomaterials used in sunscreens and other skin care products, along with ZnO. Both are colorless nanoparticles and assessed to be more effective for UV filtering than microparticles (> 100 nm). Additionally, the combination of nanoparticle-based formulation has a synergistic effect of both UV scattering and UV absorption, which provides modern sunscreens with a high sun protection factor (SPF > 30) [13]. Recently, while TiO₂ and ZnO nanoparticles have been widely applied to cosmetic formulations in sunscreens, however, safety concerns regarding the risk of their use towards human health also have surfaced. The key question is whether they are intrinsically toxic to human health.

2.2.2 Physico-chemical features relevant to Nanoparticles Toxicity

Nanotechnology is able to design and produce smaller particles by controlling shape and size at the nano-scale [14]. Characteristics of nanoparticles such as their size, shape, surface modification, and manufacturing process have an impact on particles' toxicity outcome [15-17].

- **Size.** The change from micron to nano size has enhanced the particle's surface reactivity, because the smaller the particle, the more edge and corner atoms will be exposed [18]. Particles of that smaller size may potentially penetrate the body more readily through different routes of entry (e.g. dermal penetration). They may interact with living cells at the molecular level and initiate size-dependent biochemical reactions. Particle size could be an important factor in particle-related toxicity. It was suggested that the optimum size for nanoparticle-skin penetration into hair follicles was approximately between 320 and 750 nm [19].
- **Shape.** The second aspect linked to particle toxicity is the particle shape, although very few studies have focused on the shape of nanoparticles in connection with their toxicity, but

notwithstanding, particle shape is the other important influence of nanotoxicity. For example, toxicity seems to vary among different shapes of TiO₂ nanoparticles. Rutile is a stable form of TiO₂, which has been suggested to be less toxic than anatase form [20]. Similarly, as far as particle shape is concerned, rod-shape ZnO nanoparticles were suggested to be more toxic than spherical ones [21].

- Surface modification. Surface modification of nanoparticles has been engineered in various ways for different purposes. For example, the attachment of bioactive molecules, surface coating, and modification of surface charge allow nanoparticles to serve as specific tools that interact with targeted cells [22]. The surfaces of nanoparticles have an impact on their performance because the surface area controls the reactivity and catalytic activity of nanoparticles. The surfaces of nanoparticles would interact with biological systems, and therefore, surface characteristics influence nanoparticles toxicity [22]. Various reports suggest that toxicity effects are dependent on particles' surface modification. For example, photoactivation of TiO₂ applied in sunscreens showed a particle-mediated increase in oxidative radicals and mutagenic properties, whereas surface modification of TiO₂ nanoparticles was found to quench the generated radicals, which resulted in the reduction of photocatalysis, eliminating the risk of carcinogenicity [23].
- Aggregation². Nanomaterials show a tendency to aggregate in physiologically aqueous environment. This is a phenomenon that needs to be taken into account when studying the toxicological influence of nanoparticles, whether *in vitro* or *in vivo*. It has been observed that TiO₂ nanoparticles dispersed with aggregates smaller than 200 nm exhibited no genotoxicity

² According to International Standards Organization (ISO 14887), “aggregation” suggests the assemblage of particles rigidly joined together while “agglomeration” means the assemblage of particles which are loosely coherent and transient [24].

in human lymphoblast cells (Tk6), human embryonic epithelial cells (EUE) and monkey kidney fibroblasts (Cos-1) [25]. However, larger aggregates of the same TiO₂ nanoparticles induced double-stranded DNA breaks and/or DNA oxidation lesions in the same cell types [25]. Therefore, the characterization of particle aggregation state is important in the assessment of their toxicological influence. It is critical to develop new methodologies to accurately measure the state of nanoparticle-aggregation in the environment relevant to the intended application condition.

Collectively, a number of physico-chemical features are relevant to nanoparticle-induced toxicity. Characterization of at least some physical and chemical properties of nanoparticles is therefore important for exploring their possible applications in nanotechnology as well as evaluating their safety in sunscreen formulations.

2.3 Skin Toxicity of Nanoparticles

2.3.1 Nanoparticles and Healthy Skin

Human skin is the largest organ of human body which functions as an immunity guarder to protect the body against the external environment [26]. Basically, human skin is composed of three layers: 1) the epidermis, which is made up with keratinocytes, Merkel cells, melanocytes and Langerhans cells, contains no blood vessels but serves as a physical barrier against infection. It can be further subdivided into five sublayers or strata: corneum, lucidum, granulosum, spinosum, and basale; 2) the dermis, which is beneath the epidermis, contains connective tissue, hair follicles, various glands, lymphatic and blood vessels. It can be structurally divided into two areas: papillary region, which is a superficial area adjacent to the epidermis, and reticular region, which is a deep and thick

area; 3) the hypodermis, which is below the dermis and made up of fibroblasts, macrophages, and adipocytes, serves as padding and insulation for the body [26] (**Figure 2.1**).

Figure 2.1. A Schematic cross-section of Human Skin. (Reproduced from Wikimedia [27], <https://commons.wikimedia.org/wiki/File:Skin.png>)

Since human skin has a relatively low permeability, a key factor in evaluating toxicity of nano- TiO_2 and ZnO , is whether they can penetrate the skin. Both *in vitro* and *in vivo* models are currently used, which may yield comparable results, to provide the quantity of nanoparticles present in animal or human skin samples. For example, EU Scientific Committee on Cosmetics and Non-Food Products (SCCNFP) summarized findings in different published and unpublished studies using *in vitro* human or pig models and suggested that micro- or nano- TiO_2 (size: 14 nm to 200 μm) in anatase and rutile shapes in the presence or absence of coating can penetrate only to the stratum corneum [28], while the *in vitro* penetration of ZnO of various sizes also reached only the

stratum corneum [29]. Similarly, other reports concluded that the penetration of micro- or nano-sized TiO₂ and ZnO particles reached the stratum corneum [30-32]. On the contrary, Menzel *et al.* [33] suggested that TiO₂ in various formulations (needles: 45 to 150 nm × 17 to 35) penetrated into the stratum granulosum in pig skins but not into the stratum spinosum. A series of *in vivo* studies were also performed on the penetration of TiO₂ and ZnO nanoparticles. One study first reported that TiO₂ nanoparticles with particle size of 10-50 nm were able to penetrate the stratum corneum to the dermis if tested human volunteers repeated to apply them on the skin [34]. Other similar reports applied nano TiO₂ used in sunscreens to human volunteers and suggested the location of TiO₂ was in the stratum corneum, and opening of hair follicles after 6 to 48 h exposure [35-37]. Coated TiO₂ and ZnO nanoparticles, which dispersed in hydrophobic emulsions, were found to distribute in the stratum corneum in volunteers under exaggerated UV exposure [38]. In conclusion, all these conventional *in vitro* and *in vivo* dermal penetration studies yield ample evidence that skin penetration of TiO₂ and ZnO nanoparticles can reach to the hair follicle upper regions or the outer layers of the stratum corneum in healthy skin.

2.3.2 Nanoparticles and Compromised Skin

It is to be noted that all the above results were carried out on healthy skin without the consideration of individual skin conditions. The skin has a continuous upper layer of hardened dead cells, and this forms a physical barrier, shielding the live skin cells in other layers below. Healthy and intact skin barrier possibly restricts the amount of delivered nanoparticle dosage. However, in the absence of the intact stratum corneum barrier, there is a potential for certain nanoparticles to reach viable cells and elicit an immune response. It has been observed that the removal of stratum corneum by tape stripping resulted in the deep penetration of drug into normal skin or UVB-induced sunburn skin [39]. UV irradiation can mimic the sunlight damage to skin barrier, a model

that is often employed in nanoparticle-skin penetration study. TiO₂ and ZnO nanoparticles would be photoactivated by UV and further generate reactive oxygen species (ROS). Mortesten *et al.* [40] used 30 nm quantum dots (QDs) to mimic similar sized nano-TiO₂ for their penetration in UV-damaged skin. Their results suggested a size- and surface modification-dependent skin penetration manner of nano-QDs. Miquel-Jeanjean *et al.* [41] also showed comparable results by using damaged and sun-irradiated skin-nanoparticle experimental models. They calculated and found that 0.19 ± 0.15 wt. % of TiO₂ nanoparticles can reach viable skin cells after 24 h exposure on intact skin, whereas TiO₂ penetration doubled to 0.39 ± 0.39 wt. % when skin barrier was damaged by UV irradiation [41]. These studies suggest that the delivered nanoparticle dosage is determined by skin age, body sites, and the integrity of skin barrier. Once physical skin barrier is damaged, for example, by sunburn (i.e. UV irradiation), the amount of delivered nanoparticles would certainly increase.

In fact, there are possible scenarios that a person's skin barrier can be damaged. The following example scenario can be envisaged: a person applied a heavy coat of sunscreen prior to participating in outdoor activity on a sunny day, and during the course of this activity, he sustained some abrasions with some jagged edges on the ground, or some cuts by thorns or other foliage, and these contribute to damaging parts of his skin's natural physical protection barrier. If this scenario happens, it is more likely for these small nanoparticles to penetrate into damaged skin. As emphasized by Lorenz *et al.* [9], the skin penetration of nanoparticles in real-life situations through skin cracks, minor injuries and flexed skin remains to be investigated. Therefore, whereas the prior finding that nano-TiO₂ and ZnO cannot substantively penetrate intact skin may be valid, there remains a concern of the risk if the skin's physical protection function has been compromised. Besides this, some skin diseases such as inflammation or infection may also reduce the barrier

function of the skin. Thus, the particles penetration when in contact with live skin cells should be studied – this is one of the motivations for this research.

2.4 Systemic Toxicity Studies of Nanoparticles

2.4.1 *In vitro* Toxicity Studies in Mammalian Cells and *In vivo* Clinical Safety

A number of *in vitro* studies have alluded that TiO₂ nanoparticles impact genotoxicity in various cell types. For example, Xu *et al.* [42] showed an increase in genetic mutations in MEF cells in a dose-dependent manner during the interactions with 5 nm- and 40 nm-sized anatase TiO₂. Kang *et al.* [43] showed that TiO₂ nanoparticles increased the formation of micronuclei, DNA breakages, and p53 accumulation in peripheral blood lymphocytes. Bernardeschi *et al.* [44] showed that TiO₂ nanoparticles in both anatase and rutile phases can cause DNA fragmentation in human leukocytes. Gurr *et al.* [45] observed DNA oxidative damage induced by ultrafine TiO₂ nanoparticles in human bronchial epithelial (BEAS-2B) cell line when exposed to UVA radiation. Chen *et al.* [46] exposed Chinese hamster lung fibroblasts (V79 cells) to TiO₂ nanoparticles at the concentrations of 5, 10, 20, 50, and 100 µg/mL and observed DNA damage in 100 µg/mL TiO₂ treated groups after 24 h, while TiO₂ both 20 and 100 µg/mL were found to cause gene mutation after 2 h treatment by hypoxanthine-guanine phosphoribosyl transferase (HPRT) assay. Similarly, Demir *et al.* [47] found the lowest concentration for TiO₂ nanoparticles induced genotoxicity in human peripheral blood lymphocytes and embryonic kidney cells was 100 µg/mL. Mitochondrial DNA damage was also observed in human HaCaT keratinocytes after the treatment of TiO₂ 10 µg/mL for 72 h [48]. Besides genotoxicity, the exposure of keratinocytes to TiO₂ nanoparticles have also led to inflammation and oxidative stress [49-51].

ZnO nanoparticles, on the other hand, are capable of triggering acute cytotoxicity and genotoxicity. Most *in vitro* studies show a steep curve of concentration-dependent cell viability: a small concentration range of ZnO nanoparticles in which cell viability is dropped sharply from approximately 100% to 0 [21]. For example, one cytotoxicity fluorescent screening showed ZnO nanoparticles with a diameter of 20 nm decreased the potential of mitochondrial membrane as well as disturbed the membrane integrity of BEAS-2B and RAW264.7 cells [52]. 20 nm ZnO nanoparticles were found to induce a concentration- and time-dependent toxicity in the increase of LDH release, oxidative stress, and intracellular calcium in BEAS-2B cells [53]. Studies of dermal exposure of ZnO nanoparticles showed that ZnO nanoparticles did not (or slightly) penetrate healthy human skin, but they had toxic impacts on skin cells *in vitro* [21]. ZnO nanoparticles around 20 nm can cause apoptosis with an upregulation of p53 and phosphor-38 in human dermal fibroblasts [54]. Gene profiling of HaCaT human keratinocytes and SK Mel-28 human melanoma cells exposed to ZnO nanoparticles (8-10 nm) found several changes in metal metabolism, chaperonin proteins, and protein folding genes [55]. Expressions of apoptosis and oxidative stress related genes (BNIP, PRDX3, PRNP, and TXNRD1), IL-1 β , chemokine CXCL9, and p53 were found to be induced by ZnO nanoparticles in various cell lines [56]. These *in vitro* cell studies similarly reported oxidative stress, mitochondrial dysfunction, apoptosis, and inflammation induced by ZnO nanoparticles.

However, discrepancies between *in vitro* and *in vivo* toxicological studies are well-known. As for *in vivo* clinical safety studies, EU (EU SCCP) and United States (U.S. Food and Drug Administration, FDA) have evaluated the safety of nanoparticle-based sunscreens and regulated the application of their nano-ingredients [28, 29]. A series of pre-clinical *in vivo* toxicological examinations via the topical route were performed on sunscreens containing nano-sized TiO₂ and

ZnO in volunteers with healthy skins. These results suggested that these nanoparticles did not generate much toxicity effects after dermal application to healthy human skins [28, 29].

2.4.2 *In vitro* model of Keratinocyte-Nanoparticle in Skin Toxicity Study

The current cosmetic regulations in EU, for example, do not approve *in vivo* toxicological examination of cosmetic final products, which contain different formulations in bulk, particles, or formulations with nano-sized particles [57]. Complexities of *in vivo* systems with respect to biodistribution, immunology, and metabolism in human conditions are inadequately replicated *in vitro*. However, comprehensive *in vivo* clinical safety studies are difficult to control, interpret and ethically challenging to carry out. *In vitro* cell-based toxicity characterizations are therefore more practical for preliminary examination of nanoparticles potential toxicological effects. For instance, when a significant increase in skin absorption of nanoparticles is suspected in compromised or altered skin, *in vitro* cell studies are required for verification.

Keratinocytes constitute 90% of the cells in the epidermis, with their primary function to form the skin barrier against external environmental damage. Since keratinocytes form part of skin physical barrier, the interaction between keratinocytes and nanoparticles may predict a potential risk to intact human skin. This study is going to use keratinocyte-nanoparticle model to examine some potential toxicity effects caused by TiO₂ and ZnO nanoparticles *in vitro*. Primary human keratinocytes are known to have phagocytic capacity in cell culture conditions [58]. The toxicity of TiO₂ and ZnO nanoparticles to keratinocytes is correlated with their cellular uptake. *In vitro* keratinocyte-nanoparticle model could help to identify whether ROS or lysosomal enzymes would be released to degrade the uptaken nanoparticles or attempt to convert these nanoparticles to a “safer” form.

2.5 Biological Mechanisms of Nanoparticle-induced Toxicity

Once there is nanoparticle penetration across the skin to reach viable cells and tissues, the next concern will center on whether interactions between the nanoparticles and biological systems will bring about undesirable outcomes. Nanotoxicological consequences and thereof biological mechanisms are dependent on a multitude of factors, from nanoparticle properties and concentrations to time of exposure and cell types. Essentially, the potentially toxic influences exerted by TiO₂ and ZnO nanoparticles can be broadly divided into acute and chronic toxicity. Acute toxicity includes immediate cell responses such as various modes of cell death, cell cycle modifications, reduction in metabolic activity and induction of inflammatory response. Chronic toxicity generally refers to genotoxicity and carcinogenesis.

2.5.1 Primary Genotoxicity

Nanoparticle-induced primary genotoxicity is defined as the damage of genetic DNA without the presence of inflammation [59]. There are two forms of primary genotoxicity: direct and indirect ones. Direct primary genotoxicity is a mechanism whereby nanomaterials directly interact with genomic DNA and/or affect DNA integrity [59]. Indirect primary genotoxicity is induced by the free radicals (ROS/RNS) that generated from damaged mitochondria and membrane-bound NADPH oxidases corresponding to their interaction with nanoparticles [59]. TiO₂ nanoparticles have been reported to induce micronuclei formation [60], suggesting that nano-TiO₂ is capable of causing chromosomal breakages or disrupting mitotic spindle function during cell division.

The second toxicity mechanism is the generation of ROS by nanoparticles. The possible outcome of ROS production is genotoxicity. TiO₂-generated ROS has been linked with DNA damaging and mutagenic potential [61-64] as well as other genotoxic influences [59, 60, 65-68].

Similarly, ZnO nanoparticles have been reported to cause DNA damage via the activation of p53 [54]. In summary, ROS triggered by TiO₂ and ZnO nanoparticles can cause oxidative DNA damage, which is referred to as indirect primary genotoxicity. It has been postulated that indirect genotoxic effects are caused by mitochondria-derived ROS production since damaged mitochondria can be a potent ROS producer [69-71]. The releasing of superoxide anions from the mitochondrial respiratory chain would be subjected to spontaneous or enzyme-mediated dismutation in which

giving rise to relatively stable oxidant H₂O₂, which is then converted by the Fenton reaction,

(where M represents a transition metal such as titanium or zinc) into hydroxyl radicals upon its diffusion into nucleus [72].

2.5.2 Secondary Genotoxicity

Secondary genotoxicity is considered to be caused by inflammation. Nanoparticle-induced inflammation recruits inflammatory cells to particular site to secrete various inflammatory cytokines [59]. The consequence of inflammation would lead to several oxidants (O⁻, H₂O₂ and ROS/RNS) production, which may result in unintended genotoxicity [59]. Both TiO₂ and ZnO nanoparticles have been shown to exert inflammation pressure *in vitro* and *in vivo* [73-76]. TiO₂ nanoparticles, for instance, were found to induce inflammation potently through pulmonary exposure in rats [77]. Therefore, inflammasome activation with increased levels of oxidative stress contributes to secondary genotoxicity of nano-TiO₂ and ZnO.

2.5.3 Oxidative Stress

Oxidative stress is usually associated with ROS generation and decreasing levels of antioxidants. It is suggested to be a significant contributor for nanoparticle-induced genotoxicity. Regardless of whether there is penetration of nanoparticles into nucleus, oxidative stress could induce injury to genomic DNA. Oxidative damage to DNA can be caused by surface metal ions released from nanoparticles or other small molecular weight oxidants generated through bio-interactions with nanoparticles. For example, the released α/β -unsaturated aldehyde genotoxins [4-oxo-2-nonenal, 4,5-epoxy-2(E)-decenal or 4-hydroxy-2-nonenal] from the surfaces of nanoparticles can induce genotoxicity [78]. TiO₂ nanoparticles have been reported to cause oxidative damage through the generation of a potent oxidant, peroxynitrite (ONOO⁻) [78]. Oxidative injury will eventually lead to excessive DNA damage, which may possibly overwhelm the DNA repair system.

2.6 Autophagy: A New Emerging Mechanism of Nanotoxicity

2.6.1 Autophagy Basics

Christian de Duve (1917-2013), the Nobel laureate for Physiology and Medicine in 1974, first described that lysosomes were the intracellular granules containing enzymes glucose-6-phosphatase and acid phosphatase besides a set of hydrolases [79]. He named “autophagy” for a lysosome-mediated process that was able to digest, recycle, and remove intracellular materials such as damaged organelles or invaded pathogens [80]. Autophagy (a term from Latin words, *auto* meaning ‘self’ and *phagein* meaning ‘eating’) refers to a “self-eating” pathway in which cytoplasmic contents are degraded through a lysosomal-mediated process [81]. Autophagy can be further designated as macroautophagy, microautophagy, chaperone-mediated autophagy [82], and non-canonical autophagy [83].

Macroautophagy (henceforth referred to as autophagy) is the term of “autophagy” in its strictest form and it is the focus of this work. In the process of macroautophagy shown below (**Figure 2.2**), the principal morphological feature is the formation of double-membrane structures known as autophagosomes [84]. The formation of autophagosomes depends on autophagy-related (ATG) proteins to drive the formation of isolated crescent membranes, namely phagophores in the cytoplasm [84]. Phagophores increase their sizes to sequester the captured cytoplasmic cargo and close themselves into completed autophagosomes [84]. Subsequently, the outer membrane of autophagosomes fuses with lysosomes to mature into single membrane-delimited autolysosomes, leading to the degradation of autophagosomal cargo by lysosomal enzymes under the acidification of autophagosome lumen and the acquisition of lysosomal hydrolases [84, 85]. Autophagosomes can also be converted into degradative organelles by fusion with late endosomes, or multi-vesicular bodies and major histocompatibility complex (MHC)-class-II-loading compartments [86]. At the final phase, autolysosomes are tabulated and fragmented for cellular energy renewal [84].

