Combinatorial toxicity of ZnO and TiO₂ nanoparticles in human primary epidermal keratinocytes

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2016

Kathawala, M. H. (2016). Combinatorial toxicity of ZnO and TiO₂ nanoparticles in human primary epidermal keratinocytes. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/65995>

<https://doi.org/10.32657/10356/65995>

NANYANG
TECHNOLOGICAL
UNIVERSITY

COMBINATORIAL TOXICITY OF
ZNO AND TIO₂ NANOPARTICLES
IN HUMAN PRIMARY EPIDERMAL
KERATINOCYTES

MUSTAFA HUSSAIN KATHAWALA

SCHOOL OF MATERIALS SCIENCE AND
ENGINEERING

2015

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MUSTAFA HUSSAIN KATHAWALA

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A thesis submitted to the Nanyang Technological University in
fulfillment of the requirement for the degree of
Doctor of Philosophy

2015

Dedication

I would like to dedicate this thesis to my beloved parents, Sikandar Hussain Kathawala and Shakeela Sikandar Kathawala, who have been a constant source of inspiration, an unwavering pillar of support, and a model of excellence and hard work, which laid the foundation of my entire being. Without their unconditional love and shining guidance, this work would have never existed. Every letter in this thesis is an accomplishment for them and not me.

Acknowledgements

This thesis, like every other PhD dissertation, would not have been possible without the contributions and support of a multitude of players. An exhaustive list of them is impossible to draft. Therefore, I would like to express my deepest gratitude to all involved with a special mention of a few who played the major role.

Firstly, I would like to express my genuine appreciation for my family and friends. A special mention is imperative for my siblings who lent their unconditional love and support throughout this challenge. This work is a testimony to their efforts and I hope it has made them proud. As an international student living away from family, I had been blessed with an army of shoulders to lean on throughout this incredible journey. The contribution of my friends, *has* been *immeasurable*; *this dissertation is* a timeless proof of that.

Secondly, the unwavering support of my supervisors is worthy of the highest level of appreciation. Professor Joachim Loo and Professor Ng Kee Woei were instrumental to this work with their constant guidance, encouragement, and teaching at each and every step.

Thirdly, I would like to acknowledge the School of Materials Science and Engineering for granting me the privilege to pursue PhD candidature under their esteemed institute. I deeply thank all the staff, technicians and colleagues who helped me at every stage from admission to submission.

Last but not the least, I would like to acknowledge all the members, past and present, of the Drug Delivery Systems and Nanotox Group who made the laboratory environment conducive for work, taught me all the essential techniques, and lent me advice whenever I was at dead ends.

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List of Abbreviations

AE	absolute exposure
APTES	aminopropyltriethoxysilane
BET	Brunauer-Emmett-Teller
CCK-8	cell counting kit 8
DLS	dynamic light scattering
DMEM	Dulbecco's modified eagles' medium
DSBs	DNA strand breaks
ds-DNA	double-stranded DNA
EDX	energy dispersive x-ray spectroscopy
FESEM	field emission scanning electron microscopy
FITC	fluorescein isothiocyanate
FTIR	Fourier transform infrared spectroscopy
HaCaT	Human Keratinocyte cell lines
HLFCs	Human Lung Fibroblast Cells
HPDF	Human Primary Dermal Fibroblasts
HPEKs	Human Primary Epidermal Keratinocytes
ICP-MS	inductively coupled plasma-mass spectroscopy
ISDD	In vitro, Sedimentation, Diffusion and Deposition Model
NPG	NewPort Green
NPs	nanoparticles
PBS	phosphate buffer solution
PEN	The Project on Emerging Nanotechnologies

PI (staining)	propidium iodide staining
ROS	reactive oxidative species
RTP	room temperature and pressure
SPF	sun-protection factor
ss-DNA	single-stranded DNA
TEM	transmission electron microscopy
TNP	titanium dioxide nanoparticles
TWAD	time-weighed average dosage
TWAF	time-weighted average factor
UV	ultraviolet
XRD	x-ray diffraction
XRF	x-ray fluorescence
ZI	zinc ions
ZNP	zinc oxide nanoparticles
γ -H2AX	antibody for the gene: phospho-histone 2A member X

Abstract

Nanotoxicology encapsulates studying the interactions nanomaterials can have with biological systems and the environment. Recently, this field has gained momentum due to the influx of new nanomaterials, many of which have already become popular components of consumer products like cosmetics. Some of these applications use combinations of nanomaterials: dual nanoparticle systems. However, almost all the studies to date have focused on evaluating the toxicology of single nanomaterials. Since dual nanoparticle systems are often used due to a synergistic or antagonistic relationship between the two materials, it is only logical to expect such an interplay to trickle into their combinatorial toxic influence. This study is focused on investigating the toxicity of the dual nanoparticle system of ZnO and TiO₂, which is most common in sunscreens. The combined effect of the two nanoparticles was studied using skin-mimicking human primary epidermal keratinocytes as the *in vitro* model. Various cellular processes were evaluated including cell death, ROS generation and DNA damage. These were linked to nanoparticle properties which were heavily characterized in terms of size, shape, solubility, zeta potential, etc. It was found that individual TiO₂ nanoparticles induced low cytotoxicity even at higher concentrations while ZnO nanoparticles caused a high degree of cell death even at lower concentrations. The reason behind this difference was the solubility of ZnO nanoparticles which allowed intracellular Zn²⁺ ion concentration to be imbalanced inducing cell death, ROS generation and sharp DNA damage.