The membrane dynamics of a double-membrane structure, the autophagosome, to fuse with lysosomes is stimulated by ATG genes and also involve the participation of a proteolipid molecule, LC3-II (microtubule-associated protein light chain 3) [87]. LC3-II is originated from its parent molecule, LC3-I. LC3-I is mainly generated by the action of ATG4, which cleaves LC3 to produce LC3-I [87]. During the formation of autophagosomes, cytosolic LC3-I is converted to its lipidated form, LC3-II (a component of LC3 and phosphatidylethanolamine), which is present on the membrane of autophagosomes [87].

Figure 2.2. Overview of macroautophagy. A phagophore, an isolated membrane, appears in the cytoplasm with its elongation to form the completed double-membrane autophagosome. Autophagosomes then fuse with lysosomes to mature into autolysosomes where autophagosomal cargo is degraded by lysosomal hydrolases. Term definition: 1) the phagophore is an isolated double membrane that encloses the cytoplasm in the initial stage of macroautophagy; 2) the autophagosome is formed by the elongation and completion of the phagophore. It is a double membrane-bound intracellular vacuole which contains elements of cellular cargo; 3) the autolysosome is formed by the fusion of the autophagosome and the lysosome, which contains partially digested cellular components (figure was adapted with permission from Meléndez, A. and Levine, B. Autophagy in *C. elegans*, August 24, 2009, *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.147.1, available at <http://www.wormbook.org> [88]).

2.6.2 Autophagy induced by Nanoparticles

Recently, a growing body of literature reported nanoparticle-induced autophagy [89]. The induction of autophagy by TiO₂ nanoparticles in primary human keratinocytes was reported recently [90]. It was the first report of nanoscale TiO₂ triggering autophagy in keratinocytes without other stimuli [90]. Besides TiO₂ nanoparticles, other nanoparticles have been described to induce autophagy in various types of cells [91-96].

The first series of nanoparticle-induced autophagy studies suggest autophagy as a new emerging mechanism of nanotoxicity. It is not surprising that autophagy pathway is being factored into biological mechanisms of nanotoxicity because the process of self-eating seems to be beneficial, which represents a cell's immune response to nanoparticles. To date, autophagy remains the only known mechanism that eukaryotic cells possess to dispose of intracellular invaded macromolecules and aggregates that are too large to be degraded or eliminated by the proteasome [97]. It is therefore reasonable to consider that cells can also use this evolutionarily conserved lysosomal pathway to sequester nanoparticles and degrade some organelles damaged by nanoparticles. Indeed, autophagy might be expected as an adaptation process that allows cells to adapt to the presence of nanoparticles as well as to survive the stress caused by nanoparticles. Autophagy degraded damaged cytoplasmic materials or faulty organelles for the purpose of homeostasis re-balance [90].

Currently available information of molecular mechanisms of nanoparticle-induced autophagy is limited, however, it is suggested that nanoparticle-induced autophagy is a self-defense mechanism that interfaces with most cellular stress-response pathways functioning in immunity and inflammation, since nanoparticles can be perceived by a cell as invaders, similar to micro-organisms such as viruses, bacteria, and protozoa. For instance, it has been known that cells can use autophagy pathway to target intracellular bacteria to autophagosomes through interactions between ubiquitin, p62/SQSTM-1 protein, and LC3 [98]. The adaptor protein p62 enables autophagy to designate cargo selectively to nascent LC3-positive isolation membranes [99]. Recent nanotoxic reports suggest that ubiquitination of nanoparticles directly and/or indirectly co-localize with poly-ubiquitin protein aggregates, indicating a mechanism involving ubiquitin and

p62 seems to be included in the targeting of nanoparticles to autophagosomes through a selective pathway similar to micro-organisms [100-102].

Alternatively, nanoparticle-induced autophagy might also be upregulated for the removal of damaged organelles when nanoparticles disfavor some organelles in the cell, thereby regaining metabolic energy for survival. It is postulated that this process could be referred to as selective autophagy. Damaged mitochondria, peroxisomes, and DNA have been observed to be removed by autophagy [103]. For example, one way in which damaged organelles are removed by autophagy involves a model of ubiquitinated cargo binding to LC3-interacting proteins, which subsequently bind to LC3, and thereby targeting the cargo for autophagy [103]. This study aims to examine whether TiO₂ and ZnO nanoparticles would induce selective autophagy in primary human keratinocytes. If they do, it is interesting to find out which organelles would be targeted for autophagic removal.

2.7 Summary

The unique properties of TiO₂ and ZnO nanoparticles have brought about beneficial applications to improve sunscreen formulations. Sunscreen manufacturers advertise that mineral sunscreens containing nano-TiO₂ and ZnO are comparatively safer because the new formulations utilize fewer other organic chemicals such as oxybenzone and octinoxate, which seep through the skin into the body where they function similarly as the hormone estrogen. However, the precise dosage range of nanoparticles used in the formulation as well as the exact ingredients in sunscreens have not been revealed to the public for safety testing and to check how nanoparticles administration and delivery might fare in the presence of other components in sunscreens. Therefore, safety concerns can be said to still exist over the safety of nano-TiO₂ and ZnO in sunscreens. As a result, nanotoxicity is still a health concern and many uncertain questions in this field warrant scientific

attention. On the other hand, human have evolutionarily developed unique ways such as autophagy to deal with the removal of large bio-molecules. At the same time, autophagy may also be activated to remove organelles damaged by exposure to engineered nanoparticles.

Chapter 3

Materials and Methods

3.1 Characterization of Nanoparticles

TiO₂ and ZnO nanoparticles were purchased from Meliorium Technologies Inc. (Rochester, NY, USA) and characterized by transmission electron microscope (TEM, Jeol 2010). Samples of both nanoparticles were dispersed in methanol and ultrasonicated for 30 min before proceeding with characterization. Dispersed samples were dropped onto carbon coated copper grids and viewed at an accelerating voltage of 200 keV with a LaB₆ cathode. Images were captured with between 50 – 60 particles per image to provide a sufficient number of nanoparticles that can be measured for mean particle size. ImageJ [104] software was used for the measurement of mean size of pristine nanoparticles (n=50) after TEM viewing. Hydrodynamic measurements of size distribution and zeta potential of both nanoparticles were examined by Dynamic Light Scattering (DLS) using a Zetasizer (model ZEN 3600, Malvern Instruments) with a He-Ne laser at a wavelength of 633 nm. Working solutions of both nanoparticles were dispersed in ultrapure water (Milli-Q, pH 6.0) and EpiGROTM Human Epidermal Keratinocyte Complete Media (HEKCM, pH 7.5, Millipore, USA) with proprietary supplements (Millipore, USA), respectively, followed with a 10 min bath ultrasonication (n=3). Before measurement, samples were homogenized in DLS tubes properly. The solubility of ZnO nanoparticles was measured by inductively coupled plasma-mass spectroscopy at the concentration of 50 µg/mL in HEKCM (ICP-MS, 7500 Series, Agilent Technologies).

3.2 Dosimetry

The method reported by DeLoid *et al.* [105] was used to measure effective density by volumetric centrifugation. One milliliter of 1.0 mg/mL suspensions of TiO₂ and ZnO nanoparticles were dispersed into TPP PVC tubes (Techno Plastic Products, Trasadingen, Switzerland). Tubes were put into a benchtop centrifuge at 3,000 g for 2 h to collect aggregate pellet. Aggregate pellet volumes (V_{pellet}) were measured by a slide rule-like easy-measure device provided by TPP PVC tube manufacturer. *In vitro* sedimentation, diffusion, and dosimetry (ISDD) model kindly provided by Dr. Justin Teeguarden (Pacific National Laboratory, Oregon State University Joint Faculty) was used to calculate the mass of administered TiO₂ and ZnO nanoparticles deposited in standard 96-well plates, 6-well plates, and T75 culture flasks as a function of time $M_D(t)$ [106, 107]. Briefly, effective density estimated by volumetric centrifugation, hydrodynamic diameter (d_H) measured by DLS, cell culture media volume, height, and temperature (310K), cell culture media density (1.00 g/cm³), and media dynamic viscosity (0.00074 PaS) were input in ISDD model. Results were computed by Matlab R2009a [105, 106].

3.3 Cell Culture

Human primary epidermal keratinocytes (Normal, Human Adult) were purchased from ATCC[®] (PCS-200-011) containing a minimum of 5×10^5 viable cells. Cells were cultured and maintained in EpiGRO[™] HEKCM (Millipore, USA). Cells were seeded as recommended by the manufacturer (the initial seeding density between 2,500 and 5,000 cells per cm²) and incubated in a humidified condition at 37 °C and 5% CO₂. Cells were cultured and maintained for 7 days to reach 80% confluence before passaging. Experiments were conducted on cells at passage ≤ 4 . Such a use of

young keratinocytes was to ensure that no terminal differentiation, apoptotic, necrotic and autophagic markers appeared with senescence [108].

3.4 Cell Exposure to Nanoparticles

Both TiO₂ and ZnO nanoparticles were suspended in sterile 1 × PBS (PAA Laboratories Inc.) and sterilized under UV light for 15 min followed by bath sonication for 10 min. Then, both of the stock solution were diluted in EpiGRO™ HEKCM (Millipore, USA) to obtain different concentrations. Different concentrations of nanoparticle-working dispersions were further bath sonicated for 10 min before cell treatment.

3.5 Cell Viability Assay

Cells were seeded in a 96-well plate at the density of 5×10^4 cells/well in 100 µL of culture medium. After overnight growth, cells were treated with TiO₂ or ZnO nanoparticles of different concentrations, or 4 µM tamoxifen (Cayman, USA) for 24 h in a humidified condition at 37 °C and 5% CO₂. Untreated cells were used as negative controls. 10 µL of the reconstituted WST-8 mixture (Dojindo Molecular Laboratories Inc., Japan) was added into each well and vortexed for a few minutes before incubation at 37 °C and 5% CO₂ for 2 h. Absorbance of each well was measured at 450 nm by a microplate reader (Infinite 200, Tecan Inc., Maennedorf, Switzerland).

3.6 Monodansylcadaverine (MDC) staining

Cells were treated with TiO₂ or ZnO nanoparticles of different concentrations, or 4 µM tamoxifen (Cayman, USA) for 24 h in a humidified condition at 37 °C and 5% CO₂. Untreated cells were used as negative controls. To label autophagic vacuoles, cells were stained with 1: 1000 monodansylcadaverine (MDC, Cayman, USA) in Cell-Based Assay Buffer (Cayman, USA) for 10

min at 37 °C. After incubation, cells were washed twice in Cell-Based Assay Buffer and analyzed immediately. Visualization was done under a confocal laser microscope (Leica AOBS SP2) using excitation and emission wavelengths of 405 and 525 nm, respectively. Quantitative analysis of MDC fluorescence was detected by a microplate reader (Infinite 200, Tecan Inc.) using excitation and emission wavelengths of 335 and 512 nm, respectively, according to the manufacturer's protocol.

3.7 Immunofluorescence

Cells were washed three times in $1 \times$ PBS and fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton-X 100. Upon fixation, cells were blocked with 1% BSA for 1 h at room temperature followed by incubation with 3 μ g/mL primary mouse monoclonal anti-LC3 IgG, (Nanotool, Germany) and primary rabbit polyclonal anti-phosphohistone H2AX (γ -H2AX) Ser 139 IgG (1:400 dilution, Cell Signaling Technology, USA) at 4 °C overnight. After incubation, cells were washed five times with $1 \times$ PBS and probed with an Alexa Fluor[®] 488 conjugated secondary anti-mouse IgG (Molecular Probes, USA) and Cy3[®] goat anti-rabbit IgG (Molecular Probes, USA) for 1 h in the dark at room temperature. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Thermo Scientific) at 1 μ g/mL for 10 min at room temperature. Images were captured using a fluorescence microscope (Nikon Eclipse 80i, 40 $\times$ objective, Japan). Images were captured with between 100-150 cells per image to provide data for generating positive staining cells on the sample, as well as close-up images of positive LC3- (green fluorescent signal) and γ -H2AX- (red fluorescent signal) labeled structures. Cell numbers were quantified by automated cell counting software ImageJ. The proportions of cells with autophagosomes (positive LC3 staining) and DNA damage (positive γ -H2AX staining) were shown as the percentage of total

cell population in particular images. Cells were randomly chosen and used to calculate the mean proportion of cells with autophagosomes and DNA damage, normalized to untreated negative controls.

3.8 ROS Detection

After 24 h treatment, cells were stained with 5 μ M CellROX[®] Reagent (Molecular Probes, Inc.) and incubated at 37 °C for 30 min. Then, cells were washed three times with 1 $\times$ PBS and fixed with 3.7% paraformaldehyde for 15 min at room temperature. Nuclei were counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Thermo Scientific) at 1 μ g/mL for 10 min at room temperature. Images were captured using a fluorescence microscope (Nikon Eclipse 80i, 40 $\times$ objective, Japan). Images were captured at around 50 cells per image to provide data for generating positive staining cells on the sample, as well as close-up images of positive ROS (green fluorescent signal) stained structures. Cell numbers were quantified by automated cell counting software ImageJ. The proportion of cells with positive ROS staining was shown as the percentage of total cell population in a particular image. Cells were randomly chosen and used to calculate the mean proportion of ROS positive cells, normalized to untreated negative controls.

3.9 LC3 Immunoblot

Cells were seeded in T-75 culture flasks and treated with TiO₂ nanoparticles (50 fg/mL and 500 μ g/mL), ZnO nanoparticles (50 pg/mL and 50 μ g/mL), and tamoxifen 4 μ M w/o lysosome inhibitors (NH₄Cl 10 mM/Leupeptin 50 μ g/mL) for 24 h. After treatment, cells were washed three times with D-PBS and lysed with 50 μ L lysis buffer (31.25 mM Tris-HCl, pH 6.8, 12.5% glycerol, 0.05% β -mercaptoethanol, 1% SDS, 0.005% bromophenol blue, Sigma-Aldrich) with protease inhibitors and phosphatase inhibitor cocktail (Sigma-Aldrich). Cell lysates were sonicated briefly

and harvested on ice for 2 h. All lysates were then centrifuged at 14,000 g for 10 min at 4 °C to collect supernatants. Protein concentrations were determined by 660 nm protein assay kit with the proprietary Ionic Detergent Compatibility Reagent (IDCR) (Thermo Scientific, USA). For western blotting, samples of 30 µg total protein were heated at 95 °C for 10 min. All samples were then separated by 10-20% Tris-Glycine gels (Novex®, Life Technologies) at 125 V for 120 min, followed by membrane electrotransferring with iBlot® dry blotting system onto PVDF membranes (Life Technologies). Membranes were first blocked with 50 ml 3% (w/v) BSA in PBS-T (10 mM phosphate, 150 mM NaCl, 0.03% (v/v) Tween-20) for 1 h at room temperature with rocking. After the blocking of unspecific sites, membranes were incubated with primary antibodies at 4 °C overnight with gentle agitation. The second day, membranes were washed three times with 50 ml of PBS-T, and incubated with HRP-conjugated secondary antibodies (1: 4000 – 1: 8000 dilution) for 1 h at room temperature with rocking. The visualization of protein bands was done by SuperSignal West Femto Chemiluminescent detection kit (Thermo Scientific) using gel documentation system (LAS-4000, GE Healthcare Ltd., UK). Primary antibodies were rabbit anti-LC3 (NB100-2220, Novus Biologicals; #2275 Cell Signaling, USA), and mouse anti-β-actin (AC-15, Sigma-Aldrich). Secondary antibodies were HRP-conjugated goat anti-rabbit IgG antibody (P0448, Dako, Denmark) and HRP-conjugated goat anti-mouse IgG antibody (P0447, Dako, Denmark).

3.10 Immunoblots of p62, NF-κB, and TRAF6

Cells were seeded in T-75 culture flasks and treated with different concentrations of TiO₂, ZnO nanoparticles, and tamoxifen 4 µM for 24 h. After treatment, cells were washed three times with D-PBS and lysed with 100 µL lysis buffer (31.25 mM Tris-HCl, pH 6.8, 12.5% glycerol, 0.05%

β -mercaptoethanol, 1% SDS, 0.005% bromophenol blue, Sigma-Aldrich) with protease inhibitors and phosphatase inhibitor cocktail (Sigma-Aldrich). Cell lysates were sonicated briefly and harvested on ice for 1 h. All lysates were then centrifuged at 14,000 g for 10 min at 4 °C to collect supernatants. Protein concentrations were determined by 660 nm protein assay kit with the proprietary Ionic Detergent Compatibility Reagent (IDCR) (Thermo Scientific, USA). For western blotting, samples of 10 μ g total protein were heated at 70 °C for 10 min. All samples were then separated by 4-12% SDS-PAGE gels at 125 V for 90 min, followed by membrane electrotransferring onto nitrocellulose membranes. Membranes were first blocked with 50 ml 3% (w/v) BSA in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.03% (v/v) Tween-20) for 1 h at room temperature with rocking. After the blocking of unspecific sites, membranes were incubated with primary antibodies at 4 °C overnight with gentle agitation. The second day, membranes were washed three times with 50 ml of TBS-T, and incubated with HRP-conjugated secondary antibodies (1: 4000 – 1: 8000 dilution) for 1 h at room temperature with rocking before viewing. Primary antibodies were rabbit anti-p62 (NB100-2220, Enzo Life Sciences, USA), NF- κ B (Santa Cruz SC-372X), TRAF6 (Santa Cruz SC7221) and mouse anti- β -actin (AC-15, Sigma-Aldrich). Secondary antibodies were HRP-conjugated goat anti-rabbit IgG antibody (P0448, Dako, Denmark) and HRP-conjugated goat anti-mouse IgG antibody (P0447, Dako, Denmark).

3.11 Flow Cytometry

Primary human keratinocytes were seeded in 6-well plates at the density of 1×10^5 cells and grown for 24 h. Then, keratinocytes were treated with different concentrations of TiO₂ or ZnO nanoparticles for 24 h. After treatment, cells were washed three times with $1 \times$ PBS then collected by 0.05% trypsin-EDTA. Collected cells were further gently washed with $1 \times$ PBS followed by

centrifugation (400 g, 5 min at 4 °C). Cells were stained by Dead Cell Apoptosis Kit with Annexin V/Propidium Iodide (PI) according to the manufacturer's protocol (Life Technologies). Briefly, each 100 μ L of cell suspension was incubated with 5 μ L Alexa Fluor[®] 488 Annexin V and 1 μ L (100 μ g/mL) PI working solution for 15 min at room temperature. After incubation, 400 μ L 1 $\times$ Annexin-binding buffer was added into each sample. Flow cytometry analysis was performed immediately on BD FACSCalibur Flow Cytometer, measuring the fluorescence emission at 530 nm and 575 nm using 488 nm excitation.

3.12 Transmission Electron Microscopy

After 24 h treatment, all cells were washed three times with 1 $\times$ PBS, then fixed with 2.5% glutaraldehyde (SPI, USA) in 1 $\times$ PBS at 4 °C overnight. After pre-fixation, cells were washed three times with 1 $\times$ PBS and post-fixed with 1% osmium tetroxide (OsO₄, SPI, USA) for 1 h at room temperature. Fixed cell pellets were further washed with 1 $\times$ PBS before dehydration through an increasing ethanol gradient (25%, 50%, 75%, 95%, 100%) and pure acetone, for 20 min at room temperature at each step. Infiltration in SPI-Pon[™]-Araldite[®] (SPI, USA) resin started at room temperature overnight, followed with pure resin embedding at 60 °C for 72 h. After polymerization, blocks were sectioned by an ultramicrotome (Leica Ultracut UCT) and ultrathin sections were collected on 200 mesh copper grids before stained with uranyl acetate and lead citrate. Samples were viewed under a Philips EM 208 transmission electron microscope at an accelerating voltage of 100 keV and an Advanced Microscopy Techniques (AMT) bottom mount CCD camera. Magnifications were provided from 5000x to as high as 40,000x. TEM images with micron bars were collected by AMT digital software associated with TEM. Images were automatically saved as high-resolution TIFF files. Since cells remained intact under electron beam irradiation,

microscale structures were specified as: 1) Data on morphology of each sample. Lower magnification images (5000 – 8000x magnification) were captured to show a general morphology of normal keratinocytes, keratinocytes with nanoparticle uptake, keratinocytes with autophagy, apoptosis, and necrosis; 2) Sample investigation. Images were captured at a range of higher magnifications (10,000 – 40,000x magnification) to characterize typical cellular vacuoles containing nanoparticles, autophagic vacuoles, apoptotic and necrotic structures.

3.13 Statistical Analysis

Results were presented as mean $\pm$ standard deviation (SD) of independent experiments. An unpaired Student's t-test was used to compare means of two samples while one way analysis of variance (ANOVA) was used for group means testing. In all cases, $p < 0.05$ was considered as a statistically significant difference.

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Chapter 4

TiO₂ Nanoparticles induce Autophagy in Primary Human Keratinocytes

4.1 Outline

Though not immediately obvious to the naked eye, mankind is regularly exposed to particles in everyday life. From combustion-derived particles to nanotechnology-based cosmetic powders, particles are all around us. As a result, toxicity studies of exposure to particles in a variety of situations have been documented for decades, if not longer. The employment of TiO₂ nanoparticles in sunscreens for UV absorption can be traced back to 1990 due to their superior attenuation of UVB irradiation [109]. However, subsequent concerns over the potential adverse effects of TiO₂-based sunscreen formulations are also increasing. In 2010, the International Agency for Research on Cancer (IARC) has classified nanosized TiO₂ as a “Possible Human Carcinogen” (Class 2B) [110].

In this chapter, TiO₂ nanoparticles were tested for the capability of inducing autophagy, beginning with the concentration of 10 µg/mL. Considerations regarding this choice of concentration were: the concentration should not be too low and too far away to cause toxicity; the concentration should not be too high, otherwise there would be an overwhelming cell toxicity; the concentration should cover a range such that toxic effects slowly begin to kick in and can be observed. The motivation of choosing TiO₂ 10 µg/mL was because the reported literature

suggested that TiO₂ concentrations from 10 µg/mL and beyond started inducing toxic effects in a few types of cells, including one report from the same research group showing that TiO₂ 10 µg/mL induced toxicity in BEAS-2B and RAW264.7 cells [45, 48, 49-52]. Therefore, it was surmised whether this concentration of 10 µg/mL could cause toxic effects on keratinocytes as well. Since keratinocytes form part of the skin physical barrier, the interaction between keratinocytes and TiO₂ 10 µg/mL, a moderate concentration level, may help to design the subsequent research steps as well as to predict whether it was worthwhile to study the potential risk of TiO₂ nanoparticles on human skin cells. The correlation of TiO₂-induced cytotoxicity and autophagy suggested that the induction of autophagy was not accompanied by an excessive cytotoxicity in cells exposed to TiO₂ nanoparticles.

4.2 Results

4.2.1 Characterization of TiO₂ Nanoparticles

The commercially purchased TiO₂ nanoparticles were first characterized by TEM. TEM images showed a mix of spherical and polygonal shapes of nanostructured TiO₂ (**Figure 4.1A**), with mean particle diameter at 22.07 ± 2.56 nm calculated by ImageJ software (particle number n = 50). Hydrodynamic parameters of bath ultrasonicated TiO₂ 10 µg/mL in both ultrapure water (Milli-Q, pH 6.0) and EpiGROTM serum-free HEKCM (Millipore, pH 7.5) were measured by DLS (**Figure 4.1B**).

Mean particle size = 22.07 ± 2.56 nm

B) Hydrodynamic Characterization of TiO₂ nanoparticles by DLS

Medium	Z-average size (nm)	PDI	Peak 1 Mean Intensity* (nm)	Peak 1 Area Intensity** (%)	Zeta Potential (mV)	pH
Ultrapure water	297.5 ± 23.0	0.302	243.3 ± 12.1	100	31.3 ± 0.9	6.0
EpiGRO™ HEKCM	674.8 ± 141.9	0.633	461.7 ± 13.0	92.5 ± 0.9	-12.6 ± 1.1	7.5

* Peak 1 Mean Intensity: mean particle size based on the most prominent signal peak alone

** Peak 1 Area Intensity: proportion of particles within the most prominent signal peak

Figure 4.1. Physical Characterization of TiO₂ nanoparticles. (A) TEM photomicrograph showed a mix of spherical and polygonal shapes of TiO₂ nanoparticles. (B) Hydrodynamic measurements of 10 µg/mL TiO₂ nanoparticles in ultrapure water and EpiGRO™ serum-free HEKCM. Data represent mean ± SD (n=6). (Reproduced with permission from Zhao Y., Howe J.L., Yu Z., Leong D.T., Chu J.J., Loo J.S., Ng K.W. 2013. Exposure to titanium dioxide nanoparticles induces autophagy in primary human keratinocytes. *Small*, 9, 387-392 [90])

DLS results suggested that there was the aggregation of TiO₂ nanoparticles which were not thoroughly dispersed in ultrapure water by bath ultrasonication. TiO₂ aggregation in serum-free HEKCM media was also measured by DLS. The higher degree of TiO₂ aggregation in HEKCM compared to that in ultrapure water was contrary to reports of a good dispersion of TiO₂ nanoparticles in serum-supplemented culture media [111], but consistent with the observation of poor nanoparticle-dispersion in serum-free media [112, 113]. The reason of poor nanoparticle dispersion in serum-free media is due to the absence of serum, which would otherwise function as

a coating protein, to decrease protein corona levels that act as surfactants to reduce nanoparticle-aggregation. However, TiO₂ aggregates were the stress condition that human keratinocytes were actually exposed to. As such, serum-free HEKCM was chosen for this study because it was the preferred cell culture media to sustain undifferentiated proliferation of keratinocytes [114]. The zeta potential of TiO₂ nanoparticles in ultrapure water (pH 6.0) was $+31.3 \pm 0.9$ mV while this figure changed to -12.6 ± 1.1 mV when TiO₂ nanoparticles were dispersed in HEKCM (pH 7.5).

4.2.2 Cytotoxic Effects of TiO₂ Nanoparticles on Cell Viability

After 24 h exposure to TiO₂ nanoparticles, relative cell viability was evaluated by the WST-8 assay. Cells treated with 10 µg/mL of TiO₂ nanoparticles showed a 15% reduction in cell viability ($p < 0.05$) in comparison with the negative controls. Cells treated with 4 µM tamoxifen³ did not show the statistically significant decrease in cell viability in comparison with the negative controls (**Figure 4.2**). The results suggested a relatively low toxic effect induced by TiO₂ nanoparticles at the concentration of 10 µg/mL in keratinocytes.

Figure 4.2. Relative cell viability by WST-8 assay. Keratinocytes were treated with 10 µg/mL TiO₂ nanoparticles and 4 µM tamoxifen for 24 h before analysis. Untreated cells were used as negative controls.

³ Tamoxifen has been well-recognized as a classical autophagy inducer [115]. It was used as the positive control for autophagy induction throughout this study. Autophagy induced by nanoparticles was compared with tamoxifen-triggered autophagy for confirmation. The purpose of performing toxicity analyses on tamoxifen treated keratinocytes was to examine whether autophagy response would damage cells.

WST-8 assay was performed on all cells. Data represent mean $\pm$ SD (n=3). * $p < 0.05$. (Reproduced with permission from Zhao Y., Howe J.L., Yu Z., Leong D.T., Chu J.J., Loo J.S., Ng K.W. 2013. Exposure to titanium dioxide nanoparticles induces autophagy in primary human keratinocytes. *Small*, 9, 387-392 [90])

4.2.3 Accumulation and Localization of Autophagic Vacuoles

Monodansylcadaverine (MDC)⁴ staining was used to examine whether TiO₂ 10 μ g/mL was able to induce autophagy in keratinocytes. Staining results showed MDC-positive structures accumulated in both cell groups treated with 10 μ g/mL TiO₂ nanoparticles and 4 μ M tamoxifen for 24 h, compared to negative controls (**Figure 4.3A**). The localization of these autophagic vacuoles was in the cytosol and perinuclear regions. Quantification of MDC staining confirmed that there were statistically significant increases in the number of autophagic vacuoles in TiO₂ 10 μ g/mL ($p < 0.01$) and tamoxifen treated groups in comparison with negative controls (**Figure 4.3B**). TiO₂ induced autophagy was 20% lower than tamoxifen induced autophagy ($p < 0.05$).

⁴ MDC is a specific marker of autophagic vacuoles due to its ability to incorporate into multilamellar bodies via an ion trapping mechanism and the interaction with membrane lipids [116].

Figure 4.3. Detection of autophagy induction by MDC staining. (A) Confocal microscopy images of MDC staining. MDC-positive structures were shown in keratinocytes treated with 4 μ M tamoxifen and 10 μ g/mL TiO₂ nanoparticles for 24 h. Negative controls were untreated cells. Images are representative of three independent experiments. Scale bar: 10 μ m; DIC: differential interference contrast. (B) Quantification of MDC staining. Data represent mean $\pm$ SD (n=3). * p < 0.05, † p < 0.01. (Reproduced with

permission from Zhao Y., Howe J.L., Yu Z., Leong D.T., Chu J.J., Loo J.S., Ng K.W. 2013. Exposure to titanium dioxide nanoparticles induces autophagy in primary human keratinocytes. *Small*, 9, 387-392 [90])

Autophagy induction was detected by LC3 immunofluorescence concomitantly. LC3 is known to be a specific marker for the identification of autophagosomes. During the formation of autophagosomes, cytosolic LC3-I is converted to its lipidated form, LC3-II (a component of LC3 and phosphatidylethanolamine), which is bound to the membrane of autophagosomes [87]. The accumulation of LC3-positive cytoplasmic vacuoles was observed in TiO₂ 10 µg/mL and tamoxifen treated cells, compared to negative controls (**Figure 4.4**). The increased LC3 punctate distribution in TiO₂ treated cells was consistent with MDC staining results, which suggested that autophagy was induced by TiO₂ nanoparticles at the concentration of 10 µg/mL in keratinocytes. Quantitative comparison of LC3 staining normalized to negative controls resulted in 2.57 and 2.08 times the number of cells which carried positive LC3 marker in cells exposed to tamoxifen and TiO₂ nanoparticles, respectively ($p < 0.05$).

Figure 4.4. Detection of autophagy induction by LC3 staining. (A) Epifluorescent microscopy images of LC3 immuno-labeled cells. Increased expression of LC3 protein was shown in 4 μ M tamoxifen and 10 μ g/mL TiO₂ treated groups. Images are representative of three independent experiments. *Scale bar: 10 μ m.* (B) Quantitative comparison of LC3 staining, normalized to negative controls. Data represent mean $\pm$ SD (n=3). * $p < 0.05$ compared to untreated negative controls. (Reproduced with permission from Zhao Y., Howe J.L., Yu Z., Leong D.T., Chu J.J., Loo J.S., Ng K.W. 2013. Exposure to titanium dioxide nanoparticles induces autophagy in primary human keratinocytes. *Small*, 9, 387-392 [90])

4.2.4 TEM Results

Finally, ultrastructural morphological changes in all samples from different treatment groups were examined by TEM. In comparison with untreated negative control cells (**Figure 4.5A**), keratinocytes treated with 4 μ M tamoxifen showed the accumulation of double-membrane autophagic vacuoles (**Figure 4.5B, C**). Similarly, the fine structure of autophagic vacuoles with

partially degraded electron-dense cellular debris, internalized TiO_2 aggregates and cytoplasmic contents was shown in TiO_2 10 $\mu\text{g/mL}$ treated cells (**Figure 4.5D-H**).

Figure 4.5. TEM photomicrographs. Keratinocytes were treated with 4 μ M tamoxifen and 10 μ g/mL TiO₂ nanoparticles for 24 h before TEM analysis. **(A)** Negative control cells. **(B)** Tamoxifen treated keratinocytes showed numerous perinuclear autophagic vacuoles (arrows). **(C)** High magnification image of tamoxifen treated positive control cells showed double-membrane autophagic vacuoles (arrows). **(D)** Autophagic vacuolization of cytoplasm in keratinocytes treated with TiO₂ nanoparticles. Electron-dense TiO₂ nanoparticles were visible in some vacuoles (arrow). **(E, F)** TiO₂ nanoparticles internalized by keratinocytes were found in some cytoplasmic vacuoles (arrows). **(G, H)** Late autophagic vacuoles contained cellular debris and degraded cytoplasmic contents (arrows). Images were acquired at 5,000 – 40,000 $\times$ magnification. *N: nucleus*. (Reproduced with permission from Zhao Y., Howe J.L., Yu Z., Leong D.T., Chu J.J., Loo J.S., Ng K.W. 2013. Exposure to titanium dioxide nanoparticles induces autophagy in primary human keratinocytes. *Small*, 9, 387-392 [90])

The presence of several typical autophagic structures in TiO₂ nanoparticles treated cells was shown below. These structures include: 1) a phagophore, which indicates autophagy activation when an isolated crescent membrane appears in the cytoplasm (**Figure 4.6A**); 2) double-membranous structures namely autophagosomes (**Figure 4.6B**); 3) late autophagic compartments: autolysosomes and/or amphisomes that derive from the fusion of autophagosomes with lysosomes or endosomes with cytoplasmic materials inside (**Figure 4.6C**); 4) structures of early and late autophagic vacuoles: early autophagic vacuoles look like vacuoles surrounded by cytoplasm, while late autophagic vacuoles contain partially degraded cytoplasmic cargo (**Figure 4.6D**). TEM results proved the induction of autophagy by TiO₂ 10 μ g/mL in primary human keratinocytes.

Figure 4.6. TEM photomicrographs of TiO₂ nanoparticles treated keratinocytes. (A) The formation of an autophagosome derived from a phagophore in TiO₂ nanoparticles treated cells (arrow). (B) A completed autophagosome observed in TiO₂ nanoparticles treated cells (arrow). (C) Autolysosomes/amphisomes containing cytoplasmic contents were shown in TiO₂ nanoparticles treated cells (arrows). (D) Structures of early and late autophagic vacuoles were found in TiO₂ nanoparticles treated cells (arrows). Images were acquired at 5,000 – 40,000 × magnification. *N*: nucleus. (Reproduced with permission from Zhao Y., Howe J.L., Yu Z., Leong D.T., Chu J.J., Loo J.S., Ng K.W. 2013. Exposure to titanium dioxide nanoparticles induces autophagy in primary human keratinocytes. *Small*, 9, 387-392 [90])

4.3 Discussion

In this part of work, autophagy was identified to be induced by TiO₂ nanoparticles at the concentration of 10 μg/mL in primary human keratinocytes. The correlation of TiO₂-induced cytotoxicity and autophagy suggested that the induction of autophagy was not accompanied by an excessive cytotoxicity in cells exposed to TiO₂ nanoparticles. The majority of keratinocytes seems

to survive during the interaction with TiO₂ nanoparticles in the presence of autophagy. This observation could be explained by the well-recognized cyto-protection role of autophagy, which is to help cells get rid of faulty organelles and detrimental components at times of stress [98, 117, 118]. In the field of nanotoxicology, two major biological mechanisms of nanoparticle-induced toxicity are oxidative stress and inflammation. But neither paradigm has been fully explored. It is important to explore nanoparticle-induced autophagy because autophagy can be linked to toxicity caused by nanoparticles. Autophagy pathway could become an emerging mechanism to explain and link the observed cytotoxic effects. It is postulated that autophagy allowed primary human keratinocytes to survive in response to the stress induced through the interaction with TiO₂ nanoparticles, potentially mediated by nanoparticle uptake mechanisms. Transcriptional reprogramming through the activation of stress-inducible transcription factors as well as transcription-independent adaptations are possibly involved in response to such stress [119]. The formation of autophagosomes is considered to arise from transcription-independent adaptations of keratinocytes under the exposure of TiO₂ nanoparticles [120]. It was postulated that keratinocytes with upregulated autophagy pathway were more resistant to the adversity in the interaction with TiO₂ nanoparticles. TiO₂ nanoparticles were observed in autophagic compartment, suggesting that keratinocytes rely on autophagy activation to sequester these particles, although no evidence has been seen directly proving that nanoparticles were degraded through autophagy-mediated lysosome degradation, or suggesting that nanoparticles inside autophagic vacuoles may lose their UV protective capacity. TiO₂-induced autophagy might possibly be upregulated for the removal of damaged organelles when nanoparticles disfavor some organelles in the cell, thereby regaining metabolic energy for survival. The subsequent work investigated more aspects of TiO₂-induced toxicity in keratinocytes in a dose relevance study.

Chapter 5

Exposure of TiO₂ nanoparticles to Keratinocytes: Dose Relevance of Concentration

5.1 Outline

Paracelsus (1493-1541), who founded the discipline of toxicology, once said “All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy.” His famous observation on dose has been considered as the fundament for modern toxicology. The dose issue has an important influence on the profile of nanotoxicity. Most studies used *in vitro* systems to evaluate toxic effects of TiO₂ nanoparticles. In an *in vitro* study, insoluble TiO₂ nanoparticles were dispersed in cell culture media for cell exposure. The reported administered dose is either the total particle mass, particle number, particle surface area, or particle volume per unit volume of liquid media (concentration) [105, 106, 121]. However, mass transport or the delivered dose to cells had rarely been reported until very recently. The definition of delivered dose is the total amount of particles that are deposited to cells within a certain exposure time. The transportation of nanoparticle-aggregates in media suspension determines the effective dose delivered to cells [106, 122]. DeLoid *et al.* [105] reported a simple but high-throughput method to calculate delivered dose of nanoparticles by measuring the effective density. This method was employed here to calculate delivered doses of different TiO₂ concentrations in cell culture system.

In this chapter, keratinocytes were exposed to a wide dose range of TiO₂ nanoparticles for the assessment of toxicity and autophagy. Concentrations range from femtogram/mL (fg/mL), picograms/mL (pg/mL), nanograms/mL (ng/mL) to micrograms/mL (μg/mL). The comparison of administered dose and delivered dose in TiO₂-induced cytotoxicity and autophagy was performed. Possible toxic effects and autophagy induction were evaluated in a dose relevance study of concentration.

5.2 Results

5.2.1 Physical Characterization of TiO₂ Nanoparticles

TiO₂ nanoparticles were first characterized by TEM. Nanosized TiO₂ showed a mix of spherical and polygonal shapes (**Figure 5.1A**), with a diameter at 22.16 ± 3.17 nm (Calculation was provided by ImageJ software under the measurement of 50 random TiO₂ nanoparticles). DLS measurements of both bath ultrasonicated TiO₂ in ultrapure water (pH 6.0) and serum-free HEKCM (pH 7.5) were listed in **Figure 5.1B**. The measurement of d_H results suggested that 1) TiO₂ nanoparticles formed aggregates in both ultrapure water and serum-free HEKCM; 2) With the decrease in concentration, there was an observed decrease in d_H values. Data showed that TiO₂ concentration had an impact on mean hydrodynamic diameter. There were more TiO₂ aggregates in media suspensions of high concentrations while fewer in suspensions of low concentrations. The zeta potential of TiO₂ nanoparticles in ultrapure water (pH 6.0) was $+30.8 \pm 2.1$ mV while this number dropped to -13.3 ± 2.5 mV when TiO₂ nanoparticles were dispersed in HEKCM (pH 7.5).

DLS results suggested that TiO₂ nanoparticles formed aggregates in both ultrapure water and serum-free HEKCM. With the increase in concentration, there was an observed increase in d_H values. The state of TiO₂ aggregates in serum-free HEKCM was likely to be predicted in laboratory

experiments. At physiological pH 7.4-7.5, TiO₂ nanoparticles were negatively charged and resulted in a higher tendency of aggregation. For nanotoxicity study, it has been accepted that nanoparticle-aggregates also can provide stable suspensions for a relatively correct dose. Aggregates of nanoparticles used in each batch of experiments were consistent in hydrodynamic size and surface charge. The consistency was ensured by strictly following the established protocol in the preparation of nanoparticles for each batch of experiments. Nanoparticles were prepared with fresh HEKCM. Sonication was performed on the same machine with same sonication conditions for each batch of experiments. Representative nanoparticles working solutions were measured by DLS for hydrodynamic size and zeta potential. Results obtained from different runs showed that the preparation protocols adopted ensured the consistency of experiments, where nanoparticles formed aggregates in stable suspensions for toxicity study.