Interestingly, TiO₂ nanoparticles demonstrated an ability to adsorb Zn²⁺ ions onto their negative surface and caused a reduction of intracellular Zn²⁺ ion concentration when treated alongside ZnO nanoparticles. This antagonism caused a substantial decrease in cytotoxicity and genotoxicity of the dual nanoparticle system. Intracellular TiO₂ nanoparticles were singled out as the “vigilante” causing this reduction in toxicity.

Chapter 1. Introduction

1.1. Background

The term “nano” was implied in its current connotation for the first time by Norio Taniguchi in 1974. The seed of nanotechnology was sown even before in 1959 by well-known Nobel Prize winner Richard Feynman in his talk “*There’s Plenty of Room at the Bottom*”. Considered pure science fiction at that point, the idea gained popularity. Scientists became obsessed with pushing the size barrier to as small as possible and with the advent of electron microscopy it became possible to characterize ever shrinking sizes. It was only a matter of time before a plethora of new materials were invented exhibiting unprecedented properties; the era of nanotechnology had officially begun.

Or had it? As much as it is believed that nanomaterials are a recently developed class of materials, in truth they have been around for a while, longer than one would guess. The earliest known usage of nanoparticles dates back to medieval times, 4th Century AD, when Romans made the famous Lycurgus Cup using silver, gold and copper nanoparticles to exploit the size effect on the energy of Plasmon resonance (Summers 2013). Although unaware of this special class of materials, artisans in history have used nanoparticles unknowingly in order to cultivate similar optical masterpieces e.g. Chinese *famille rose*, the medieval stained glass (Chang 2005), etc. It is only recent examinations of these masterpieces using advanced material characterization techniques which have revealed the presence of nanoparticles. Nonetheless, their true potential has only

been realized after their identification through advanced characterization techniques.

Nanomaterials have been a major focus of scientific investigation in recent times. The drape over nanomaterial-properties is being quickly removed with the hopes of discovering advantageous properties. However, at the same time, there are also concerns of revealing attributes which would make nanomaterials unsafe. The recent and unfortunate case of asbestos has further prompted awareness that before flooding the market with new materials, their effects, desired and unintended, upon human and environmental exposure must be well understood. This is the principle on which the field of nanotoxicology is founded, aiming to increase the knowledge of “nanosafety”.

Two nanomaterials which have received special consideration and shown prodigious potential are ZnO and TiO₂. Their optical properties make them lucratively marketable and since their micron-sized counterparts were already in use, they made the natural transition to nanometric dimensions. The minute size brought transparency to the table, a delightful property when used in cosmetics especially sunscreens. Indeed many sunscreens (and other topically applied products) have started using nanomaterials copiously. The FDA allows up to 25% of ZnO or TiO₂ to be present in sunscreens whether alone or as an active combination.

Concerns over the possibility of nanoparticles penetrating the skin barrier have been raised and nanotoxicologists have been kept busy. Researchers have raced

to quantify absorption of nanoparticles through skin under different conditions and studying the biological responses of nanoparticles in the event of cellular contact (Labouta and Schneider 2013). While studies have evaluated nanotoxicity from the perspective of both cellular and material behavior, the scenario of dual nanoparticle exposure has been ignored. The work done in this study was to evaluate the safety aspects of ZnO and TiO₂ nanoparticles in terms of their combinative effects on skin cells. This is important as nanoparticles can interact with the cells in intricate ways and disturb cellular homeostasis, metabolic pathways and other cellular activities. In dual nanoparticle exposure, these permutations are further complicated as interactions between nanoparticles also affect the net toxicity profile of the dual nanoparticle system. While real life scenarios formed the basis of designing the study, the focus was on dissecting the mechanisms influencing their combined cellular response.

1.2. Hypothesis

ZnO nanoparticles have been classified as a highly toxic species in many cell types including human keratinocyte cell lines (HaCaT) (Kathawala et al. 2013; Ng et al. 2014). In contrast, TiO₂ nanoparticles do not induce strong cellular death despite their ability to be taken up by cells and to interfere in many cellular functions (Kathawala et al. 2013; Ng et al. 2014). It is hypothesized that a similar trend in the relative cytotoxic potential of ZnO and TiO₂ nanoparticles would be experienced by human primary epidermal keratinocytes (HPEKs). The most

commonly cited route of ZnO nanoparticles' toxicity is through zinc (ZI) which can overload the cells homeostatic capacities. Interestingly, TiO₂ nanoparticles, according to many studies, have shown to possess a negative surface charge and the ability to adsorb other molecules, especially charged ones. It is, therefore, hypothesized that when exposed together to cells *in vitro*, natural electrostatic attraction between the negative TiO₂ surface and positive ZI would scavenge these ions intracellularly and alleviate the toxic effects induced by an overload of ZI.

1.3. Objectives

The abovementioned hypotheses required a systematic approach starting off with understanding dual particle interactions in an acellular environment followed by their behavior *in vitro*. The objectives of this study in the order of investigation was as follows:

- Perform comprehensive material characterization on ZnO and TiO₂ nanoparticles used in sunscreens including their size, shape, crystal structure, solubility, surface properties, etc.
- Examine interactions, including aggregation, ion adsorption, etc., between the two nanoparticles in various environments including water, phosphate buffer and cell culture media.

- Evaluate the response of single nanoparticles (ZnO **OR** TiO₂) on human primary epidermal keratinocytes (the major type of skin cells) in order to establish the primary effects. The avenues of investigation included:
 - Cytotoxicity rendered to the cells in terms of metabolic activity, DNA quantitation, cellular uptake and oxidative stress.
 - Genotoxicity, primary and secondary, induced to cells as a result of oxidative stress or nuclear penetration or other mechanisms.
- Compare the above to the response of dual nanoparticles (ZnO **AND** TiO₂) on human primary epidermal keratinocytes to test the hypothesis.