Mean particle size = 22.16 ± 3.17 nm

B) Hydrodynamic Characterization of TiO₂ nanoparticles by DLS

Ultrapure Water	Z-average size (nm)	PDI	Peak 1 Mean Intensity* (nm)	Peak 1 Area Intensity** (%)	Zeta Potential (mV)	pH
500 µg/mL	5456 ± 266.2	0.418 ± 0.02	2494.3 ± 236.0	100	+30.8 ± 2.1	6.0
100 µg/mL	2876.3 ± 153.6	0.526 ± 0.05	1775 ± 309.6	98.2 ± 1.3	/	6.0
50 µg/mL	1463 ± 17.0	0.534 ± 0.12	1049.1 ± 279.9	100	/	6.0
10 µg/mL	706.5 ± 19.2	0.502 ± 0.04	343.3 ± 28.3	100	/	6.0
5 µg/mL	677.1 ± 84.4	0.447 ± 0.00	289.4 ± 27.0	100	/	6.0
50 ng/mL	544.7 ± 51.7	0.661 ± 0.09	168 ± 34.4	99.4 ± 1.0	/	6.0
50 pg/mL	461.9 ± 24.9	0.431 ± 0.02	169.7 ± 24.8	91.9 ± 7.0	/	6.0
50 fg/mL	363.9 ± 99.1	0.707 ± 0.10	167.6 ± 24.8	93.7 ± 10.9	/	6.0
EpiGRO™ HEKCM	Z-average size (nm)	PDI	Peak 1 Mean Intensity* (nm)	Peak 1 Area Intensity** (%)	Zeta Potential (mV)	pH
500 µg/mL	2663.9 ± 12.8	0.329 ± 0.05	2042 ± 27.3	100	-13.3 ± 2.5	7.5
100 µg/mL	2075 ± 26.5	0.502 ± 0.06	985.8 ± 47.9	100	/	7.5
50 µg/mL	949.9 ± 52.7	0.386 ± 0.07	657.3 ± 91.6	93.6 ± 2.69	/	7.5
10 µg/mL	774.8 ± 41.3	0.433 ± 0.03	561.2 ± 63.8	95.4 ± 2.7	/	7.5
5 µg/mL	535.1 ± 32.6	0.439 ± 0.05	449.4 ± 48.9	100	/	7.5
50 ng/mL	238.7 ± 14.9	0.293 ± 0.06	179.7 ± 23.0	98.4 ± 2.8	/	7.5
50 pg/mL	163.6 ± 16.4	0.555 ± 0.07	122.4 ± 16.9	87.9 ± 3.9	/	7.5
50 fg/mL	133.3 ± 12.9	0.575 ± 0.06	103.4 ± 4.7	84.4 ± 4.5	/	7.5

* Peak 1 Mean Intensity: mean particle size based on the most prominent signal peak alone

** Peak 1 Area Intensity: proportion of particles within the most prominent signal peak

Figure 5.1. Physical characterization of TiO₂ nanoparticles. (A) TEM photomicrograph showed morphology and pristine size of nano-TiO₂ in a mix of spherical and polygonal shapes. (B) Hydrodynamic measurements of different concentrations of TiO₂ nanoparticles in ultrapure water and EpiGRO™ serum-free HEKCM. Data represent mean $\pm$ SD (n=3).

5.2.2 Delivered dose of TiO₂ Nanoparticles in media suspensions

Nanoparticles in suspension behave differently from chemicals in solution in that they will aggregate and sediment. It is therefore necessary to model and calculate how much of these particles in suspension (administered dose) also sediment over time to come into direct contact with cells sitting at the bottom of culture vessels (delivered dose). Delivered doses of nanoparticles were calculated based on effective density (ρ_{EV}) estimated by volumetric centrifugation, hydrodynamic diameter (d_H) measured by DLS, media volume, height, temperature (310K), density (1.00 g/cm³), and dynamic viscosity (0.00074 PaS), and mass concentration (i.e. γ : $\sim$ μ g/mL). The *in vitro* sedimentation, diffusion, and dosimetry (ISDD) model was used to calculate numerically for each nanoparticle-media suspension [105, 106, 121, 123]. ISDD model calculated the delivered dose in terms of mass, M_D (i.e. μ g), as:

$$\text{Administered dose } (M_A) = \text{mass concentration } (\gamma_A) \times \text{media volume } (V) \quad (5.1)$$

$$M_D(t) = f_D(t) \times M_A \quad (5.2)$$

The fraction of administered particles that would be delivered onto cells as a function of time $f_D(t)$ was measured by the Gompertz sigmoidal equation [121] as:

$$f_D(t) = 1 - e^{-\alpha t} \quad (5.3)$$

where t was the time (h) and α was a particle- and media-specific deposition fraction constant (h^{-1}). The time t required for the delivery of administered dose within 24 h was calculated as:

$$t = - \ln (1 - f_D(t)) / \alpha \quad (5.4)$$

The effective density (ρ_{EV}) value for TiO_2 agglomerates was $1.188 \pm 0.002 \text{ g/cm}^3$. The value for ρ_{EV} is lower than the value for TiO_2 density which is 4.23 g/cm^3 , indicating that TiO_2 aggregates in media suspension are substantially less dense than bulk TiO_2 . The sedimentation rate of TiO_2 nanoparticles in media suspensions, thus, is expected to be much slower. ISDD simulations predicted the fraction of TiO_2 nanoparticles (f_D) deposited within 24 h treatment. The value for f_D was plotted against TiO_2 treatment time. The integral or the area under the curve (AUC) of this profile provided a time-weighted average factor (fracAUC). fracAUC is a dimensionless number calculated by ISDD simulations. ISDD simulations predicted the maximum mass of the administered TiO_2 dose ($M_{\text{D max}}$) delivered to cells within 24 h. Representative results, illustrated in **Figure 5.2**, showed the delivery model of TiO_2 administered dose at $500 \mu\text{g/mL}$ within 24 h.

The modelling of TiO_2 delivery suggested the contribution of ρ_{EV} , d_H , treatment time, and cell culture conditions to the deposition of maximum TiO_2 administered dose to cells. TiO_2 nanoparticles (administered dose: $500 \mu\text{g/mL}$) were completely deposited onto the cell monolayer within 18 h, 12 h, and 11 h for cells cultured in 96-well plates, 6-well plates, and T75 flasks, respectively (Figure 5.2A). Delivered dose ($M_{\text{D max}}$) and time-weighted average dose (TWAD) over 24 h exposure were normalized to average cell yield of different culture conditions to calculate mean $M_{\text{D max}}$ and TWAD per cell. Figure 5.2B showed that TiO_2 delivery over 24 h by the fraction of the administered dose, f_D . f_D went to 1, suggesting $M_D = 500 \mu\text{g/mL}$ when the

administered particles were delivered onto cells completely. In addition, the calculation in ISDD template required the input of media volume as one parameter. Media volume of cells cultured in different conditions such as 96-well plates, 6-well plates, and T75 flasks varied greatly. According to the calculation, the same administered dose resulted in different $f_D(t)$ under the input of different media volume. Figure 5.2C illustrated the differences in M_D per cell of the same administered dose 500 $\mu\text{g/mL}$ under different culture conditions.

A) Cell culture conditions, maximum TiO_2 deposition time and mass, and time-weighted average dosage

Culture Conditions	Media Height (m)	Media Volume (mL)	Average Cell Yield	$T_{\max}$ (h)	$M_{D \max}$ (μg)	$M_{D \max}$ per cell (μg)	fracAUC	TWAD ($\mu\text{g} \cdot \text{h}$)	TWAD per cell ($\mu\text{g} \cdot \text{h}$)
96-well plate	0.003242	0.1	3.2×10^4	18	50	1.56×10^{-3}	16.482	824.1	0.026
6-well plate	0.002071	2	9.5×10^5	12	1000	1.05×10^{-3}	19.188	19188	0.020
T75 flask	0.002	15	7.5×10^6	11	7500	1.00×10^{-3}	19.355	145162.5	0.019

$T_{\max}$, the time required for the maximum deposition of TiO_2 nanoparticles in EpiGRO™ media suspension; $M_{D \max}$, the maximum mass of the administered dose deposited within 24 h treatment; $M_{D \max}$ per cell, $M_{D \max}$ / average cell yield; fracAUC, time-weighted average factor at the treatment time of 24 h; TWAD, time-weighted average dosage after 24 h treatment, $\text{TWAD} = \text{fracAUC} \times M_{D \max}$; TWAD per cell = $\text{fracAUC} \times M_{D \max}$ per cell.

Figure 5.2. Dosimetry of TiO_2 nanoparticles in media suspension. (A) Dosimetry parameters for keratinocytes cultured in different conditions. (B) Modelling of TiO_2 delivery over 24 h indicated by the fraction of the administered dose, f_D . (C) Modelling of TiO_2 delivery in different culture conditions over 24 h indicated by M_D per cell.

However, the delivery curve for different TiO_2 administered dose varied greatly. Representative dosimetry curves in **Figure 5.3** showed the delivered dose in mass of TiO_2 at different administered concentrations to cells cultured in 96-well plates over 24 h. For TiO_2 concentrations below 100 $\mu\text{g/mL}$, the calculated delivered doses were lower than the administered doses. While TiO_2 concentrations within (100 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$], the calculated delivered doses were close to or equal to the administered doses. The calculated delivered doses corresponding to each administered dose of TiO_2 nanoparticles were shown in **Table 5.1**.

Figure 5.3. Delivery of TiO_2 at different administered doses to keratinocytes cultured in 96-well plate.

(A, B, C) Delivered dose in mass was plotted against TiO_2 treatment time. Over 24 h treatment period, the delivered dose increased over time. Low concentrations of TiO_2 50 fg/mL, 50 pg/mL, and 50 ng/mL did not manage to reach the maximum dose to be equivalent to the administered dose, even at the end of 24 h. (D) While for the high concentrations of TiO_2 nanoparticles (i.e. 500 $\mu\text{g/mL}$), the maximum delivered dose was equivalent to administered dose over 24 h, whereby the delivered dose sat on a plateau from 18 h onwards.

Table 5.1 TiO₂ administered dose and corresponding calculated delivered dose over 24 h

Administered dose (24 h)	Delivered dose (24 h)
50 fg/mL	11.35 fg/mL
100 fg/mL	22.70 fg/mL
250 fg/mL	56.74 fg/mL
500 fg/mL	113.48 fg/mL
1 pg/mL	0.21 pg/mL
10 pg/mL	2.06 pg/mL
50 pg/mL	10.29 pg/mL
100 pg/mL	20.58 pg/mL
250 pg/mL	51.44 pg/mL
500 pg/mL	102.89 pg/mL
1 ng/mL	0.21 ng/mL
10 ng/mL	1.74 ng/mL
50 ng/mL	7.69 ng/mL
100 ng/mL	15.38 ng/mL
250 ng/mL	36.27 ng/mL
500 ng/mL	72.54 ng/mL
1 µg/mL	0.145 µg/mL
10 µg/mL	1.62 µg/mL
50 µg/mL	24.48 µg/mL
100 µg/mL	95.25 µg/mL
250 µg/mL	250 µg/mL
500 µg/mL	500 µg/mL

ISDD applies well-established principles of diffusional and sedimentational (i.e. gravitational) transport of particles in viscous media to calculate the movement of particles from the media to the bottom of the vessel, where the cells reside [106]. The particle transport to cells in the ISDD model is calculated by simultaneous solution of Stokes Law (for sedimentation) and the Stokes-Einstein Equation (for diffusion) [106]. According to the calculation, the time t required for the delivery of administered dose in high dose ranges was shorter than that of the delivery in low dose ranges. For TiO₂ administered dose [50 fg/mL, 100 µg/mL], the time t required for the complete delivery of administered particle onto cells was more than 24 h, suggesting the delivered dose in

mass (M_D) was lower than the administered dose at the end of 24 h. In contrast, when TiO_2 administered dose reached (100 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$], the administered particles were completely delivered onto cells during 24 h treatment duration.

5.2.3 Cytotoxicity Characterization of TiO_2 Nanoparticles

Phase contrast images of cell culture were observed after 24 h exposure to different concentrations of TiO_2 nanoparticles (**Figure 5.4**). Keratinocytes exposed to TiO_2 administered concentrations of 50 fg/mL, 50 pg/mL, 50 ng/mL, and 50 $\mu\text{g/mL}$ showed typical pointy star-like shape with a high mitotic index as similar to negative controls. Whereas cells under the exposure of 500 $\mu\text{g/mL}$ reflected morphological changes into flat and grainy cells covered by TiO_2 aggregates in comparison with negative controls.

Figure 5.4. Light microscopy images of keratinocytes at high density in EpiGRO™ serum-free HEKCM. (A) Untreated keratinocytes showed typical pointy star-like shape with a large percentage of healthy, refractile cells. **(B, C, D, E)** Cells showed similar morphology and maintained a high mitotic index

in the presence of 50 fg/mL, 50 pg/mL, 50 ng/mL, and 50 µg/mL of TiO₂ nanoparticles after 24 h in comparison with negative controls. **(F)** In contrast, keratinocytes treated with 500 µg/mL TiO₂ nanoparticles after 24 h were flat and grainy. TiO₂ aggregates were visible in the culture media. *Scale bar: 50 µm.*

Cytotoxicity was examined by WST-8 assay. WST-8 results showed that there was a reduction in relative cell viability observed in keratinocytes under the exposure of TiO₂ nanoparticles **(Figure 5.5)**. Keratinocytes exposed to TiO₂ administered concentrations of 10 µg/mL and beyond showed a statistically significant reduction in cell viability over 24 h, compared to negative controls ($p < 0.05$). Cells treated with TiO₂ administered concentrations below 10 µg/mL as well as 4 µM tamoxifen, did not show the statistically significant decrease in cell viability in comparison with negative controls.

Figure 5.5. Relative cell viability by WST-8 assay. Primary human keratinocytes were incubated with different concentrations of TiO₂ nanoparticles and 4 µM tamoxifen for 24 h before analysis. Untreated cells

were used as negative controls. WST-8 assay was performed on all cells. Data represent mean $\pm$ SD (n=3).

* $p < 0.05$.

The comparison of cytotoxicity caused by TiO₂ administered dose versus delivered dose showed that the toxic dose range of TiO₂ administered dose was [10 μ g/mL, 500 μ g/mL] while this range changed to [1.62 μ g/mL, 500 μ g/mL] in TiO₂ delivered dose over 24 h (**Figure 5.6**). TiO₂ nanoparticles started to induce cytotoxicity at the calculated delivered dose of 1.62 μ g/mL over 24 h, but the observed corresponding toxic administered dose was much higher at 10 μ g/mL. When TiO₂ concentrations reached over 100 μ g/mL, the maximum administered dose was almost totally delivered to cells over 24 h, according to dosimetry calculation. TiO₂ administered dose of 250 and 500 μ g/mL, which was equal to delivered dose over 24 h, induced approximately 50% reduction in cell viability.

Figure 5.6. The comparison of cell viability between TiO₂ administered dose and delivered dose.

Primary human keratinocytes were incubated with different concentrations of TiO₂ nanoparticles for 24 h before analysis. Untreated cells were used as negative controls. WST-8 assay was performed on all cells. Delivered dose was calculated by ISDD model. **(A)** Cell viability of TiO₂ administered dose. **(B)** Cell viability of TiO₂ delivered dose. Data represent mean $\pm$ SD (n=3). * $p < 0.05$.

5.2.4 Profile of Autophagy induced by TiO₂ Nanoparticles

TiO₂-induced autophagy was first quantified by MDC staining. MDC data showed that there were statistically significant increases in the number of autophagic vacuoles triggered by TiO₂ nanoparticles within the administered dose range, [100 fg/mL, 250 μ g/mL], as well as 4 μ M tamoxifen treated groups, in comparison with negative controls ($p < 0.05$) (**Figure 5.7**).

Figure 5.7. Autophagy induction by TiO₂ nanoparticles. Keratinocytes were treated with different concentrations of TiO₂ nanoparticles and 4 μ M tamoxifen for 24 h before analysis. Cells were stained with MDC and fluorescence was quantified by a microplate reader. Data represent mean $\pm$ SD (n=3). * $p < 0.05$.

The comparison of autophagy induced by TiO₂ administered dose versus delivered dose showed that TiO₂ nanoparticles were able to trigger autophagy within administered dose range, [100 fg/mL, 250 µg/mL], corresponding to the calculated delivered dose range, [22.7 fg/mL, 250 µg/mL], over 24 h (**Figure 5.8**). The threshold of autophagy was induced by TiO₂ administered dose from as low as 100 fg/mL, corresponding to the calculated delivered dose of 22.7 fg/mL.

Figure 5.8. The comparison of autophagy between TiO₂ administered dose and delivered dose.

Primary human keratinocytes were incubated with different concentrations of TiO₂ nanoparticles and 4 µM tamoxifen for 24 h before analysis. Untreated cells were used as negative controls. MDC staining was performed on all cells. Delivered dose was calculated by ISDD model. **(A)** Autophagy induced by TiO₂ administered dose. **(B)** Autophagy induced by TiO₂ delivered dose. Data represent mean ± SD (n=3). **p* < 0.05.

MDC results indicated that autophagy can be triggered by TiO₂ nanoparticles at a relative lower administered dose range approximately of 100 fg/mL, while the calculated delivered dose range for this threshold value was reduced to 22.7 fg/mL. Besides these, there was also an observed decrease in the number of autophagic vacuoles in cells exposed to toxic TiO₂ concentrations beyond 250 µg/mL. Results suggested that TiO₂ nanoparticles within a certain administered and delivered dose range can induce autophagy in keratinocytes. The observed autophagy induction in TiO₂ administered dose range, [100 fg/mL, 10 µg/mL), corresponding to calculated delivered dose range, [22.7 fg/mL, 1.62 µg/mL), was not accompanied with acute cytotoxicity.

Secondly, LC3 immunoblotting and its turnover assay were used to confirm the threshold of TiO₂-induced autophagy. Endogenous LC3 can be detected as two bands by immunoblot analysis: one band is corresponded to cytosolic LC3-I (molecular weight 14 kDa), and the other represents LC3-II (molecular weight 16 kDa) which is conjugated with phosphatidylethanolamine (PE) [87]. The conversion from LC3-I to LC3-II is an indicator of autophagosome formation because LC3-II is present on isolated membranes through the formation of double-membrane autophagosomes, but much less on autolysosomes [87]. Therefore, the amount of LC3-II is suggested to be closely correlated with the number of autophagosomes [82]. Cells were treated in T-75 flasks with autophagy positive control tamoxifen 4 µM, negative control media, 100 fg/mL or 500 µg/mL of TiO₂ administered dose in the presence and absence of lysosome inhibitors for 24 h before analysis. Lysosome inhibitors, NH₂Cl/Leupeptin, served as LC3 turnover agents. LC3 turnover is also one of the principal methods to measure autophagic flux. If cells are treated with lysosome inhibitors, which either inhibit acidification inside lysosome or inhibit autophagosome-lysosome fusion, the degradation of LC3-II is blocked, thus leading to the accumulation of LC3-II [82]. Accordingly, the level of LC3-II is supposed to increase after the treatment with lysosome inhibitors. The

differences in total LC3-II amount between samples in the presence and absence of lysosome inhibitors correspond directly with the amount of LC3 that would be delivered to lysosomes for degradation if lysosome inhibitors are absent⁵.

Results showed the migration of LC3-I was slower than that of LC3-II (**Figure 5.9A**) and suggested that the treatment of 4 μ M tamoxifen and TiO₂ administered dose of 100 fg/mL induced the upregulation of LC3-II expression. Samples treated by tamoxifen, TiO₂ 100 fg/mL and 500 μ g/mL in the presence of lysosome inhibitors showed an increased level of LC3-II, which was due to the blockage of autophagic degradation. Densitometry values for LC3-II/LC3-I ratio and the amount of LC3-II were shown below the immunoblotting (**Figure 5.9B, C**). TiO₂ administered dose of 100 fg/mL and tamoxifen 4 μ M treatment in the presence and absence of lysosome inhibitors resulted in greater LC3-II/LC3-I ratio as well as the amount of LC3-II than untreated negative control ($p < 0.05$) and TiO₂ 500 μ g/mL treatments. LC3 immunoblotting and its turnover analysis confirmed MDC results that TiO₂ nanoparticles at the administered dose of 100 fg/mL (calculated delivered dose of 22.7 fg/mL) was sufficient to induce autophagy in keratinocytes.

⁵ How to interpret LC3 immunoblotting: During autophagy, the amount of LC3-I decreases and that of LC3-II increases. In Figure 5.9B, the ratio of LC3-II/LC3-I showed the decrease of LC3-I and the increase of LC3-II in each sample. If cells were treated with lysosomal inhibitors such as NH₄Cl/Leupeptin, the degradation of LC3-II was partially inhibited, whereas the amount of LC3-I was not much affected. These suggest that the differences in the amount of LC3-II between samples in the presence and absence of lysosomal inhibitors more accurately represent the total autophagic flux rather than the differences in the total amount of LC3 (i.e. LC3-I + LC3-II). In LC3 immunoblotting analysis, the amount of LC3-II, the ratio of LC3-II/LC3-I, or the ratio of LC3-II/(LC3-I + LC3-II) could be used for data interpretation. However, it has been recognized that LC3-II tends to be more sensitive than LC3-I in immunoblotting, simple comparison of LC3-I and LC3-II, or summation of LC3-I and LC3-II (i.e. LC3-I + LC3-II), is not appropriate. The more accurate indicator is the comparison of the amount of LC3-II among samples. In Figure 5.9C, the comparison of the amount of LC3-II (i.e. LC3-II/ β -actin) more accurately showed the autophagic flux among different samples [124].