1.4. Scope

This study considered two broad categories of cellular toxicity which are cytotoxicity and genotoxicity. The focus was on establishing the combinatorial effects of ZnO and TiO₂ nanoparticles using well established assays for cellular toxicity. Since these nanoparticles are found in sunscreens and other topically applied consumer products, human primary epidermal keratinocytes (HPEKs) were the cellular model of choice. *In vitro* 2D cultures were utilized.

The study did not directly focus on the effect of size, shape, crystal structure and other physicochemical properties on the cellular effects although these physiochemical properties were studied to explain the effects observed. The nanoparticles used were commercially purchased to represent the most widely

used kinds in order to maintain their relevance to consumer products as well as to cater to uniformity and quantity needed for experimentation.

1.5. Novelty

1.5.1. Human Primary Epidermal Keratinocytes

To model topical exposure to nanoparticles, skin cells are the logical choice. It is therefore that most studies have focused on various skin fibroblasts. However, another major cell type which makes up majority of the skin has been neglected. These are keratinocytes which make up 90% of the cells. Very few studies have focused on keratinocytes and out of those most have used immortalized cell lines. This is the first study to focus on TiO₂ and ZnO induced nanotoxicity on human primary epidermal keratinocytes. Similarly, the genotoxicity of ZnO and TiO₂ nanoparticles on HPEKs has also remained unstudied and this project is the first to address this issue.

1.5.2. Combinatorial Nanotoxicity

No other study to date has focused on the combinatorial effects of nanoparticles in the context of toxicity. This is the first study which delves into the unexplored area. The combination of ZnO and TiO₂ nanoparticles has become commonplace in topical applications. Therefore this thesis focused the pair's combined toxic

effect with an emphasis on their interplay and antagonistic (or synergistic) interactions.

1.5.3. Relative Exposure Scenarios

A novel approach was adopted in order to understand the source of any dual nanoparticle effect. The most basic approach to study dual nanoparticle system is to simultaneously treat HPEKs with the two species of nanoparticles and measure the cellular influences. However, such an approach provides a holistic examination. To probe deeper, it was necessary to single out the specific effects of the various species in the dual nanoparticle and HPEKs system. These include intracellular and extracellular nanoparticles, and solubilized ions and insoluble particulate. By varying the relative exposure times of the two nanoparticles, a variety of scenarios were replicated which could single out such specie specific effect. E.g. by pre-exposing HPEKs to TiO₂ nanoparticles, cells which contained intracellular TiO₂ nanoparticles were prepared. These could then be subsequently treated with ZnO nanoparticles in order to single out the influence of intracellular TiO₂ nanoparticles in mitigating the ZnO nanoparticles induced toxicity.

Chapter 2. Literature Review

2.1. Nanomaterials

Nanomaterials are a family of materials which are less than 100 nm (nano-range) in at least one dimension. Those which have a single dimension in the nano-range include nano-sheets and nano-belts, those with two nanometric dimensions include nano-rods and nano-cylinders, and those which have all three dimensions below 100 nm include quantum dots and nanoparticles.

As previously mentioned, nanomaterials though recently identified, have been around for a while. Nonetheless, true power lies in knowledge and the true potential of nanomaterials has only recently been recognized. At this stage, there is a great drive for science to push the limits with nanomaterials and achieve what was previously impossible. The application of nanomaterials, owing to their unique properties, is fast growing in fields including automobiles, drug delivery, therapeutics, diagnostics, optics, electronics, cosmetics, and many more. The capabilities to visualize the minute sizes and the increased understanding of the unique properties which these materials offer have spurred a spike in research and development of nanomaterials. There has been a steady and sharp rise in the number of articles in the last decade (Figure 2-1). Not surprisingly, there has also been a steady rise in the number of consumer products which use nanomaterials.

Figure 2-1 – The era of Nanotechnology. The chart depicts the increase in the number research articles in the Web of Science database from the category “Nanoscience and Nanotechnology”(Web of Science Database 2015), and the increase in the number of commercial products contain nanomaterials(The Project on Emerging Nanotechnologies 2015). * indicates extrapolated values due to missing data in the database.

2.2. Nanomaterials in Commercial Products

According to the Nanowerk Nanomaterials DatabaseTM, there are now more than 3000 nanomaterials which have been developed (Nanowerk Nanomaterials DatabaseTM 2015). This includes organic, carbon-based as well as metallic nanomaterials. The real value of nanomaterials is due to their minute size which tilts the balance between surface area and volume in favor of the surface area. As a result, surface properties dominate leading to unique properties being exhibited. Materials generally established as “nonreactive”, start becoming active in the

nano regime. One simple example is the enhanced solubility which many nanomaterials exhibit due to an increased surface.

Owing to their superlative physico-chemical properties, nanomaterials have found applications in various fields including biomedicine, cosmetics, automobiles, aerospace, electronics, food and drugs, and many others (Staggers et al. 2008; Wagner et al. 2006; Zhang et al. 2013). The uses of nanomaterials are not just confined to theories of the lab either. The Project on Emerging Nanotechnologies (PEN) database reports a rise in consumer products containing nanomaterials every year since 2005 (Figure 2-1). As of October 2013, there were 1628 consumer products which were known to contain nanomaterials; a massive increase since March 2005 when there were only 54 such products (The Project on Emerging Nanotechnologies 2015).

The leading category to which almost half (788) of these consumer products belong is Health and Fitness. This includes cosmetics, sunscreens and other topically applied products which is a huge market for nanomaterials. The small size makes such ingredients aesthetically more favorable as they are not visible to the naked eye. Photoactive nanomaterials have been especially successful in this application. Two such materials are TiO₂ and ZnO (Kathawala et al. 2013). Not surprisingly titanium and zinc based nanomaterials are both in the top five most used nanomaterials in commercial products (The Project on Emerging Nanotechnologies 2015).

2.3. Zinc Oxide and Titanium Dioxide in Sunscreens

The main function of sunscreens is to absorb potentially harmful UV radiation. While many organic formulations are used, inorganic filters have become increasingly popular due to their superior SPF values. TiO₂ and ZnO are classified as inorganic semiconductors which exhibit UV-absorptive properties (Carp et al. 2004; Hernández-Alonso et al. 2009; Moezzi et al. 2012). TiO₂ occurs as numerous allotropes; tetragonal rutile, tetragonal anatase and orthorhombic brookite (Carp et al. 2004). Rutile and anatase are of the highest commercial importance representing 90% and 10% of the market, respectively (Park et al. 2011a). The band gap energy for rutile and anatase is 3.2 eV and 3.0 eV respectively (Fujishima and Honda 1972); this corresponds to a wavelength of 385nm and 400nm, respectively. On the other hand, ZnO has at least three known crystal structures: hexagonal wurtzite, cubic zinc-blende structure and cubic rock-salt (NaCl-type). The most common of them is the wurtzite form which has a broad adsorption spectrum with a band gap energy of 3.2 eV (Hernández-Alonso et al. 2009) similar to TiO₂; this energy corresponds to a wavelength of 385nm. In addition to the ability to absorb UVA and UVB (Martorano et al. 2010) ZnO possesses a high refractive index of 1.95-2.10 (Moezzi et al. 2012)

With properties like these, both TiO₂ and ZnO fit the bill perfectly for UV-absorptive applications. Interestingly, some studies have even shown that when the two are put together they become even more efficient (Dunford et al. 1997; Nesseem 2011). This was confirmed by our test trials measuring UV-Vis

absorbance of the two NPs (Supplementary Materials: Figure S 1). In fact many sunscreens, especially those which boast of super high SPF, use both TiO₂ and ZnO together in their formulation as active sunscreen agents.

2.4. Nanotoxicity

Nanotoxicology is a branch of the broader field called particle toxicology. As the name suggests, it focuses on nanomaterials. With the rising popularity of nanomaterials and their usage, it is essential that knowledge about their safety is also found. Unfortunately, due to the lack of knowledge there is an air of uncertainty associated with nanomaterials. It is imperative that the charm of nanomaterials is maintained because clearly they possess capabilities which make unprecedented applications possible. However, the negative stigma, which is sometimes associated with them because of their heightened activity and potential of human and environmental exposure, needs to be addressed so that science is not held back due to safety concerns. This puts a great emphasis on research to be directed to study the applications and toxicity of nanomaterials so as to enable the progress to continue without compromising safety.

2.4.1. Zinc Oxide

ZnO is a classic example of nanomaterials differing from bulk materials. At micron size, it has long been deemed non-toxic and used for many years.

However, when reduced to nanometric dimensions it demonstrates acute cytotoxicity (Carp et al. 2004; Horie et al. 2009; Kao et al. 2011) and severe genotoxic effects (Dufour et al. 2006; Ng et al. 2011; Sharma et al. 2011). It can readily be taken up by cells (Sharma et al. 2011), release ZI (David et al. 2012; Xia et al. 2008) and cause mitochondrial depolarization and ROS spike (Bae et al. 2012; Kao et al. 2011; Ma et al. 2011; Martorano et al. 2010; Park et al. 2011b; Xia et al. 2008). While zinc is one of the most important metallic elements for the functionality of cells, most heavily involved in protein folding, an excessive amount of zinc can be equally disruptive to normal cellular functions (Kao et al. 2011). Their sharp toxicity killed both human mesothelioma MSTO-211H and rodent 3T3 fibroblast cells exposed to more than 15 ppm (Brunner et al. 2006). Their cytotoxicity has been shown to be time and concentration dependent (Sharma et al. 2009). Sharma et al. also observed that ZnO nanoparticles induce oxidative stress measured by an increase in hydro peroxide as well as a decrease in glutathione and catalase activity; all of these lead to cell death. Similarly, another study suggested cytotoxicity caused by oxidative stress on human colon cancer LoVo cells (De Berardis et al. 2010). Again an increase in hydrogen peroxides and hydroxyl radicals along with a decrease in molecular oxygen, glutathione and interleukin-8 (IL-8) was measured. There is also strong evidence suggesting ZnO NPs' toxicity to aquatic organisms (Ma et al. 2013).

There is also a strong genotoxic potential of ZnO NPs especially stemming from oxidative stress. ZnO NPs cause an imbalance in the oxidant and anti-oxidant processes. The resulting oxidative stress trickles a number of downstream effects.

Excessive stress can modify proteins, lipids and nucleic acids, all of which trigger an additional anti-oxidant response and even lead to cell death (Chang et al. 2012). ROS can trigger DNA damage something which is classified as primary indirect genotoxicity (Ng et al. 2014; Yang et al. 2009). It can trigger an over expression of death receptors. Lysosomal ROS can cause DNA point mutations or single and double strand breaks (Singh et al. 2009). A separate response to ROS is intracellular Ca²⁺ release (Xia et al. 2008). This can lead to mitochondrial perturbations and eventually cell death.