Figure 5.9. Expression levels of LC3-I and LC3-II in keratinocytes under TiO₂ treatments. (A) Immunoblotting results of LC3 protein. Positions of LC3-I and LC3-II were indicated. Wild-type of primary human keratinocytes were treated with 4 μ M tamoxifen, 100 fg/mL and 500 μ g/mL of TiO₂ nanoparticles for 24 h before being lysed for western blot analysis. Media treatments served as negative controls. **(B)** Densitometry readings of LC3-II/LC3-I ratio for experimental treatments in the presence and absence of lysosome inhibitors were compared independently to untreated negative controls w/o lysosome inhibitors. Data represent mean $\pm$ SD (n=3). * $p < 0.05$ compared to untreated negative controls without lysosome inhibitors. # $p < 0.05$ compared to untreated negative controls with lysosome inhibitors. **(C)** Densitometry values of LC3-II for experimental treatments in the presence and absence of lysosome inhibitors were compared independently to untreated negative controls w/o lysosome inhibitors. Data represent mean $\pm$ SD (n=3). * $p < 0.05$ compared to untreated negative controls without lysosome inhibitors. # $p < 0.05$ compared to untreated negative controls with lysosome inhibitors.

5.2.5 Oxidative Stress induced by TiO₂ Nanoparticles

CellROX[®] oxidative stress green reagent was used to examine whether TiO₂ nanoparticles would generate ROS in keratinocytes (**Figure 5.10**). Results showed that there was no significant increase in ROS levels in cells under the exposure of subtoxic TiO₂ administered concentrations of 50 fg/mL, 50 pg/mL, and 500 ng/mL, compared to negative controls. In contrast, ROS levels dramatically increased in cells exposed to TiO₂ administered dose of 50 µg/mL and beyond in comparison with negative controls. ROS signal was localized in the cytoplasm and nucleus. Quantitative comparison of ROS staining was normalized to untreated negative controls. Data showed a statistically significant increase in ROS-positive living cells when keratinocytes were treated with 50 µg/mL ($p < 0.05$), 200 µg/mL ($p < 0.05$), and 500 µg/mL ($p < 0.01$) of TiO₂ administered concentrations in comparison with negative controls.

Figure 5.10. Detection of TiO_2 nanoparticles induced oxidative stress. (A) Fluorescent microscopy analysis of ROS induced by different concentrations of TiO_2 nanoparticles measured with 5 μM CellROX[®]

green reagent in primary human keratinocytes. Increased production of ROS was shown in 50 µg/mL, 200 µg/mL, and 500 µg/mL TiO₂ treated groups. Images are representative of three independent experiments. *Scale bar: 10 µm.* **(B)** Quantitative comparison of ROS-positive living cells, normalized to negative controls. Data represent mean ± SD (n=3). **p* < 0.05, ***p* < 0.01 compared with negative controls.

5.2.6 DNA damage and Autophagy induced by TiO₂ Nanoparticles

ROS triggered by TiO₂ nanoparticles at toxic dose range can further induce DNA damage. DNA dysfunction is associated with the appearance of nuclear foci of an early DNA damage responsive protein marker, phosphorylated histone H2AX (γH2AX) [125]. γH2AX immunofluorescent staining was performed on keratinocytes exposed to different administered concentrations of TiO₂ nanoparticles and 4 µM tamoxifen after 24 h, as well as cells exposed to UV light (a positive control for DNA damage) for 15 min (**Figure 5.11**). An increase in γH2AX signal was found in the nuclei of cells under UV and TiO₂ 500 µg/mL treatments, compared to untreated negative controls. The increase of γH2AX foci was also shown in cells exposed to TiO₂ 50 µg/mL in comparison with negative controls. There was no significant increase of γH2AX signal in cells treated with 4 µM tamoxifen and TiO₂ administered concentrations of 100 fg/mL, 50 pg/mL, 50 ng/mL, and 10 µg/mL, compared to negative controls. Quantification of staining images showed that UV light, TiO₂ 50 µg/mL and 500 µg/mL resulted in 1.78, 1.34, and 1.65 times the number of cells which carried positive γH2AX marker, respectively (*p* < 0.05), compared with negative controls.

Concomitantly, TiO₂-induced autophagy was examined by LC3 staining (**Figure 5.11**). LC3-positive signal was observed in keratinocytes exposed to TiO₂ administered concentrations of 100 fg/mL, 50 pg/mL, 50 ng/mL, 10 µg/mL, 50 µg/mL, as well as 4 µM tamoxifen for 24 h. There was no increased LC3 signal shown in untreated cells, cells under UV light and TiO₂ 500 µg/mL

treatments. Quantitative comparison of LC3 staining normalized to negative controls confirmed that there was a statistical increase of LC3-positive cells in keratinocytes treated with 4 μ M tamoxifen and TiO₂ administered concentrations of 100 fg/mL, 50 pg/mL, 50 ng/mL, 10 μ g/mL, and 50 μ g/mL.

In summary, TiO₂ administered dose was identified to cause DNA damage at approximately 50 μ g/mL and beyond. Administered concentrations of TiO₂ 100 fg/mL, 50 pg/mL, 50 ng/mL, and 10 μ g/mL did not carry detectable DNA damage marker but showed autophagy positive marker. Both DNA damage and autophagy markers were observed in TiO₂ 50 μ g/mL treated groups. However, no autophagy but DNA damage was observed in cells exposed to TiO₂ 500 μ g/mL. Results suggested that autophagy induced by TiO₂ nanoparticles within the administered dose range, [100 fg/mL, 50 μ g/mL], in keratinocytes was not accompanied with detectable genotoxicity. TiO₂-induced autophagy within the administered dose range, [50 μ g/mL, 500 μ g/mL], was accompanied with genotoxicity.

Figure 5.11. Detection of DNA damage and autophagy by γ -H2AX and LC3 staining. (A) Epifluorescent microscopy images of γ -H2AX and LC3 immuno-labeled human keratinocytes. Increased expression of γ -H2AX protein was shown in UV treated groups, TiO₂ administered concentrations of 50 μ g/mL and 500 μ g/mL treated groups. Increased expression of LC3 protein was shown in 4 μ M tamoxifen, TiO₂ administered concentrations of 100 fg/mL, 50 pg/mL, 50 ng/mL, 10 μ g/mL and 50 μ g/mL treated groups. Images are representative of three independent experiments. *Scale bar: 10 μ m.* (B) Quantitative comparison of γ -H2AX staining, normalized to negative controls. Data represent mean $\pm$ SD (n=3). * $p < 0.05$ compared to untreated negative controls. (C) Quantitative comparison of LC3 staining, normalized to negative controls. Data represent mean $\pm$ SD (n=3). # $p < 0.05$ compared to untreated negative controls.

5.2.7 Apoptosis and Necrosis induced by TiO₂ Nanoparticles

Besides autophagy, apoptosis and necrosis are two other major responses that could be induced through the interaction between keratinocytes and TiO₂ nanoparticles. Necrotic cells usually lack the integrity of plasma membrane, whereas apoptotic cells have an intact plasma membrane. The translocation of membrane phosphatidylserine from the inner side of the plasma membrane to the surface is one of the characteristics of apoptosis [126]. Flow cytometry can measure necrosis and apoptosis. Fluorescent dyes can bind with different cellular components such as DNA, RNA as well as specific proteins on cell membranes or inside cells. The loss of integrity of the plasma membrane can be demonstrated by the fluorescent dye, propidium iodide (PI) which is excluded by the intact plasma membrane, while the dye of Annexin V has a high affinity for binding with phosphatidylserine. Therefore, FITC-labeled Annexin V/PI staining was performed to analyze the percentage of viable, apoptotic, and necrotic cells induced by different administered dose of TiO₂ nanoparticles via flow cytometry.

Flow cytometry plots of keratinocytes exposed to different TiO₂ administered concentrations showed varying degree of viable, apoptotic, and necrotic cells (**Figure 5.12**). The majority of cells were viable in untreated negative controls (93.76%) and TiO₂ 50 ng/mL treated groups (92.93%). The magnitude of late apoptotic or necrotic cells varied between groups treated with 500 ng/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, and 20 µg/mL of TiO₂ nanoparticles in the range from 4.75% to 5.94%. The magnitude of early apoptotic cells in these five groups increased from 4.5% to 7.66%. No particular cell death was predominant in these five groups with more than 10% early apoptotic or 10% late apoptotic or necrotic cells. Cell viability of these five groups was over 80%. The comparison of flow cytometric data from all experimental groups demonstrated that there was a

decrease in living cells, and an increase in early apoptotic cells and late apoptotic/necrotic cells with increased administered dose of TiO₂ nanoparticles.

Figure 5.12. FITC-labeled Annexin V/PI analysis of viable, apoptotic and necrotic keratinocytes after 24 h exposure to different concentrations of TiO₂ nanoparticles. (A) Flow cytometry scatterplots showed Annexin V/PI staining of untreated keratinocytes and cells exposed to 50 ng/mL, 500 ng/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, and 20 µg/mL TiO₂ nanoparticles. Numbers in each quadrant indicate the

percentage of total cells for the respective quadrant. V = viable cells, A = apoptotic cells, N = necrotic cells.

(B) The comparison of flow cytometric data from different experimental groups.

5.2.8 TiO₂-induced Toxicity is associated with p62, TRAF6, and NF-κB activation

p62, also known as sequestosome 1, is a signaling adaptor protein that coordinates the processes required for metabolic homeostasis and inflammation, including: a scaffold in nuclear factor κB (NF-κB) activation and tumor necrosis factor (TNF) receptor associated factor (TRAF) mediated cellular signal transduction [127, 128]. p62 is found to bind autophagy regulator Atg8/LC3 through LC3-interacting region (LIR) for autophagy formation as well as to regulate the packing and delivery of cellular cargo for their clearance by autophagy [129]. It also interacts with NF-κB and TRAF6 in the recruitment of pro-inflammatory, DNA damage repair, and autophagy induction [129-131]. Immunoblotting of these three proteins was performed to examine whether their expression varied in keratinocytes treated with different administered dose of TiO₂ nanoparticles.

Firstly, an upregulation of p62 expression associated with the increased concentrations of TiO₂ nanoparticles was observed (**Figure 5.13**). There was a statistically significant increase in p62 expression at TiO₂ 500 µg/mL, compared to untreated negative controls ($p < 0.05$). While p62 protein levels did not vary greatly in untreated negative controls, 4 µM tamoxifen, TiO₂ subtoxic administered dose treated groups. Secondly, expressions of TRAF6 and NF-κB subunit p65/RelA were observed to increase in TiO₂ treated samples in an administered dose-dependent manner (**Figure 5.14**).

Figure 5.13. p62 expression of keratinocytes exposed to different concentrations of TiO₂ nanoparticles. (A) Immunoblotting results of p62 protein. Wild-type of primary human keratinocytes were treated with 4 μ M tamoxifen and different concentrations of TiO₂ nanoparticles for 24 h before being lysed for western blot analysis. (B) Densitometry readings for tamoxifen and TiO₂ nanoparticles treated keratinocytes were compared independently to untreated negative controls. Data represent mean $\pm$ SD (n=3). * $p < 0.05$ compared to untreated negative controls.

Figure 5.14. TRAF6 and NF- κ B (p65/RelA) expression of keratinocytes exposed to different concentrations of TiO₂ nanoparticles. Immunoblotting results of TRAF6 and NF- κ B (p65/RelA) proteins. Wild-type of primary human keratinocytes were treated with 4 μ M tamoxifen and different concentrations of TiO₂ nanoparticles for 24 h before being lysed for western blot analysis (Credits: Dr. Esther Wong, Yu Chye Yun).

5.2.9 TEM Results

Finally, morphological changes of keratinocytes of all treatments were determined using transmission electron microscopy. Untreated negative controls displayed the structures of a normal keratinocyte (**Figure 5.15A**), while tamoxifen treated cells showed the presence of membrane-bound compartments such as autophagosomes and autolysosomes that contained cytoplasmic materials (**Figure 5.15B, C**). A few of autophagic vacuoles appeared in keratinocytes exposed to TiO₂ administered dose of 50 fg/mL (**Figure 5.15D-F**). One piece of small TiO₂ aggregates was shown to localize in the nucleus (Figure 5.15E). With the increasing concentrations of TiO₂ nanoparticles, more and more autophagic vacuoles appeared in keratinocytes exposed to TiO₂ administered concentrations of 100 fg/mL, 50 pg/mL, and 50 ng/mL (**Figure 5.15G-L**). A number of double-membrane autophagosomes contained undegraded cytoplasmic contents. In some images, ribosomes were seen inside or near autophagosomes. Some autophagosomes were

observed in fusion profiles with lysosomes to form into late autophagic vacuoles. Partially degraded electron-dense materials identified the degradation of cytoplasmic contents. Besides autophagy structures, other observed structures in these subtoxic administered dose treated groups were shown in Appendix A. TiO₂ uptake and aggregates can be seen in keratinocytes treated with concentrations of 50 µg/mL and 500 µg/mL (**Figure 5.15M-R**). In TiO₂ 50 µg/mL, some TiO₂ aggregates attached on the cellular membrane, while other aggregates inside different sizes of cellular vacuoles were found to localize nearby the nucleus (Figure 5.15M, N, O). One autophagic vacuole contained TiO₂ aggregates and a damaged mitochondrion (Figure 5.15O), suggesting autophagic removal of damaged mitochondria. Necrotic cells were also found at the administered dose of 50 µg/mL (Figure 5.15P), suggesting necrotic cell death happened to cells under the treatment of toxic TiO₂ administered dose. When the concentration reached 500 µg/mL, the majority of keratinocytes showed necrosis and apoptosis (**Figure 5.15Q, R**). One typical apoptotic cell showed the uptake of TiO₂ aggregates and the generation of numerous compact dense micronuclei and engulfed apoptotic bodies.

Figure 5.15. TEM photomicrographs of primary keratinocytes exposed to different concentrations of TiO₂ nanoparticles. (A) Untreated keratinocytes served as negative controls. (B) Autophagic compartments in tamoxifen 4 μ M treated keratinocytes (arrows). (C) Autophagosome-like compartments in keratinocytes treated by tamoxifen (arrows). (D) Overview of TiO₂ 50 fg/mL treated cells showing a few of intracellular vacuoles (arrows). (E) One small piece of TiO₂ aggregates localized in the nucleus of keratinocytes exposed to 50 fg/mL of TiO₂ nanoparticles (arrow). (F) Double-membrane structures were found near a damaged mitochondrion in cells treated with 50 fg/mL of TiO₂ nanoparticles (arrows). (G) Overview of TiO₂ 100 fg/mL treated keratinocytes with the increase in numbers of vacuoles. (H) A small autophagosome and later autophagic vacuoles were visible in cells treated with 100 fg/mL of TiO₂ nanoparticles (arrows). (I) Double-membrane structures of autophagic vacuoles were found in cells exposed to 50 pg/mL of TiO₂ nanoparticles (arrow). (J) Different stages of autophagic compartments were observed in cells treated with 50 pg/mL of TiO₂ nanoparticles. The presence of ribosomes inside an autophagosome-like structure (black arrow), an autolysosome (white arrow), and a later autophagic compartment (black arrowhead). (K) Keratinocytes exposed to 50 ng/mL of TiO₂ nanoparticles showed the structure of various autophagic vacuoles (arrows). (L) Two later autophagic compartments were shown in TiO₂ 50 ng/mL treated cells (arrows). (M) Overview of keratinocytes exposed to 50 μ g/mL of TiO₂ nanoparticles showing TiO₂ aggregates inside a cell (arrow). Some TiO₂ nanoparticles attached on the cellular membrane. (N) TiO₂ aggregates were found inside intracellular vacuoles near the nucleus in a cell treated with TiO₂ 50 μ g/mL (arrows). (O) The presence of TiO₂ aggregates and a damaged mitochondrion in an autophagic vacuole was shown in a cell exposed to TiO₂ 50 μ g/mL (arrow). (P) A necrotic cell was shown after the treatment of TiO₂ 50 μ g/mL. (Q) A necrotic cell indicated by a star and an apoptotic cell indicated by an arrowhead were shown in cells exposed to 500 μ g/mL of TiO₂ nanoparticles. TiO₂ aggregates were found in both programmed death cells. Compact dense micronuclei were shown in the apoptotic cell (arrows). (R) An apoptotic body was shown in keratinocytes exposed to TiO₂ 500 μ g/mL (arrow). Images were acquired at 5,000 – 40,000 $\times$ magnification. *N*: nucleus.

5.3 Discussion

In this part of work, TiO₂-induced toxicity and autophagy were profiled in a wide dose range. Cytotoxicity caused by TiO₂ nanoparticles could be considered as a multi-step particle deposition and delivery to the cells. The toxicity of TiO₂ nanoparticles is suggested to be determined by their transportation processes as well as biological processes. The concept of dose is not simple in nanotoxicological study. Most reported work focused on administered dose but underestimated the delivered dose that actually caused toxicity. Herein, the dosimetry ISDD model of TiO₂ deposition time and delivered dose suggested that TiO₂ nanoparticles were completely delivered onto cells at the administered dose range beyond 100 µg/mL. While the calculated delivered dose was lower than TiO₂ administered dose within [100 fg/mL, 100 µg/mL]. A summary of observations in this part was listed in **Table 5.2**, according to TiO₂ administered dose and calculated delivered dose.

Table 5.2 TiO₂ concentration range in the induction of toxicity and autophagy

Administered dose range	Delivered dose range	Cytotoxicity	Autophagy	ROS generation	DNA damage
50 fg/mL	11.35 fg/mL	-	-	-	-
[100 fg/mL, 10 µg/mL)	[22.7 fg/mL, 1.62 µg/mL)	-	+	-	-
10 µg/mL	1.62 µg/mL	+	+	-	-
(10 µg/mL, 250 µg/mL]	(1.62 µg/mL, 250 µg/mL]	+	+	+	+
(250 µg/mL, 500 µg/mL]	(250 µg/mL, 500 µg/mL]	+	-	+	+

(-) negative observation, (+) positive observation

TiO₂ nanoparticles were first characterized to form aggregates in serum-free HEKCM at pH 7.5. For nanotoxicity study, it has been accepted that nanoparticle-aggregates also can provide stable suspensions for a relatively correct dose. Additionally, TiO₂ nanoparticles form aggregates in sunscreen formulations as well [8, 10]. Therefore, it is biologically relevant to observe toxicity caused by TiO₂ aggregates in the interaction with skin keratinocytes. Nonetheless, it is recognised

that *in vitro* studies such as these do not replicate the complexities of real skin exposure scenarios, which will need more realistic models to fully simulate.

The focus of this study was the final toxic effects caused by TiO₂ nanoparticles of different administered concentrations at the end of 24 h treatment duration. TiO₂ aggregates, whether they were uptaken by cells or just delivered onto cell surface, caused direct injury to cells as well as indirect stress in the cell growth environment. The purpose of this study was to observe certain cellular damage in keratinocytes in the presence of TiO₂ nanoparticles regardless of whether nano-TiO₂ particles were fully uptaken into cells. Cellular uptake validation experiments such as ICP-MS require the minimum amount of measured dose (above ~ µg/mL), therefore, it is difficult to measure cellular uptake when the administered dose is in the lower range (fg/mL ~ ng/mL). Whereas ISDD calculation is capable of predicting the maximum administered particles delivered to keratinocytes in any dose range, which is more suitable for this wide dose relevance study. This simulation considers a number of empirically determined parameters that had been used in the experiments. It has been validated against empirical results over the parameter ranges of interest, and it attains good accuracy of results for cell culture systems.

ISDD calculation was supported by TEM observation. The delivery curve for high and low TiO₂ administered dose varied greatly. After 24 h treatment, the calculated delivered dose of high concentrations above 100 µg/mL almost equal to administered dose, whereas that of low concentrations below 100 µg/mL was lower than the administered dose. The delivered dose calculation showed that more TiO₂ nanoparticles were delivered to cells under the high dose regime at the end of 24 h, suggesting more TiO₂ aggregates could be endocytic uptaken into cells. TEM results were consistent with this calculation: more visible TiO₂ aggregates were found inside endocytic vesicles in the cytoplasm with the increase in TiO₂ administered dose. In comparison,

ISDD calculation showed that fewer TiO₂ nanoparticles were delivered to cells under the low dose regime at the end of 24 h, indicating fewer particles could enter cells. Thus, fewer visible TiO₂ nanoparticles inside cells were captured by TEM. Even under the low dose regime, however, TiO₂ nanoparticles were still capable of inducing autophagy in keratinocytes.