Much has been made out of the solubility of ZnO. Although insoluble in water, it has some solubility in culture mediums, possibly due to the presence of proteins. Furthermore it has also been suggested that ZnO NPs are more soluble than bulk ZnO (Borm et al. 2006; Yang and Xie 2006). This solubility may vary depending on conditions although it is widely believed that if ZnO NPs are endocytosed the acidic lysosomes can greatly enhance the solubility of ZnO. The type of medium also plays an important role as each medium has its own unique mix of ions, proteins and sugars (Midander et al. 2009). Once dissolved, the Zn²⁺ ions (ZI) essentially cause an imbalance in the ZI levels in the cells and can trigger a host of responses by disrupting the homeostasis in human cells and even bacteria (Kao et al. 2011; Kasemets et al. 2009). Song et al. correlated, at different concentrations, the solubility of ZnO NPs to the cytotoxicity and ROS generation caused (Song et al. 2010). They found good agreement between the expected amount of ZI and the level of response but there were no measurements or observations made on intracellular ZI. Some studies negate this correlation

stressing on particle chemistry rather than its solubility and resulting ions as the key players (Gojova et al. 2007; Moos et al. 2010).

2.4.2. Titanium dioxide

Numerous studies have investigated TiO₂ nanoparticles focusing on their cyto-, geno- and photo- toxic effects. Physio-chemical properties like size, crystal structure, surface area and shape play a critical role as does the synthesis procedure. This is also why comparing studies which use a different type of nanoparticle becomes a challenge. TiO₂ nanoparticles (30-60 nm; 20 µgml⁻¹) synthesized by a sonomechanical method induced loss in cell viability, compromised antioxidant system, morphological alterations, intracellular ROS production and significant DNA damage in human amnion epithelial (WISH) cells (Saqib et al. 2012). A mixture of anatase and rutile of spherical shape TiO₂ nanoparticles also caused a drop in cell viability when exposed in moderate concentrations (50ppm) to HaCaT cells (Park et al. 2011a). Similarly, Kang et al. observed apoptosis induced through caspase-8 and caspase-3 in lymphocytes (Kang et al. 2009) as well as genotoxic effect in both micronucleus and comet assays (Kang et al. 2008). Zhao et al. showed that commercially produced TiO₂ nanoparticles induce autophagy in primary human keratinocytes (Zhao et al. 2013). In one study, human fibroblasts also suffered from DNA damage monitored by γ-H2AX Immunofluorescence (Setyawati et al. 2013a). The same group demonstrated another interesting phenomenon. They observed endothelial

cell leakiness caused by physical interaction between the TiO₂ nanoparticles and the endothelial cells' adherens junction protein VE-cadherin (Setyawati et al. 2013b). The leakiness was visualized as increased gaps between adjacent endothelial cells which otherwise form a tightly packed layer. Most studies, however, have shown TiO₂ nanoparticles to have low potential to induce cell death in the absence of UV exposure until very high doses (Fujishima and Honda 1972; Peters et al. 2004; Yamamoto et al. 2004; Zhang et al. 1998).

There was also ample literature which suggested genotoxic potential of TiO₂ NPs. Both anatase and rutile crystal structured nanoparticles (12-140nm) induced single strand breaks, oxidative lesions and oxidative stress in A549 cells (Jugan et al. 2012). Furthermore it was shown that they impair cellular mechanisms to repair faulty DNA by suppressing the nucleotide excision repair (NER) and base excision repair (BER). ROS once again seems a central theme in studies focusing on DNA damage. It was shown that TiO₂ NPs could increase extracellular ROS, HO-1 and NOS mRNA expression as well as the release of TNF- α in NR8383 rat lung alveolar macrophages (Scherbart et al. 2011). Human amnion (WISH) cells suffered 1.87 fold increase in intracellular ROS and 7.3% increase in G₂M cell cycle arrest suggesting DNA repair activities (Saqib et al. 2012). Finally, it was shown that NAC, an anti-oxidant, reduced DNA damage in human lymphocytes which otherwise showed increased micronuclei formation, DNA breakages and ROS (Kang et al. 2008). On the other side of the coin, there were plenty of studies which supported the contrary as well. Wan et al. showed that there was no ROS or increase in γ -H2AX in A549 cells (Wan et al. 2012). Others

also showed that TiO₂ nanoparticles were not genotoxic under Ames and Comet assay (Woodruff et al. 2012), did not induce any chromosome aberration to Chinese hamster ovary cells (Hussain et al. 2011) and prove negative in the micronuclei assay on rat liver epithelial cells (Linnainmaa et al. 1997). The conflicting results can be attributed to the variety of parameters across these studies. Indeed one study showed that cell lines mattered as primary human lung fibroblasts were more sensitive than BEAS-2B cells (Wang et al. 2007). Other differences like exposure metrics, crystalline structure, particle dispersion and NP sizes could also impact (Shi et al. 2013).

Photo-toxicity, toxicity under UV exposure, has also been a topic of many studies and the effect is thought to be significant (Dodd and Jha 2009; Dunford et al. 1997; Park et al. 2011a). Although micro-particles of TiO₂ are considered very safe (Baan et al. 2006; Fryzek et al. 2003), under UV exposure their nano-scale counterparts exhibit cell penetrative (Carp et al. 2004) as well as enhanced ROS production capabilities (Jang et al. 2001). It is hence no surprise that nano-TiO₂ is considered a potential candidate as an effective bactericide, anti-viral agent, anti-tumor agent and anti-fungal (Markowska-Szczupak et al. 2011; Sunada et al. 2003).

2.4.3. Penetration through the Skin

With the increase in the usage of NPs in products like cosmetics and sunscreens, there is a rising concern that these nanoparticles may gain entry into the body

through the skin. Skin is the largest organ of the body and acts as the first line of defense against foreign invaders. It is believed with reason that lipophilic particles bigger than 600 Da are effectively stopped by the skin (Barry 2001). However, smaller particles can penetrate the skin. A recently published review on skin penetration by NPs comprehensively discusses the current state of knowledge related to the subject (Labouta and Schneider 2013). 49% of the articles which had been published by then had reported NP penetration with or without mechanical or chemical enhancement. Interestingly, some articles suggested penetration across the entire length of the skin. However, 51% of the articles showed no penetration. After further scrutiny, it was found that the size range suggesting penetration and no penetration were indistinguishable from each other even though there were suggestions in the same review that NP penetration should increase with increasing exposure times and decreasing particle size. There have also been numerous studies which suggest that the penetration of NPs is only until the hair follicles or assisted by them (Nohynek et al. 2007; Smijs and Bouwstra 2010).

Another review published by our group earlier argued the role that skin integrity and health could play (Zhao and Ng 2014). Whether nanoparticles can penetrate in substantial quantities remains inconclusive, however, it is obvious that unhealthy skin with compromised integrity would enhance the penetration. UV exposure can serve as one of the agents which can cause this effect. Indeed quantum dots penetrated deep into the epidermis and dermis via the stratum corneum of UV damaged murine skin.(Mortensen et al. 2008; Mortensen et al.

2010). It was calculated that the penetration of TiO₂ NPs to the viable epidermis and dermis was 0.19 ± 0.15 wt.% and 0.39 ± 0.39 wt.% in healthy and UV treated skin (Miquel-Jeanjean et al. 2012). Another trend that was noticed in some studies was that *in vitro* models suggested a lower penetration than *in vivo* models (Wu et al. 2009).

Chapter 3. Materials and Methods

This section describes the materials and key methodologies used during the course of this study. This section is not an exhaustive list of protocols for the entire thesis. Only key techniques are described here. Specialized techniques and methods are described at the beginning of their specifically relevant chapter.

3.1. Nanoparticles Characterization

All nanoparticles used for toxicology studies were purchased from commercial sources. P25 TiO₂ and ZnO NPs were purchased from Evonik Degussa (Essen, Germany) and Meliorum Technologies (USA) respectively. These nanoparticles were heavily characterized in terms of size, shape, surface, etc.

Transmission electron microscopy (TEM; JEOL 2010) at an accelerating voltage of 200 kV with a Lanthanum Boride (LaB₆) cathode was performed to evaluate size and shape. Samples were prepared by mixing a small quantity of NPs in methanol followed by 30 min of ultrasonic treatment, and then dropped onto carbon coated copper grids. Size of NPs was measured from their TEM micrographs using the ImageJ software.

In order to study size and morphology, electron microscopy was performed using a field emission scanning electron microscope (FESEM; Joel JSM 7600F). Nanoparticles dispersed in ethanol were loaded onto the sample holder and allowed to dry, followed by platinum coating before viewing. In conjunction with

imaging, energy dispersive x-ray analysis was also performed to study the elemental distribution.

The elemental and oxide composition of the powder was measured with an X-ray fluorescence system (XRF; PW 2400) whereas the oxides and their crystal structures were analyzed by X-ray diffraction technique (XRD; Bruker D8 Advance). Both these techniques did not require any sample preparation except loading the powder into the respective sample holders.

Dynamic light scattering (DLS; Malvern Co., UK) was utilized to measure hydrodynamic sizes and zeta potentials. The NP samples (100 µgml⁻¹) were dispersed in Milli-Q water or cell culture medium and ultra-sonicated for 5 mins to form colloidal suspensions. Immediately after, the readings were taken using 12mm square polystyrene cuvettes (Malvern; DTS0012) and disposable capillary cell (Malvern; DTS1070) for size and zeta potential measurements respectively. Each sample was tested in triplicates.

For Brunauer–Emmett–Teller (BET) surface area measurements, nitrogen adsorption/desorption isotherms were measured at 77 K using ASAP2000 adsorption apparatus from Micromeritics. The samples were degassed at 200 °C for 4 h under vacuum before analysis.

3.2. Solubility of Nanoparticles

10 mg of ZNP and TNP were weighed separately in an enclosed environment, suspended in 1 ml of cell culture medium (or other media), and vortexed for a minute to get 10 mgml⁻¹ stock suspensions. From these, three categories of suspensions were prepared targeting a final concentration of 50 µgml⁻¹ for each NP: ZNP only, TNP only, and ZNP and TNP combined. The time at which the target concentration was prepared was marked as the starting point of dissolution. Aliquots were drawn out at various time points and centrifuged instantly to remove any undissolved NPs. Samples were stored in 4°C for mass spectrometry.

3.3. Ion Adsorption

50 µgml⁻¹ of ZNP in medium was left overnight for maximal dissolution. For control, 75 µgml⁻¹ of the Zn soluble salt, ZnSO₄·7H₂O was also prepared (the concentration was chosen to achieve comparable concentrations of ZI in both the solutions). The next day undissolved ZNP were removed by centrifugation and TNP at various concentrations (0 – 400 µgml⁻¹) were introduced to both solutions. After 10 minutes of equilibration time, centrifugation was performed to spin down the TNP and any adsorbed ions. Aliquots of the supernatant were stored in 4°C for mass spectrometry.