TiO₂ nanoparticles were observed to induce autophagy in keratinocytes at approximately 100 fg/mL of administered dose. But the calculated delivered dose was much lower as 22.7 fg/mL for autophagy induction. No significant cytotoxicity was observed in autophagy activated cells within subtoxic TiO₂ administered dose range, [100 fg/mL, 10 µg/mL). Notably, autophagy was upregulated before cytotoxicity was observed in subtoxic TiO₂ concentrations treated cells. Autophagy is known to be an essential defense mechanism that maintains cellular homeostasis in the skin [132, 133]. Some skin pathological conditions such as infection could reduce skin barrier functions. Recent findings suggest that autophagy contributes to control the spread of infection and some skin diseases [134, 135]. Autophagy has been observed to defend the skin against pathogens by enveloping invaded bacteria into autophagosomes and eliminating them through the fusion of vacuoles with lysosomes [136]. It is possible that autophagy is a reaction of keratinocytes to such ultrafine TiO₂ particles. There remains the likely possibility that autophagy induced by TiO₂ nanoparticles in keratinocytes may be an attempt to degrade those particles that have been perceived as “aberrant bodies”. TEM results showed that TiO₂ nanoparticles were mostly observed in autophagic compartments, suggesting that keratinocytes attempted to sequester these nanoparticles by autophagy even before cytotoxicity was generated. Skin cells may upregulate autophagy to target nanoparticles entered into cytoplasm through a pathway similar to pathogens [80, 136]. Whether nanoparticles would be eventually degraded by autophagy, requires further

exploration. But once the skin barrier is compromised, the increased penetration of nanoparticles may probably upregulate autophagy.

Within administered dose range, (10 $\mu\text{g/mL}$, 250 $\mu\text{g/mL}$], TiO_2 -induced autophagy was accompanied by cytotoxicity, oxidative stress, and genotoxicity. When TiO_2 administered concentrations reached over 100 $\mu\text{g/mL}$, the maximum administered particles were completely delivered to cells at the end of 24 h, inducing approximately 50% reduction in cell viability at the highest concentration of 500 $\mu\text{g/mL}$. Results suggested that TiO_2 nanoparticles did not cause a severely toxic effect on cell viability, which was consistent with other reports of low toxicity induced by TiO_2 nanoparticles. Autophagy was also reduced in samples treated with TiO_2 highest concentration of 500 $\mu\text{g/mL}$. The complete delivery of administered particles resulted in necrosis, apoptosis, and the increased level of p62 expression possibly due to the maximum cellular uptake of TiO_2 aggregates. Therefore, the decreased level of autophagy in TiO_2 highest concentration treated cells was not because of the acute toxicity due to which the cell's metabolism and ability to upregulate autophagy was impaired, but probably due to the increase in the proportion of necrotic and apoptotic cells. The reduced autophagy induction did not necessarily imply that autophagy did not happen to those cells during earlier treatment time points before they underwent apoptosis and necrosis, as pointed out in the literature review. There is a complex crosstalk between autophagy, apoptosis, and necrosis. Autophagy is reported to play an anti-necrosis function in cellular injury and inflammation [137]. But this role of anti-necrosis may indirectly act as an enabler of apoptosis which eventually results in cell death [137]. Autophagy might first happen to keratinocytes under highest TiO_2 concentration treatments to serve as a pro-survival mechanism via its suppressive role on necrosis during earlier treatment time points. But eventually,

the existence of autophagy as a protective mechanism failed to prevent cell death from necrosis and apoptosis after certain hours of treatment.

Besides these, toxic dose range of TiO₂ nanoparticles generated cellular ROS. ROS production is potentially deleterious and excessive ROS will damage mitochondria and eventually lead to cell death. At toxic TiO₂ dose range, autophagy may be potently induced through the oxidative stress mechanism [138], such as the accumulation of damaged mitochondria, or endoplasmic reticulum stress. TEM results identified the presence of damaged mitochondria in autolysosomes with TiO₂ aggregates in keratinocytes. This observation suggested that mitochondria damaged by TiO₂ nanoparticles were removed via autophagy, probably for the purpose of nutrient and energy recycling [139]. Autophagy is suggested to act as a survival mechanism that prevents the accumulation of damaged mitochondria as well as reduces ROS accumulation [140]. Excessive ROS accumulation would irreversibly oxidize DNA, thereby causing genotoxicity in keratinocytes. CellROX[®] data showed the localization of ROS signal was in the cytoplasm and nucleus areas, suggesting that TiO₂-generated ROS further caused secondary genotoxicity via oxidative stress at toxic dose range. TEM images showed the localization of one small piece of TiO₂-aggregates in the nucleus of one keratinocyte under the exposure of administered dose of 50 fg/mL. Smaller than some critical size, TiO₂ aggregates may penetrate into nucleus to induce primary genotoxicity through the direct interaction with genomic DNA.

Finally, the expression of p62, TRAF6, and NF-κB in cells under the exposure of toxic TiO₂ administered doses may suggest the possible interplay of autophagy with inflammation. Autophagy has been recognized to play a role in inflammation [141]. The p62 protein can bind LC3 to serve as a selective substrate of autophagy. The level of LC3-II decreased in samples treated with the highest concentration of TiO₂ 500 µg/mL. While the level of p62 increased in the

same samples, but not in samples treated with low TiO₂ concentrations, suggesting that the regulation of p62 was mediated by autophagy. Autophagy was reduced in samples treated with TiO₂ 500 µg/mL, thus, the accumulation of p62 in the highest concentration of TiO₂ 500 µg/mL could be a good indicator of autophagy suppression. Lee *et al.* [142] showed autophagy negatively regulated p62 expression in keratinocytes. The genetic knockdown of p62 gene resulted in the reduction of inflammatory cytokines, suggesting a linear model of which p62 levels upregulated in autophagy-deficient cells, which then promoted inflammation [142]. In addition, the expression level of p62 can also be regulated independent of autophagy. It is suggested that p62 coordinates the processes required for metabolic homeostasis and inflammation, including NF-κB activation and TRAF mediated cellular signal transduction [127, 128]. It interacts with NF-κB and TRAF6 in the recruitment of pro-inflammatory, DNA damage repair, and autophagy induction [129-131]. In this study, the increased level of p62 expression, which accumulates in autophagy-deficient cells, may activate the pro-inflammatory transcription factor NF-κB through a mechanism involving TRAF6 oligomerization [143]. However, because of the complexity of this system, more protein markers require further elucidation.

Taken together, all these data suggested that TiO₂ nanoparticles were capable of inducing autophagy within subtoxic administered dose range, [100 fg/mL, 10 µg/mL), which was not accompanied with acute cytotoxicity and genotoxicity. The interaction of TiO₂ nanoparticles with autophagy pathway seems to be an advantageous scenario. Results showed that autophagy itself did not cause excessive cytotoxicity or DNA damage in keratinocytes. Autophagy activation appearing in advance of cell death, oxidative stress, and genotoxicity were seen in subtoxic TiO₂ concentrations treated cells. It has been suggested that cells can phagocytose small insoluble particles, which release ROS [144]. Lysosomal enzymes would subsequently be activated in order

to degrade these insoluble particles, or attempt to convert them to a safer form [144]. When too many insoluble particles are phagocytosed into the cells, the accumulation of these particles would result in oxidative damage, cell cycle disruption, and ultimately cytotoxicity [145]. Therefore, autophagy might be a cyto-protective immune response of keratinocytes to sequester phagocytosed TiO₂ nanoparticles, as well as to remove proteins and organelles damaged by TiO₂ nanoparticles. Of note, as autophagy is also related to skin homeostasis and pathological conditions, autophagy ought to be considered in TiO₂-induced toxicity in skin keratinocytes. TiO₂-induced autophagy in keratinocytes ought to be given attention when applying TiO₂ nanoparticles in skin products.

Chapter 6

ZnO Nanoparticles induce Autophagy and Toxicity in Primary Human Keratinocytes

6.1 Outline

Historical documents provide the evidence that even early human civilizations used various methods of skin protection from sunlight damage. Ancient Greeks, for instance, chose olive oil while ancient Egyptians recommended extracts of a few plant products for this purpose [146]. ZnO paste has been found in skin care and protection products for thousands of years [147]. Nowadays, ZnO nanoparticle powders are widely used in sunscreens, and skin exposure has become the predominant consumer exposure to final ZnO products [148]. However, the safety of nano-ZnO compound for human health is still under debate. Although most studies regarding dermal exposure of ZnO nanoparticles show that there is no penetration of ZnO nanoparticles in sunscreens beyond the stratum corneum or only a small extent that crossed the skin [149], there are remaining safety concerns towards the exposure of ZnO nanoparticles to damaged skin or long-term exposure of nano-ZnO.

In this chapter, potential toxic effects caused by ZnO nanoparticles in keratinocytes were explored in a wide concentration range. A dosimetry model was also used to compare certain toxicity between administered dose and delivered dose.

6.2 Results

6.2.1 Physical Characterization of ZnO Nanoparticles

Morphology and pristine particle size evaluated by TEM as well as DLS measurements of ZnO nanoparticles were shown in **Figure 6.1**.

Figure 6.1. Physical characterization of ZnO nanoparticles. (A) TEM documentation of morphology and pristine particle size of nano-ZnO. **(B)** DLS measurements of different concentrations of ZnO nanoparticles in ultrapure water and EpiGRO™ serum-free HEKCM. Data represent mean $\pm$ SD (n=3).

The particles were mostly shown in a near-spherical shape with a mean diameter at 21.46 ± 3.17 nm (Calculation was provided by ImageJ software under the measurement of 50 random ZnO nanoparticles). The mean hydrodynamic diameter (d_H) of different ZnO concentrations in ultrapure

water (Milli-Q, pH 6.0) and in EpiGRO™ serum-free HEKCM were listed above. Values of d_H suggest that in ultrapure water, ZnO nanoparticles found in agglomerates⁶. d_H values for the same ZnO concentrations in EpiGRO™ serum-free HEKCM were not decreased greatly due to the lack of serum to help in good ZnO dispersal in an aqueous condition. The zeta potential of ZnO nanoparticles in ultrapure water (pH 6.0) was $+13.7 \pm 1.4$ mV while this value changed to -24.6 ± 2.6 mV when ZnO nanoparticles were dispersed in HEKCM (pH 7.5).

6.2.2 Delivered Dose and Released Zn^{2+} of ZnO Nanoparticles

Details of ISDD calculation of delivery dose has been described in Chapter 5, section 5.2.2. Herein, the effective density (ρ_{EV}) value for ZnO nanoparticles was 2.26 ± 0.001 g/cm³. ISDD simulations predicted the fraction (f_D) of ZnO nanoparticles delivered to cells within 24 h. The value of f_D was plotted against ZnO treatment time. The area under the curve (AUC) showed a time-weighted average factor ($fracAUC$). Delivered dose ($M_{D \max}$) was the maximum mass of administered ZnO dose delivered to cells within 24 h. Representative dosimetry results of ZnO administered dose at 50 µg/mL were shown in **Figure 6.2**. The delivery curve of ZnO administered dose was determined by ρ_{EV} , d_H , treatment time, and cell culture conditions. Administered ZnO dose was completely delivered to keratinocytes cultured in 96-well plates, 6-well plates, and T75 flasks at the end of 12 h, 8 h, and 8 h, respectively. Delivered dose ($M_{D \max}$) and time-weighted average dose (TWAD) at the end of 24 h treatment were normalized to average cell yield of different culture conditions to calculate mean $M_{D \max}$ and TWAD per cell.

⁶ According to International Standards Organization (ISO 14887), “aggregation” suggests the assemblage of particles rigidly joined together, while “agglomeration” suggests the assemblage of particles which are loosely coherent and transient [24].

A) Cell culture conditions, maximum ZnO delivery time and mass, and time-weighted average dosage

Culture Conditions	Media Height (m)	Media Volume (mL)	Average Cell Yield	$T_{\max}$ (h)	$M_{D \max}$ (μg)	$M_{D \max}$ per cell (μg)	fracAUC	TWAD ($\mu\text{g}\cdot\text{h}$)	TWAD per cell ($\mu\text{g}\cdot\text{h}$)
96-well plate	0.003242	0.1	3.2×10^4	12	5	1.56×10^{-4}	17.845	89.225	2.78×10^{-3}
6-well plate	0.002071	2	9.5×10^5	8	100	1.05×10^{-4}	20.001	2000.1	2.1×10^{-3}
T75 flask	0.002	15	7.5×10^6	8	750	1.00×10^{-4}	20.189	15141.75	2.02×10^{-3}

$T_{\max}$, the time required for the maximum delivery of ZnO nanoparticles in EpiGRO™ media suspension; $M_{D \max}$, the maximum mass of the administered dose deposited within 24 h treatment; $M_{D \max}$ per cell, $M_{D \max}$ / average cell yield; fracAUC, time-weighted average factor at the treatment time of 24 h; TWAD, time-weighted average dosage after 24 h treatment, $\text{TWAD} = \text{fracAUC} \times M_{D \max}$; TWAD per cell = $\text{fracAUC} \times M_{D \max}$ per cell.

Figure 6.2. Delivery dose of ZnO nanoparticles in media suspension. (A) Dosimetry parameters for keratinocytes cultured in different conditions. **(B)** Modeling of ZnO delivery over 24 h treatment time indicated by the fraction of the administered dose, f_D . **(C)** Modeling of ZnO delivery in different culture conditions over 24 h treatment time indicated by M_D per cell.

Figure 6.2B showed the modelling of ZnO delivery over 24 h by the fraction of the administered dose, f_D . The administered dose for this figure was 50 $\mu\text{g}/\text{mL}$. f_D went to 1 (equal to $M_D =$ administered dose) when the administered particles were delivered to cells completely. Dosimetry data suggested that the delivered dose at the end of 24 h was equal to the administered ZnO dose in all concentration groups investigated. ZnO nanoparticles are unable to remain a stable state in cell culture media suspensions [150]. ICP-MS was used to measure ZnO solubility by quantifying

for Zn^{2+} ions. ICP-MS data suggested the solubility of ZnO nanoparticles achieved a substantial level with the increased time in EpiGRO™ serum-free HEKCM (**Figure 6.3**). The concentration of Zn^{2+} from ZnO 50 $\mu\text{g/mL}$ reached approximately 16 $\mu\text{g/mL}$ in media at the end of 24 h.

Figure 6.3. Measurement of free Zn^{2+} ions by ICP-MS. The released Zn^{2+} from ZnO nanoparticles at the concentration of 50 $\mu\text{g/mL}$ in EpiGRO™ serum-free HEKCM (Credits: Mustafa Hussain Kathawala).

6.2.3 Cytotoxicity induced by ZnO Nanoparticles

Morphology changes of keratinocytes exposed to different concentrations of ZnO nanoparticles at the end of 24 h were shown in **Figure 6.4**. Untreated keratinocytes grew in a typical pointy star-like shape and formed homogenous monolayers. Concentrations of ZnO nanoparticles below 5 $\mu\text{g/mL}$ did not cause morphology changes in keratinocytes. Those cells still grew in regular shape and size with a large percentage of healthy cells. With the increase in concentration up to 5 $\mu\text{g/mL}$, keratinocytes gradually lost their regular shape and enlarged in size for differentiation. When the concentration reached 10 $\mu\text{g/mL}$ and above, keratinocytes retracted into spherical shape, leaving detached dead cells on the top of cell culture media.

Figure 6.4. Phase contrast images of keratinocytes at high density in EpiGRO™ serum-free HEKCM.

(A) Untreated negative control showed typical pointy star-like shape of small polygonal cells forming homogenous monolayers. (B, C, D) Cells showed similar morphology and maintained a high mitotic index in the presence of 50 pg/mL, 50 ng/mL, and 1 µg/mL of ZnO nanoparticles for 24 h in comparison with negative controls. (E) Cells exposed to ZnO 5 µg/mL for 24 h became irregular in size and shape with the enlargement of senescent cells in culture accompanied by differentiation. (F) Cells treated with ZnO 10 µg/mL for 24 h showed apoptotic and necrotic aspect swollen out and detached from the culture plate. *Scale bar: 50 µm.*

Cytotoxicity was observed to be induced in cells under the exposure of ZnO nanoparticles (Figure 6.5). Cells under the exposure of ZnO administered concentrations of 5 µg/mL, 10 µg/mL, and 50 µg/mL for 24 h showed a statistically significant reduction in cell viability in comparison with untreated negative controls ($p < 0.05$). ZnO treatments at the administered dose of 1 µg/mL and below, as well as 4 µM tamoxifen treatment did not cause the statistical decrease in cell viability, compared to negative controls.

Figure 6.5. Relative cell viability by WST-8 assay. Primary human keratinocytes were incubated with different concentrations of ZnO nanoparticles and 4 µM tamoxifen for 24 h before analysis. Untreated cells were used as negative controls. WST-8 assay was performed on all cells. Data represent mean ± SD (n=3). * $p < 0.05$, ** $p < 0.01$.

The comparison of cytotoxicity caused by ZnO administered dose and delivered dose showed the same toxic dose range from 5 µg/mL to 50 µg/mL because the maximum administered ZnO dose was completely delivered to cells in all treatment groups at the end of 24 h (**Figure 6.6**). Cytotoxicity data suggested that there was a concentration range as small as between 1 and 5 µg/mL of ZnO nanoparticles in which relative cell viability decreased sharply from 86.69% to 47.78%. This value greatly dropped to 3.77% at the concentration of 10 µg/mL and continued dropping to 1.75% at the concentration of 50 µg/mL. It was suggested that both ZnO administered and delivered doses beyond 10 µg/mL induced complete cytotoxicity in keratinocytes at the end of 24 h exposure. Low concentrations [50 pg/mL, 1 µg/mL) can be considered as subtoxic concentration range.

Figure 6.6. The comparison of cell viability between ZnO administered dose and delivered dose.

Primary human keratinocytes were incubated with different concentrations of ZnO nanoparticles for 24 h before analysis. Untreated cells were used as negative controls. WST-8 assay was performed on all cells. Delivered dose was calculated by ISDD model. **(A)** Cell viability of ZnO administered dose. **(B)** Cell viability of ZnO delivered dose. Delivered dose at the end of 24 h was equal to maximum ZnO administered dose. Data represent mean $\pm$ SD (n=3). * $p < 0.05$, ** $p < 0.01$.

The reduction in cell viability between ZnO 1 and 10 $\mu\text{g/mL}$ was further analyzed by flow cytometric FITC-labeled Annexin V/PI staining of cell populations (**Figure 6.7**). Flow cytometry diagrams showed that 94.91% of keratinocytes were viable in negative controls. Cells exposed to ZnO 1 $\mu\text{g/mL}$ displayed 94.1% of living cells, while with the increase in ZnO concentrations up

to 10 $\mu\text{g/mL}$, a massive shift was observed towards increased values of apoptotic cells (40.62%) and necrotic cells (22.46%), corresponding to morphological changes of cell shape and size as well as the reduction in relative cell viability.

Figure 6.7. FITC-labeled Annexin V/PI analysis of viable, apoptotic and necrotic keratinocytes after 24 h exposure to different concentrations of ZnO nanoparticles. Flow cytometry scatterplots showed Annexin V/PI staining of untreated cells and cells exposed to 1 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$ of ZnO nanoparticles. Numbers in each quadrant indicate the percentage of total cells for the respective quadrant. V = viable cells, A = apoptotic cells, N = necrotic cells.

6.2.4 Profile of Autophagy induced by ZnO Nanoparticles

ZnO-associated autophagy was identified by MDC staining. MDC results quantified autophagic vacuoles in cells exposed to a wide concentration range of ZnO nanoparticles for 24 h (**Figure 6.8**). There were statistically significant increases in the number of autophagic vacuoles in cells treated with ZnO nanoparticles within the administered concentration range from 50 $\mu\text{g/mL}$ to 1 $\mu\text{g/mL}$, and 4 μM tamoxifen ($p < 0.05$), compared to untreated negative controls. There were also statistically sharp decreases in the number of autophagic vacuoles in cells exposed to ZnO administered concentrations of 10 and 50 $\mu\text{g/mL}$ ($p < 0.01$) in comparison with untreated cells.

No statistical increase or decrease in MDC signal was found in cells exposed to ZnO 1 pg/mL, 10 pg/mL, and 5 µg/mL.

Figure 6.8. ZnO nanoparticles induced Autophagy. Keratinocytes were treated with different administered concentrations of ZnO nanoparticles and 4 µM tamoxifen for 24 h before analysis. Cells were stained with MDC and fluorescence was quantified by a microplate reader. Data represent mean ± SD (n=3). * $p < 0.05$, ** $p < 0.01$.

As mentioned above, the maximum administered ZnO dose was completely delivered to keratinocytes in all treatment groups at the end of 24 h, therefore, there was no difference in ZnO delivered dose induced autophagy, compared to administered dose induced autophagy (**Figure 6.9**). MDC data suggested that ZnO nanoparticles were capable of triggering autophagy in keratinocytes at the concentration of approximately 50 pg/mL. Autophagy induction was found in cells under the treatment of ZnO concentrations [50 pg/mL, 1 µg/mL]. The statistical decrease in MDC signal in cells exposed to ZnO 10 and 50 µg/mL was likely attributed to the sharp reduction

in relative cell viability to 1.75%. The majority of keratinocytes underwent apoptosis and necrosis when ZnO concentration reached 10 $\mu\text{g/mL}$ and beyond. No autophagy induction was observed at the end of 24 h treatment, but this did not imply that autophagy did not happen to cells during earlier treatment time points before dying. There is a complex crosstalk between autophagy, apoptosis, and necrosis. Autophagy was reported to play an anti-necrosis function in cancer, injury, and inflammation [137]. Autophagy might first happen to keratinocytes under high ZnO dose treatments to serve as a pro-survival mechanism via its suppressive role on necrosis during earlier treatment time points. But eventually, autophagy failed to prevent cell death from necrosis and apoptosis after certain hours of treatment.

Figure 6.9. The comparison of autophagy between ZnO administered dose and delivered dose.

Primary human keratinocytes were incubated with different concentrations of ZnO nanoparticles and 4 μ M tamoxifen for 24 h before analysis. Untreated cells were used as negative controls. MDC staining was performed on all cells. Delivered dose was calculated by ISDD model. **(A)** Autophagy induced by ZnO administered dose. **(B)** Autophagy induced by ZnO delivered dose. Delivered dose at the end of 24 h was equal to maximum ZnO administered dose. Data represent mean $\pm$ SD (n=3). * $p < 0.05$, ** $p < 0.01$.

LC3 immunoblotting and its turnover assay were further used to examine the presence of autophagosomes in cells exposed to ZnO 50 pg/mL, 10 μ g/mL, and 4 μ M tamoxifen. LC3 immunoblot can detect two bands of LC3 protein, LC3-I and LC3-II. The conversion from LC3-I to LC3-II indicates the formation of autophagosome as the presence of LC3-II is on the membrane of double-membrane autophagosomes. The amount of LC3-II is roughly correlated with autophagosome numbers. Cells were exposed to 4 μ M tamoxifen, which served as autophagy positive control, negative media control, 50 pg/mL and 10 μ g/mL of ZnO nanoparticles in the presence and absence of lysosome inhibitors (NH₂Cl/Leupeptin, used for LC3 turnover assay) for 24 h before analysis.

LC3 immunoblot results showed the migration position of LC3-I and LC3-II, and further suggested the upregulation of LC3-II protein in cells treated with 4 μ M tamoxifen and ZnO 50 pg/mL (**Figure 6.10A**). Samples treated with 4 μ M tamoxifen and ZnO 50 pg/mL in the presence of lysosome inhibitors showed an increase in LC3-II expression in comparison with samples without inhibitors, which was due to the blockage of autophagosome-lysosome fusion, resulting in the accumulation of autophagosomes. Densitometry readings for LC3-II/LC3-I ratio and the amount of LC3-II were shown below the immunoblotting (**Figure 6.10B, C**). In the presence and absence of lysosome inhibitors, 4 μ M tamoxifen and ZnO 50 pg/mL resulted in greater LC3-

II/LC3-I ratio and LC3-II amount in keratinocytes than those of untreated negative controls ($p < 0.05$) as well as ZnO 10 $\mu\text{g/mL}$ treated groups. LC3 immunoblotting and its turnover results were consistent with MDC quantification data, suggesting ZnO nanoparticles induced autophagy in keratinocytes at the concentration of approximately 50 pg/mL .

Figure 6.10. Expression levels of LC3-I and LC3-II in keratinocytes under ZnO treatments. (A) Immunoblotting results of LC3 protein. Positions of LC3-I and LC3-II were indicated. Wild-type of keratinocytes were treated with 4 μM tamoxifen, 50 pg/mL and 10 $\mu\text{g/mL}$ of ZnO nanoparticles for 24 h before being lysed for western blot analysis. Media treatments served as negative controls. (B) Densitometry readings of LC3-II/LC3-I ratio for experimental treatments in the presence and absence of lysosome inhibitors were compared independently to untreated negative controls w/o lysosome inhibitors. Data represent mean $\pm$ SD ($n=3$). * $p < 0.05$ compared to untreated negative controls without lysosome inhibitors. # $p < 0.05$ compared to untreated negative controls with lysosome inhibitors. (C) Densitometry

values of LC3-II for experimental treatments in the presence and absence of lysosome inhibitors were compared independently to untreated negative controls w/o lysosome inhibitors. Data represent mean $\pm$ SD (n=3). * $p < 0.05$ compared to untreated negative controls without lysosome inhibitors. # $p < 0.05$ compared to untreated negative controls with lysosome inhibitors.

6.2.5 ZnO Nanoparticles caused Oxidative Stress in Keratinocytes

CellROX[®] staining was used to examine whether the exposure of ZnO nanoparticles to keratinocytes would result in ROS generation and oxidative stress. Staining results suggested that ZnO nanoparticles did not increase ROS levels in cells at subtoxic concentrations: 50 pg/mL, 50 ng/mL, and 500 ng/mL. While ROS signal was predominantly detectable in cells under ZnO treatments at 1, 5, and 10 μ g/mL in comparison with untreated negative controls (**Figure 6.11**).

Figure 6.11. Oxidative stress induced by ZnO nanoparticles. (A) Fluorescent microscopy analysis of ROS induced by different concentrations of ZnO nanoparticles measured with 5 μ M CellROX[®] green

reagent in keratinocytes. Increased production of ROS was shown in 1 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, and 10 $\mu\text{g/mL}$ ZnO nanoparticles treated groups. Images are representative of three independent experiments. *Scale bar: 10 μm .* **(B)** Quantitative comparison of ROS-positive cells, normalized to negative controls. Data represent mean $\pm$ SD (n=3). * $p < 0.01$ compared with negative controls.

The detected ROS signal in cells treated with ZnO 1 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, and 10 $\mu\text{g/mL}$ was localized in the cytoplasm and nucleus areas. Quantitative comparison of ROS staining among different ZnO concentration groups was normalized to negative controls. There were statistically significant increases in ROS-positive living cells in ZnO 1, 5, and 10 $\mu\text{g/mL}$ treatment groups ($p < 0.01$), compared to negative controls. Data showed ZnO nanoparticles (21.46 nm) induced a concentration-dependent toxicity by generating ROS and oxidative stress. Oxidative stress and ROS triggered by ZnO nanoparticles may be the key route in the induction of cytotoxicity in keratinocytes.

6.2.6 Genotoxicity induced by ZnO Nanoparticles accompanied by Autophagy

ZnO nanoparticles were expected to cause genotoxicity in keratinocytes, as they induced oxidative injury to genomic DNA. DNA damage was examined by γ -H2AX immunofluorescent staining in cells treated with different ZnO concentrations and 4 μM tamoxifen for 24 h, as well as cells exposed to UV light for 15 min (**Figure 6.12**). The exposure of cells to UV light was used as a positive control for DNA damage because UV light directly induced DNA damage. There was a dramatic increase in γ -H2AX protein expressed in the nucleus of keratinocytes treated with UV light for 15 min and ZnO 10 $\mu\text{g/mL}$ for 24 h, compared to negative controls. There was no significant elevated γ -H2AX signal in cells under subtoxic ZnO concentration treatments but only a small micronucleus was found in ZnO 10 ng/mL treated cells (arrow). The signal of γ -H2AX

was also found at ZnO 1 $\mu\text{g/mL}$ and beyond. Quantification of $\gamma\text{-H2AX}$ staining showed that UV light 15 min exposure, ZnO 1 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, and 10 $\mu\text{g/mL}$ resulted in 1.70, 1.25, 1.31, and 1.62 times the number of cells which carried positive $\gamma\text{-H2AX}$ marker, respectively ($p < 0.05$), compared to negative controls. ZnO 10 $\mu\text{g/mL}$ treatment showed substantially extensive DNA damage in comparison with UV light treatment.

Concomitantly, LC3 immunofluorescent staining showed the expression of LC3 protein in cells treated with ZnO subtoxic concentrations below 5 $\mu\text{g/mL}$, and 4 μM tamoxifen for 24 h, compared to negative controls. In contrast, cells under UV light treatment, ZnO 5 and 10 $\mu\text{g/mL}$ treatments did not show LC3 expression. Quantitative comparison of LC3 staining normalized to negative controls showed that 4 μM tamoxifen, ZnO subtoxic concentrations: 50 pg/mL , 10 ng/mL , 100 ng/mL , and 1 $\mu\text{g/mL}$ resulted in 3.03, 1.87, 2.09, 2.28, and 2.29 times the number of LC3-positive cells, respectively ($p < 0.05$) in comparison with negative controls.

The comparison of LC3 staining with $\gamma\text{-H2AX}$ staining suggested that autophagy occurred to cells exposed to subtoxic ZnO concentrations below 1 $\mu\text{g/mL}$ while DNA damage was not severe within the same dose range. Both $\gamma\text{-H2AX}$ and LC3 markers were observed in ZnO 1 $\mu\text{g/mL}$ treated groups. LC3 expression reduced while $\gamma\text{-H2AX}$ levels dramatically increased in cells exposed to ZnO nanoparticles within the dose range between 5 and 10 $\mu\text{g/mL}$. Results showed that ZnO nanoparticles caused genotoxicity in a concentration-dependent manner in keratinocytes. ZnO-induced DNA damage was accompanied with autophagy within the concentration range [1 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$).

Figure 6.12. Detection of DNA damage and autophagy by γ -H2AX and LC3 staining. (A) Epifluorescent microscopy images of γ -H2AX and LC3 immuno-labeled human keratinocytes. Increased expression of γ -H2AX protein was shown in UV treated groups as well as 1 μ g/mL, 5 μ g/mL, and 10 μ g/mL ZnO treated groups. Increased expression of LC3 protein was shown in 4 μ M tamoxifen and ZnO concentrations below 5 μ g/mL treated groups. Images are representative of three independent experiments. Scale bar: 10 μ m. (B) Quantitative comparison of γ -H2AX staining, normalized to negative controls. Data represent mean $\pm$ SD (n=3). * p < 0.05 compared to untreated negative controls. (C) Quantitative comparison of LC3 staining, normalized to negative controls. Data represent mean $\pm$ SD (n=3). # p < 0.05 compared to untreated negative controls.

6.2.7 ZnO-induced Toxicity is associated with p62, TRAF6, and NF- κ B activation

p62 protein functions as a hub in the connection of inflammation and autophagy. It also mediates the activation of NF- κ B signaling, as well as interacts with another signaling modulator, TRAF6. The expression of p62, TRAF6, and NF- κ B (p65/RelA) was examined by immunoblotting in cells exposed to ZnO nanoparticles.

As shown below in **Figure 6.13**, a dramatic increase in p62 expression was observed in cells treated with 10 μ g/mL of ZnO nanoparticles, compared to negative controls ($p < 0.05$). On the contrary, there was a statistically significant decrease in p62 level in cells exposed to 50 μ g/mL of ZnO nanoparticles in comparison with untreated cells ($p < 0.05$). There was no overexpression of p62 in cells exposed to subtoxic ZnO concentrations of 50 pg/mL, 500 ng/mL, 1 μ g/mL, 5 μ g/mL as well as 4 μ M tamoxifen, compared to negative controls.

Figure 6.13. p62 expression of keratinocytes exposed to different concentrations of ZnO nanoparticles.

(A) Immunoblotting results of p62 protein. Wild-type of keratinocytes were treated with 4 μ M tamoxifen and different concentrations of ZnO nanoparticles for 24 h before being lysed for western blot analysis. (B)

Densitometry readings for tamoxifen and ZnO nanoparticles treated keratinocytes were compared independently to untreated negative controls. Data represent mean $\pm$ SD (n=3). * $p < 0.05$ compared to untreated negative controls.

An upregulation of TRAF6 expression was observed in keratinocytes exposed to ZnO nanoparticles with the increased concentrations from 50 pg/mL (**Figure 6.14**). Immunoblotting results showed the trend of an increase in TRAF6 level with the increase in ZnO concentrations, possibly suggesting a concentration-dependent upregulation of TRAF6 protein in ZnO treated samples. However, there was no substantial difference in the expression of NF- κ B in all experimental groups (Figure 6.14).

Figure 6.14. TRAF6 and NF- κ B (p65/RelA) expression of keratinocytes treated with different concentrations of ZnO nanoparticles. Immunoblotting results of TRAF6 and NF- κ B (p65/RelA) protein. Wild-type of keratinocytes were treated with 4 μ M tamoxifen and different concentrations of ZnO nanoparticles for 24 h before being lysed for western blot analysis (Credits: Dr. Esther Wong, Yu Chye Yun).

6.2.8 TEM Results

Ultrastructural changes of keratinocytes of all treatment groups were finally examined by transmission electron microscopy. Untreated cells showed the structure of a typical keratinocyte

(**Figure 6.15A**), while 4 μ M tamoxifen treated cells showed double-membrane autophagosomes and late autophagic compartments (**Figure 6.15B, C**). Double-membrane bound autophagic compartments contained cytoplasmic contents appeared in cells exposed to ZnO 50 μ g/mL (**Figure 6.15D-F**). Keratinocytes under the treatment of ZnO 50 μ g/mL displayed a few of early and/or late autophagic vacuoles (**Figure 6.15G, H**). With the increase in ZnO concentrations up to 1 μ g/mL, more autophagic vacuoles appeared in keratinocytes (**Figure 6.15I, J**). Late autophagic vacuoles contained partially degraded electron-dense contents. In cells treated with 5 μ g/mL of ZnO nanoparticles, few cells also showed autophagic vacuoles in the cytoplasm (**Figure 6.15K, L**). At the concentration of 5 μ g/mL, however, 50% of cells under TEM viewing were found to show necrosis with lesions appeared on the cell surface, disruption of plasma membrane and organelles as well as swelling and cytoplasm vacuolization (**Figure 6.15M**). But double-membrane autophagosomes and late autolysosomes found in ZnO 5 μ g/mL treated groups suggested that autophagy may had once occurred to these cells before they underwent cell death. When ZnO concentration reached 10 μ g/mL, the majority of keratinocytes underwent apoptosis and necrosis (**Figure 6.15N**). In one dying cell, an atypical ring-shape structure located in the nucleus was visible, suggesting the nucleus of this cell was damaged by ZnO nanoparticles (**Figure 6.15O**). One necrotic cell showed a relative preservation of damaging nucleolus (**Figure 6.15P**). One apoptotic cell evidently displayed surface blebbing, apoptotic nuclear morphology, and vacuolization of cytoplasm (**Figure 6.15Q**), while the other apoptotic cell retracted into the spherical shape and displayed a characteristic chromatin aggregation which was strongly different from its normal organization, with a good preservation of cell membrane (**Figure 6.15R**). TEM images confirmed that ZnO nanoparticles induced autophagy, apoptosis, and necrosis in primary human keratinocytes at different concentrations.

Figure 6.15. TEM photomicrographs of keratinocytes under exposure of different concentrations of ZnO nanoparticles. (A) Untreated keratinocytes served as negative controls. (B) Autophagic vacuoles in tamoxifen 4 μ M treated keratinocytes (arrows). (C) Autophagosomes in keratinocytes treated with 4 μ M tamoxifen (arrows). (D) Overview of ZnO 50 pg/mL treated cells showing a few of intracellular vacuoles. (E) Autophagic compartments in cells exposed to 50 pg/mL of ZnO nanoparticles (arrows). (F) Autophagosomes and autolysosomes were found in cells treated with 50 pg/mL of ZnO nanoparticles (arrows). (G) Overview of ZnO 50 ng/mL treated keratinocytes with the increased numbers of vacuoles. (H) An autophagic vacuole was indicated in cells treated with 50 ng/mL of ZnO nanoparticles (arrow). (I) Overview of ZnO 1 μ g/mL treated cells with late autophagic vacuoles (arrow). (J) Different stages of autophagic compartments were observed in cells treated with 1 μ g/mL of ZnO nanoparticles. The presence of an autophagosome was indicated (arrow). (K) One keratinocyte exposed to 5 μ g/mL of ZnO nanoparticles showed the structure of some autophagic vacuoles (arrows). (L) Two early autophagic compartments were shown in ZnO 5 μ g/mL treated cells (arrows). (M) Necrotic cells were found in keratinocytes treated with 5 μ g/mL of ZnO nanoparticles. (N) Overview of ZnO 10 μ g/mL treated cells. One necrotic cell was in the center of the image with two apoptotic cells besides (arrows). (O) A cell with an atypical ring-shape structure (arrow) in the nucleus and cytoplasmic vacuolization was found in keratinocytes exposed to ZnO 10 μ g/mL. (P) A necrotic cell was shown after the treatment of ZnO 10 μ g/mL with a relative preservation of nucleolus (arrow). (Q) One apoptotic cell was shown in keratinocytes exposed to ZnO 10 μ g/mL with cell surface blebbing, apoptotic nuclear morphology, and cytoplasmic vacuolization. (R) A second apoptotic cell was shown in keratinocytes exposed to ZnO 10 μ g/mL. This cell retracted into the spherical shape with chromatin aggregation. Images were acquired at 5,000 – 40,000 $\times$ magnification. *N*: nucleus.

6.3 Discussion

In the light of commercial application of ZnO nanoparticles and potential dermal exposure in sunscreens, a dose relevance study was performed on primary human keratinocytes to explore the potential toxicity induced by ZnO nanoparticles across a wide concentration range. The most recognized cause for ZnO induced toxicity is through the released Zn^{2+} ions [150]. ZnO nanoparticles formed agglomerates in ultrapure water and serum-free HEKCM. However, no ZnO agglomerates were observed in the cells, by TEM at the end of 24 h treatment in all groups. The dosimetry (ISDD model) study of ZnO delivery time and delivered dose suggested that ZnO delivered dose at the end of 24 h was equal to the maximum administered dose in the particular concentration groups investigated. The measurement of ICP-MS suggested that the release of Zn^{2+} from ZnO nanoparticles had reached a significant level over time in EpiGRO™ serum-free HEKCM. The released Zn^{2+} ions from ZnO nano-compound possibly played a main role in causing toxicity. It was suggested that the release of Zn^{2+} ions rather than ZnO agglomerates caused different toxic effects on keratinocytes. According to the observed results, it can be suggested that high dose regime of ZnO with more released Zn^{2+} ions showed a larger effect on cell viability, oxidative stress, DNA damage, and p62 related inflammation when compared with low dose regime.

In this study, near-spherical ZnO nanoparticles (21.46 nm) were found to cause toxicity accompanied with autophagy activation in keratinocytes. There was no observed difference in ZnO-induced cytotoxicity and autophagy levels between administered dose and delivered dose, because the maximum ZnO administered dose managed to be completely delivered to cells at the end of 24 h with an increase in released Zn^{2+} over time. With even slightly increased concentration

of released Zn^{2+} , keratinocytes failed to restore intracellular homeostasis, resulting in observed increased ROS production followed by oxidative DNA damage. ZnO nanoparticles at the concentration of 1 $\mu\text{g/mL}$ was sufficient to generate ROS and cause DNA damage. When the concentration reached 5 $\mu\text{g/mL}$ and beyond, ZnO nanoparticles potentially induced apoptosis and necrosis, as well as nuclear atypia in keratinocytes. All of these observation results suggested that ZnO nanoparticles induced injury to keratinocytes in a concentration-dependent manner, mostly within the concentration range starting from as small as between 1 to 10 $\mu\text{g/mL}$.

Physiologically, very little free Zn^{2+} is usually present in the cytosol of healthy cells [21], therefore, these observed toxic effects is likely to be linked to the released Zn^{2+} ions from ZnO nanoparticles. The upregulation of pro-inflammatory cytokines and mitochondrial damage induced by ZnO nanoparticles were observed in RAW 264.7 and BEAS-2B [151]. It is suggested that the releasing of Zn^{2+} into cell culture media is related to ZnO-induced toxicity [152]. The accumulation of released Zn^{2+} over time in the cytosol is associated with organelle damage, oxidative stress, oxidative DNA damage, intracellular Ca^{2+} release, mitochondrial damage, and cytotoxicity [150]. Herein, ZnO nanoparticles generated ROS in mitochondria and nucleus. The increased ROS level is deleterious and further prevents mitochondria from energy supply for cellular activities and eventually may lead to cell death. Additionally, mitochondrial membranes are sensitive to the change of cytosol ions. The released Zn^{2+} from ZnO nanoparticles followed by the intracellular Ca^{2+} release are suggested to cause depolarization of inner mitochondrial membranes leading to apoptosis [153]. Excessive ROS generated by toxic ZnO concentrations beyond 1 $\mu\text{g/mL}$ resulted in oxidative DNA damage. Cytotoxicity, ROS generation, and DNA damage were found to be co-related at corresponding exposure levels in keratinocytes.

Autophagy is an additional finding in the assessment of ZnO induced toxicity in keratinocytes. Autophagy induction was found in ZnO subtoxic concentrations [50 pg/mL, 1 µg/mL] of which no significant cell death was observed within these dose range. Since no ZnO agglomerates were found in cells at the end of 24 h treatment, autophagy might be living keratinocytes' adaptation to the increased Zn²⁺ concentrations during the interaction with ZnO nanoparticles. Cytotoxicity, ROS generation, and DNA damage caused by ZnO 1 µg/mL were accompanied with autophagy induction. TEM results showed the presence of autophagic vacuoles in few living cells after 24 h exposure of ZnO 5 µg/mL, but no autophagic vacuoles were observed in dying cells when the concentration reached 10 µg/mL. These observations suggested that autophagy may play a pro-survival role in cells under exposure of subtoxic ZnO nanoparticles. The development of autophagosomes by keratinocytes is possibly an immune response to the accumulation of Zn²⁺ ions, ROS production and oxidative damage caused by ZnO nanoparticles.