3.4. Mass Spectrometry

The samples were filtered using a 0.45 µm filter and diluted in 5% nitric acid. The MERCK (UN2031) standard was used by diluting it in 5% nitric acid to give 1 to 10 µgml⁻¹ concentrations. A standard curve was plotted using those values for Zn and Ti. Inductively coupled plasma-mass spectroscopy (ICP-MS) was performed using the 7500 Series ICP-MS systems (Agilent Technologies) at the National University of Singapore. Each sample was measured 5 times to ensure stable readings and the tubes were washed with 5% nitric acid and Milli Q water between each sample to ensure accurate readings.

3.5. Dosimetry

Dosimetry was performed using the In vitro Sedimentation, Diffusion and Dosimetry (ISDD) model (Hinderliter et al. 2010). Effective density of the nanoparticle aggregates were measured using the protocol proposed by DeLoid et al. (DeLoid et al. 2014). Briefly, 250 µgml⁻¹ of NP were subjected to volumetric centrifugation using packed cell volume tubes (TPP, product number 87005) at 1000g for an hour in a swing-out bucket centrifuge. The pellet volume was read off using the TPP “easy read” measuring device (TPP, product number 87010). The pellet volume was then used to calculate the effective density (DeLoid et al. 2014). ISDD simulations were run using MATLAB R2013a starter application for the concentrations tested. The simulation program was kindly provided by Dr

Justin Teegarden from Pacific Northwest National Laboratory, Oregon State University Joint Faculty.

The simulation predicts the fraction of nanoparticles which deposits over time. This fraction is plotted against incubation time, so that the integral or the area under the curve (AUC) of this profile gives a time-weighted average (TWA) factor. The TWA factor is a dimensionless number which can be converted to the TWA dosage (TWAD) in terms of particle surface area, mass, number, etc. by simply multiplying it by the absolute exposure in terms of particle surface, mass, number, etc. respectively. The TWA dosage is therefore a function of (a) incubation time and (b) concentration. Wherever appropriate and useful, both absolute exposure in terms of particle mass per media volume (μgml^{-1}) and the TWAD in terms of particle mass per area of cell growth (μgcm^{-2} or mgcm^{-2}) are reported.

3.6. Fluorescent tagging of nanoparticles

TNP were tagged using Fluorescein Isothiocyanate (FITC) following a condensation reaction as previously reported (Zhao et al. 2012). Briefly, 100 mg of TNP were dispersed in 100 mL of anhydrous ethanol (analytical grade) by sonication for 10 mins and then refluxed under nitrogen. 5 mL 3-aminopropyltriethoxysilane (APTES) was then added drop-wise and the solution refluxed under nitrogen for three hours with continuous stirring. Next, 0.05g FITC was added to the mixture and left to react overnight at 70°C. The step

reaction enabled FITC to form a stable thiourea linkage with the NP surface. The NPs were collected through centrifugation and washed several times with anhydrous ethanol and deionized water to ensure complete removal of unattached FITC before freeze drying. Fourier Transform Infrared spectroscopy (FTIR) was performed to confirm the presence of the thiourea linkage.

3.7. Nanoparticle suspensions for cell culture tests

As mentioned earlier, only commercially purchased nanoparticles were used for toxicity testing. Except for cellular uptake, non-tagged (FITC free) NPs were used. In order to prepare the target concentrations of nanoparticle suspensions, the following procedure was used. First, 5-10 mg of NPs were weighed and suspended in 1 ml of culture medium to prepare stock suspensions. These were exposed to UV radiation for sterilization for 10 mins followed by vortex for 10 s and sonication for 10 mins. The now-dispersed suspension was diluted sequentially in cell culture medium to prepare the target concentrations typically in the range of 0-20 μgml^{-1} for ZNP and 0-400 μgml^{-1} for TNP. These concentrations were chosen as they covered the expected threshold/onset of cytotoxicity induced by the two NPs. Furthermore, the concentrations of NPs were within the realistic concentration of NPs used in sunscreens (Supplementary Materials: Table S 1).

3.8. Cell culture & maintenance

Human primary epidermal keratinocytes (HPEKs; ATCC, USA) were cultured and maintained in serum-free EpiGRO™ Human Epidermal Keratinocyte Complete Medium kit (Chemicon, USA). Human primary dermal fibroblasts (HPDF; ATCC, USA) were cultured and maintained in Dulbecco's Modified Eagles' Medium (DMEM; Gibco) supplemented with 10% fetal bovine albumin (FBS; Gibco), 1% L-glutamine and 1% penicillin-streptomycin (Gibco). Cells were cultured in standard culture conditions (37°C; 95% humidity; 5% CO₂) for up to 7 days. After 80% confluence was reached, the cells were passaged.

3.9. Cell viability assay

3.9.1. Metabolic Assay

In order to measure cytotoxicity, a cell viability assay based on measuring cell metabolic activity was used (CCK-8; Dojindo Molecular Laboratories Inc., Japan). This contains WST-8 salt (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium) which is reduced by dehydrogenase in the cell to a yellow colored medium/water soluble formazan dye. The absorbance from the dye can be measured by a microplate reader at a wavelength of 450 nm. The absorbance indirectly depends on the amount of metabolic activity and thus the number of living cells in the sample. In a typical

experiment, HPEKs were seeded in a 96-well plate at a density of 10^4 cells/well in culture medium. After allowing cells to equilibrate overnight, they were treated with ZNP or TNP of different concentrations, while negative control cells were treated with fresh culture medium. Pure medium and medium with NPs (without cells) were used as blank controls. After 24 hours of treatment, reconstituted WST-8 mixture was added to each well and left to homogenize for a few minutes on an orbital shaker. Cells were incubated for up to two hours after which the absorbance was measured.