A few of signal adaptor proteins are suggested to be involved in ZnO-associated toxicity and autophagy. Autophagy is reported to negatively regulate inflammation in keratinocytes through the signal adaptor protein, p62 [142]. The upregulation of p62 protein in ZnO 10 µg/mL treated cells is probably associated with p62 inflammasome activation, which is consistent with the linear model of p62 overexpression in autophagy-deficient cells. Other evidence has suggested the expression of p62 also plays a role in cell survival [154]. Cells with the expression of p62 protein are more likely to be alive than dead, but cells lacking p62 expression are more likely to be dead already [154]. The lack of p62 expression in keratinocytes exposed to ZnO 50 µg/mL was mostly due to the large percentage of cell death. The expression of TRAF6 protein seemed to show an upregulation in cells under the exposure of ZnO nanoparticles with increased concentrations. TRAF6 participates in autophagy pathway associated with p62. The recruitment of p62 in

association with TRAF6 contributes to the ubiquitination of Beclin 1 to trigger and control autophagy against inflammasome activation [155]. The expression of NF- κ B (p65/RelA) did not show a concentration-related change in ZnO treated cells. Pujalté *et al.* [156] reported that ZnO nanoparticles induced the translocation of NF- κ B in HK-2 cells. NF- κ B is also reported to regulate cell survival and apoptosis in cases of inflammation [157] as well as in the activation of autophagy [158]. Therefore, the expression of NF- κ B in ZnO-induced toxicity and ZnO-associated autophagy in keratinocytes requires future exploration.

In summary, ZnO nanoparticles induced toxicity and autophagy was identified within a wide dose range:

Table 6.1 ZnO concentration range in the induction of toxicity and autophagy

ZnO concentration range	Cytotoxicity	Autophagy	ROS generation	DNA damage
[1 pg/mL, 10 pg/mL]	-	-	-	-
[50 pg/mL, 1 μ g/mL)	-	+	-	-
1 μ g/mL	-	+	+	+
(1 μ g/mL, 5 μ g/mL)	+	+	+	+
[5 μ g/mL, 50 μ g/mL]	+	-	+	+

ZnO administered dose equalled to delivered dose after 24 treatment. (-) negative observation, (+) positive observation

Autophagy can be considered as another feature in ZnO-induced toxicity. Autophagy might be a kind of cellular adaptation to the increased Zn²⁺ concentrations during the interaction with ZnO nanoparticles. Signal adaptor protein p62 and TRAF6 are involved, and both interacted in ZnO-induced toxicity with autophagy and inflammation through a concentration-dependent manner. Future studies ought to continue elucidating more details of ZnO-associated autophagy.

Chapter 7

Conclusions and Recommendations

7.1 Conclusions

Nanotechnology, long seen to be an important emerging area, is finally making an impact. With the advent of TiO₂ and ZnO nanoparticles in cosmetics, sunscreens, and other over-the-counter topical products, human end-consumers are coming into close contact with engineered nanomaterials. The field of nanotoxicology needs to advance alongside these developments, so as to ensure that human safety and health concerns are properly addressed in the continued exploitation of nanotechnology. This research focused on the exposure of TiO₂ and ZnO nanoparticles to primary human keratinocytes and the exploration of their possible toxicity in a dose relevance of concentration study.

TiO₂ has been considered as a relatively low toxic nanomaterial. Results from this study suggested that TiO₂ did not cause acute cytotoxicity in keratinocytes but they did generate genotoxicity, oxidative stress and inflammasome activation. TiO₂ nanoparticles have been reported to induce genotoxicity such as genetic mutation, micronuclei formation, and DNA oxidative damage in various cell types. The reported dose from most studies suggested that TiO₂ administered concentrations from 10 µg/mL and beyond started inducing genotoxicity. Results from this study were consistent with these reported work. It was observed within the administered dose range, [10 µg/mL, 500 µg/mL], TiO₂ nanoparticles were able to cause cytotoxicity, oxidative stress, and genotoxicity in keratinocytes. Notably, this study supplemented the deposition time and

delivered dose of TiO₂ nanoparticles that actually caused toxicity, which had been overlooked by most previous work.

Additionally, TiO₂ nanoparticles formed aggregates in serum-free HEKCM. With the increase in concentration, there was an observed increase in aggregate size. The size of TiO₂ aggregates impacts their reactivity, nanoparticle-cellular interactions, and toxic consequences. The state of TiO₂ aggregates in serum-free HEKCM was likely to be predicted in laboratory experiments. At physiological pH 7.4-7.5, TiO₂ nanoparticles were negatively charged and resulted in a higher tendency of aggregation. For nanotoxicity study, it has been accepted that nanoparticle-aggregates also can provide stable suspensions for a relatively correct dose. The dose issue is found to be the characteristic that is damaging keratinocytes. Increasing dose will result in corresponding changes in physical properties of nanoparticles, such as aggregation. The hydrodynamic size (d_H) increased with the increase in nanoparticle concentrations, as nanoparticles would always form aggregates or agglomerates in serum-free media such as HEKCM. The reason of poor nanoparticle dispersion in serum-free media is due to the absence of serum, which would otherwise function as a coating protein, to decrease protein corona levels that act as surfactants to reduce nanoparticle aggregation. In contrast, the hydrodynamic size would be much smaller (but larger than primary size) if nanoparticles are dispersed in serum-supplemented media such as DMEM containing 10% FBS. However, it is the concentration of nanoparticles that causes the stress condition to cells. A high nanoparticle-concentration dispersed in serum-free media would lead to a relatively large d_H value; if dispersed in serum-supplemented media would lead to a smaller d_H value. But this high nanoparticle-concentration, regardless of the dispersion media and the hydrodynamic size, would cause toxicity to damage cells. Observations from this study suggest that the concentration issue has direct impact on toxicity consequences. It is possible that secondary effects of increasing

concentration, such as changes of hydrodynamic sizes, may have contributed to the observed toxicity, but these effects are not separated out in this study.

On the other hand, according to data from this study, it was not due to the released free Ti ions, but nano-TiO₂ particles themselves, causing toxicity in keratinocytes. It can be suggested that higher concentrations of TiO₂ with large visible aggregates showed a larger effect on cell viability, oxidative stress, DNA damage, and p62 related inflammation when compared with lower concentrations with small aggregates. In comparison, the concentration range of ZnO nanoparticles associated with toxicity in keratinocytes was as small as between 1 to 10 µg/mL. ZnO nanoparticles were capable of triggering acute cytotoxicity, oxidative stress, genotoxicity, and p62 related inflammation within a small concentration range. Nano-ZnO particles were unable to remain a stable agglomerate state in serum-free HEKCM, and were found to release Zn²⁺ ions, as measured by ICP-MS. It was supposed that the concentration of released Zn²⁺ ions may cause toxic effects on keratinocytes.

Notably, this is the first study to identify that TiO₂ and ZnO nanoparticles induce autophagy in primary human keratinocytes in a wide dose relevance study. It is important to make this link, because these types of nanoparticles are found widely in today's sunscreen products and have not been previously studied in this light. The reported literature has showed that autophagy was induced by a few of nanoparticles including TiO₂ in certain other cells. But the role of nanoparticle-induced autophagy is still not clear in nanotoxicity research. It is suggested that nanoparticle-induced autophagy is in a size-/shape-dependent manner. For example, if the primary particle size is smaller than a critical size, nanoparticles are able to induce autophagy; while if the primary particle size exceeds a particular critical size, certain large-sized nanoparticles do not trigger an autophagic response. This study first identified that TiO₂ and ZnO nanoparticles with primary

particle sizes around 20 nm, which was considered to be smaller than a critical size, were capable of activating autophagy in keratinocytes at relatively lower administered doses. TiO₂- and ZnO-induced autophagy also takes place in a concentration-dependent manner. This study profiled the exact concentration ranges of TiO₂- and ZnO-induced autophagy for a better understanding.

Nano-TiO₂ can be considered as a potent autophagy inducer as it triggered autophagy in keratinocytes at the administered dose range as low as approximately 100 fg/mL. TEM results from this study showed the localization of ER besides autophagosomes containing TiO₂ nanoparticles and damaged mitochondria. Autophagy was upregulated before cytotoxicity was observed in subtoxic TiO₂ and ZnO concentrations treated cells. These observations suggest that autophagy is upregulated in keratinocytes as an immune response to such ultrafine nanoparticles. Furthermore, autophagy also plays a role in inflammation. This study attempted to show the expression of pro-inflammatory mediators such as p62, NF-κB, and TRAF6 increased in TiO₂ and ZnO treated keratinocytes in a concentration-dependent manner. Both TiO₂ and ZnO nanoparticles were observed to activate p62-mediated inflammasomes, therefore, autophagy upregulation might be a response to the elevated inflammatory with oxidative stress signaling, the accumulation of damaged mitochondria, or ER stress.

Collectively, nanoparticle-induced autophagy is suggested to be initially pro-survival and cytoprotection in keratinocytes under the exposure at subtoxic concentration ranges. From these investigations it can be deduced that autophagy induction figures into TiO₂- and ZnO-induced toxicity. A scheme illustrating the postulated cause of TiO₂- and ZnO-induced toxicity and autophagy is shown in **Figure 7.1**.

Figure 7.1. Putative cause of *in vitro* toxicity and autophagy induced by TiO₂ and ZnO nanoparticles.

The uptake of insoluble TiO₂ nanoparticles by endocytosis resulted in the formation of autophagosomes. ZnO nanoparticles dissolved in culture media and resulted in an increased extracellular Zn²⁺, which in turn caused an increase in intracellular Zn²⁺. TiO₂ nanoparticles and the released Zn²⁺ from ZnO nanoparticles generated ROS cause oxidative damage to genomic DNA at toxic dose range. TiO₂ nanoparticles and impaired mitochondria were enveloped in autophagosomes. ROS generation and oxidative stress further lead to the expression of signal adaptor protein p62, NF-κB, and TRAF6 in inflammasomes activation. Insoluble nanoparticles and damaged organelles might be degraded by autophagy for energy recycling. The released hydrolytic enzymes from autolysosomes plus intralysosomal Zn²⁺, however, would probably result in cytosolic acidification and lysosomal destabilization, which may worsen oxidative stress and mitochondrial dysfunction that potentially lead to necrosis and apoptosis.

In summary, the hypotheses of this study have been proved throughout the experiments and concluded as:

- TiO₂ and ZnO nanoparticles are capable of activating autophagy in primary human keratinocytes.
- TiO₂ and ZnO nanoparticles cause a number of toxicity effects such as cell death, oxidative stress, genotoxicity, and inflammasome activation in primary human keratinocytes.
- The differences in the dose range of TiO₂- and ZnO-induced autophagy and toxicity effects in primary human keratinocytes have been identified in a dose relevance of concentration study.

The novelty and contributions of this study are:

- It is the first report of autophagy induced in primary human keratinocytes by a group of inducers widely found in sunscreens – TiO₂ and ZnO nanomaterials.

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- It conducted and combined several bio-assays with TEM observations to profile the triggering doses of autophagy and toxicity by TiO₂ and ZnO nanoparticles in primary human keratinocytes.
 - It employed a recently developed dosimetry model to identify TiO₂ and ZnO levels causing toxicity and autophagy in a dose relevance of concentration study. The low triggering dose range of autophagy found in keratinocytes can provide useful data points and serve as an impetus for the research community to look more into safe limits of nanomaterials applied into skin products.

At the conclusion of this study, the low triggering concentration levels of nano-TiO₂ and ZnO found are surprising results, and the findings were made possible because a wide concentration range was purposefully investigated in this dose-relevance study. The results strongly suggested in fact that the research community, or the regulatory authorities such as FDA, could take a harder look at prevalent concentration guidelines for human safety, going forward.

7.2 Recommendations

This research has yielded some promising findings in several areas. On the basis of these findings, possible future directions of research moving forward are suggested, as below.

7.2.1 Size- and Shape-dependent Toxicity Study

Toxicity of nanoparticles varies significantly with their size and shape. This study examined TiO₂ and ZnO nanoparticles both in near-spherical shape with average diameters about 20 nm. It is reported that nanoscale ZnO is much more toxic for the same weight than bulk ZnO in human epithelial A549 cells [21]. Different shapes also lead to different types of cytotoxic effects. ZnO

nanoparticles in rod-shape are suggested to cause greater toxicity than the corresponding spherical ones partly because rod-shaped ZnO has a larger contact area with the cell membrane receptors than spherical-shaped ZnO as well as partly because of the more released Zn^{2+} from rod-shaped nanoparticles [21]. Future work can attempt to treat keratinocytes with different sizes or shapes of nanoparticles (Free metal concentration, i.e. ZnSO_4 , is recommended as a control for ZnO study). Exposure to nanoparticles with different sizes and shapes may result in different intracellular responses that lead to the disruption of cell functions for size- or shape-dependent toxicity. Two issues: (i) cell sensitivity and cell response; (ii) cellular force in the interaction with lengthwise-oriented nanoparticles could be evaluated if cells are treated with nanoparticles in different sizes and shapes.

7.2.2 Particle Aggregation Study

Aggregation of nanoparticles in HEKCM was observed in this study. Nanoparticles show a tendency to aggregate in physiological, aqueous environment. This is a phenomenon that needs to be taken into account in future work. The aggregation of nanoparticles can be measured by fluorescence correlation spectroscopy (FCS). FCS is based on the statistical analysis of fluctuations of fluorescence intensity $I(t)$ originating from a small detection volume (typically approximately $1\text{ }\mu\text{m}^3$) defined by point spread function of a confocal microscope [159]. The analysed fluctuations in fluorescence intensity are caused by temporal variations in the number of fluorophores contributing to the intensity collected from the detection volume. Continual diffusion of fluorescent particles (the fluorescently labelled TiO_2 nanoparticles in this case) in and out of the detection volume is usually the dominant source of those variations. The autocorrelation function $G(\tau)$ of the intensity fluctuations (7.1), then, decays on a time-scale defined by the average time a

fluorescent particles spends diffusing within the detection volume (the so-called diffusion time τ_D) [160].

$$G(\tau) = \frac{\langle I(t) I(t+\tau) \rangle}{\langle I(t) \rangle^2} \quad (7.1)$$

In the case of a free Brownian diffusion, the decay of the autocorrelation function is described by equation (7.2). N is the number of independently diffusing particles present on average within the detection volume and ω_0 and ω_z are the characteristic dimensions of the detection volume in lateral and in axial direction respectively [161].

$$G(\tau) = 1 + \frac{1}{N} \frac{1}{1 + (\tau/\tau_D)} \left(\sqrt{\frac{1}{1 + (\tau/\tau_D)(\omega_0/\omega_z)^2}} \right) \quad (7.2)$$

If intersystem crossing of the fluorophore to a non-fluorescent triplet state happens, it represents an additional source of intensity fluctuation on a characteristic time-scale τ_T (usually on μ s scale). The decay of autocorrelation can be then described by (7.3) where F_T is the average fraction of molecules of fluorophore in triplet state [161].

$$G(\tau) = 1 + [1 - F_T + F_T \exp(-\tau/\tau_T)] \frac{1}{N(1 - F_T)} \frac{1}{1 + (\tau/\tau_D)} \sqrt{\left(\frac{1}{1 + (\tau/\tau_D)(\omega_0/\omega_z)^2} \right)} \quad (7.3)$$

The diffusion time τ_D is related to the diffusion coefficient D of the particles and the dimension of the detection volume via (7.4). ω_0 is usually determined though a calibration FCS measurement of diffusion of a standard dye with a well-known diffusion coefficient (such as rhodamine 6G) [162].

$$D = \frac{\omega_0^2}{4\tau_D} \quad (7.4)$$

The hydrodynamic radius r_H of the nanoparticles is obtained from D using (7.5), where T is thermodynamic temperature, k Boltzmann constant and η viscosity of the medium.

$$r_H = \frac{kT}{3\pi\eta D} \quad (7.5)$$

In this way, FCS can be used to determine the size of nanoparticles and study their aggregation state. It has been already shown that FCS is a powerful tool to study size and aggregation of TiO₂ nanoparticles [163-166]. Since aggregation of nanoparticles depends on many factors, such as their size, concentration or ionic strength of the solution [165, 166] and at the same time it can have a profound effect on their penetration to cells and their cytotoxic effects as well, the aggregated state of TiO₂ nanoparticles could be investigated by FCS in detail.

7.2.3 *In vitro* Mechanism Study

This study has preliminarily explored the underlying mechanisms of TiO₂- and ZnO-induced toxicity. Certain toxicity effects involve various cell signaling pathways. Nanoparticle-induced autophagy is a convergence of different modulators. It remains a challenging but useful task to uncover the nanotoxicological mechanisms. It is suggested to employ autophagy protein synthesis and degradation assays to explore the mechanisms for the production and destruction of autophagy related proteins in keratinocytes. Click-iT[®] technology is able to track autophagy pathway with LC3, p62, and other autophagy markers simultaneously to investigate their role in maintaining intracellular homeostasis. It is also suggested using R&D system[™] proteome profiler antibody arrays to measure multiple protein expression in one single sample to eliminate the need for multiple immunoblots. Human phospho-MAPK antibody array, cell stress array, and apoptosis

antibody array will help to determine the role of Akt, ERK, JNK, p38, p53, NF- κ B, and caspase-3 families in nanoparticle-induced toxicity, inflammation, and autophagy.

7.2.4 Toxicity in Compromised Skin Model

This study used *in vitro* keratinocytes model to demonstrate autophagy and toxicity induced by TiO₂ and ZnO nanoparticles. But it remains unclear whether TiO₂ and ZnO nanoparticles could penetrate and cross the healthy and/or compromised skin barrier to interact with skin keratinocytes. It is suggested to employ an *in vivo* and UV irradiation exposure model in future research. Technically, nanoparticles for *in vivo* study could be prepared in a vehicle appropriate for skin application with its material property quantification (i.e. a vehicle contains 75% glycerol, which is commonly present in commercial sunscreens, combined with 25% nanoparticle-stock solution). Animal model is recommended to use 6-week-old outbred SKH-1 wild-type mice weighing 25 – 30g. SKH-1 wild-type mice carry an autosomal recessive hypomorphic mutation called *hr* in the gene *Hairless* [167], which has been well accepted as a model system for the study of skin barrier function and UV irradiation induced skin diseases [168]. The experimental design could use n = 3 mice at different UV radiation exposure conditions, UV-exposed (UVA: 320 – 400 nm, UVB: 290 – 320 nm) and unexposed, at the time point of 24 h after nanoparticle application. Fluorescent microscopy and TEM could be used to examine structural details of nanoparticle-skin penetration through mouse skin and their end locations at the cellular level. Potential results would provide more insights into the capability of nanoparticles found in sunscreens to penetrate intact and UV irradiation compromised skin barrier as well as toxicity thereof.

These directions for future ongoing work in TiO₂ and ZnO nanoparticles related nanotoxicological study are recommended. With the increasing development of highly engineered nanoparticles with

various applications, nanotoxicology will continue to be a prospective and important research area. Interactions at nano-scales are complex and would become quite unlike those at the larger scales that we are typically more familiar with. More specific studies, perhaps employing multi-disciplinary techniques would be required for a sufficient understanding of the physico-chemical-biological nature of particular engineered nanoparticles and underlying toxicity mechanisms. Nanotoxicology remains a young, dynamic research field with a future worth looking forward to.

Appendix A

Appendix A supplements data for Chapter 5 and 6.

A.1 TEM Results of Keratinocytes on exposure of TiO₂ and ZnO Nanoparticles

A microglandular cavity was observed in one keratinocyte under the exposure of TiO₂ 50 ng/mL for 24 h (Figure A.1A). This microglandular hyperplasia-like change suggested a bad cell differentiation. A double-membraneous like structure was found in cells exposed to 50 fg/mL of TiO₂ nanoparticles (Figure A.1B). It looks like the elongation of ER membrane. Two cells in mitosis were observed in ZnO 50 pg/mL (Figure A.1C) and 1 µg/mL (Figure A.1D) treated groups.

Figure A.1. TEM photomicrographs of primary keratinocytes under exposure of different concentrations of TiO₂ and ZnO nanoparticles. (A) A microglandular cavity was shown in one cell exposed to TiO₂ 50 pg/mL, and a damaged mitochondrion was shown in a nearby cell (arrows). **(B)** The formation of a double-membraneous like structure was shown in TiO₂ 50 fg/mL treated keratinocytes (arrow). **(C, D)** Mitosis was shown in cells under the exposure of ZnO 50 pg/mL and 1 μg/mL, respectively (arrows).

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