3.9.2. DNA Quantitation Assay

DNA quantitation is another method to measure cell proliferation and viability. Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen, CA, USA) is a fluorescent stain specific for double stranded DNA. For quantitation, the procedure suggested by the supplier was followed. After treatment with nanoparticles, cells were lysed with 0.5% (v/v) Triton X-100 in deionized water for 30 min with agitation at 80 rpm on the orbital shaker and gentle pipetting. The working solution of the dsDNA reagent was prepared by 200 times dilution in TE buffer. The lysate and diluted reagent was mixed in a 1:1 ratio and incubated in 96-well plates for 5 mins in the dark. After incubation, fluorescence was recorded using a microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.

3.9.3. Propidium Iodide Staining

Propidium Iodide (PI) staining is a commonly employed viability test. The PI dye is unable to permeate an intact cell membrane. Therefore it only stains dead cells which usually possess a compromised cytoplasmic barrier. After treatment with nanoparticles, cells were washed thoroughly with PBS. Then the cells were harvested by treatment with trypsin and then incubated with PI dye for a few minutes. The cells are then re-dispersed in filtered PBS to run flow cytometric analysis to quantify the dead cells.

3.10. Cellular uptake of TNP

3.10.1. Confocal Microscopy

Confocal microscopy was used to determine cellular uptake and NP localization. Cells were grown on 15mm square no. 1 cover slips (thickness ~ 160 µm) inside 6-well plates to confluency. The cells were treated with FITC tagged TNP (AE:50 µgml⁻¹; TWAD: see Supplementary Materials: Figure S 1) for different times (up to 24 hours) after which they were washed with cold PBS and fixed using cold 4% paraformaldehyde in PBS for 10 minutes. Once fixed, the cell membranes and nuclei were stained using Wheat Germ Agglutinin, Alexa Fluor 350 Conjugate (Molecular Probes, Catalogues number W11263) and Hoechst 33342 (Molecular Probes, Catalogue number H3570), respectively, according to the manufacturer's instructions. The cells were washed thoroughly with PBS and

the cover slips were flipped and mounted onto glass microscope slides assisted by mounting medium and sealed using transparent nail polish. The cells were viewed instantly or stored in the dark at 4°C for up to a week before being viewed. Microscopy was performed either on Leica TCS SP5 Broadband Confocal microscope using an oil lens at magnifications up to 60x or Nikon A1-Rsi Confocal Microscope using an oil lens up to 100x. Post-image processing was performed using ImageJ 1.47v.

3.10.2. Flow Cytometry

For flow cytometry analysis, 3×10^5 HPEKs were seeded in 6-well plates and maintained until confluency was reached. The cells were treated with TNP at 50 μgml^{-1} (TWAD: 116.6 μgcm^{-2}). After 4 or 24 hours of incubation, the cells were washed with filtered PBS, collected by trypsin and washed again before dispersing in filtered PBS. Quenching was performed using trypan blue to discern internalized TNP from cell bound TNP. This has been shown to suppress fluorescent signals from NPs on the membrane and therefore measures fluorescence only from internalized NPs (Loike and Silverstein 1983). TNP treated cells dispersed in 1 mL filtered PBS were mixed with diluted trypan blue. After 10-20 minutes incubation, the cells were washed with and dispersed in filtered PBS. Flow cytometry was performed by FACSCalibur Flow Cytometer (BD Biosciences) and analysis was performed using WinMDI 2.9.

3.11. Intra-cellular ZI

3.11.1. Microplate reader

Control, TNP-exposed and TNP-loaded cells were treated using non-tagged nanoparticles in 96-well plates. After ZNP exposure of 24 hours, cells were treated with 5 μ M Newport Green™ DCF Diacetate (Molecular Probes, Catalogue number N-7991) for 1 hour. Cells were washed and incubated for another 30 mins in fresh medium. Cells were washed again and then fluorescence measurements were taken at excitation/emission wavelengths of 505/535 nm using a microplate reader.

3.11.2. Confocal microscopy

Control, TNP-exposed and TNP-loaded cells were treated using non-tagged nanoparticles on cover slips in 6-well plates. ZNP concentration tested was 5, 10 and 20 μ gml⁻¹. Samples containing only ZNP (5, 10 and 20 μ gml⁻¹) and TNP (100 μ gml⁻¹) were also tested for comparison. After fixation, cells were stained for free and unbound intracellular ZI using Newport Green™ DCF Diacetate (Molecular Probes, Catalogue number N-7991) according to the manufacturer's instructions. Cell nucleus was stained using Hoechst 33342 (Molecular Probes, Catalogue number H3570). Imaging was performed within 24 hours and all images were taken at the same instrument settings to allow for post image analysis. Semi-quantitative analysis was done using ImageJ 1.47V. Color

threshold was adjusted to suppress background and cells were counted. The green fluorescence was quantified in terms of the number of bright green specks observed in the cells.

3.11.3. ICP-MS

Cells were seeded in 6-well plates at 1.5×10^4 cells per well and maintained for 2 days. After treatment with NP, the cell monolayer was washed using filtered PBS, harvested using trypsin and then permeabilized by 0.5% w/v Triton X-100 treatment with simultaneous sonication of the cell suspension for 1 hour. The cell lysate was viewed under a microscope using a haemocytometer with trypan blue to ensure that lysis was complete. TNP were removed from the cell lysate by centrifugation before the samples were diluted with 5% nitric acid and the Zn²⁺ ion concentration was measured using the same ICP-MS used for solubility studies.

3.12. Statistical analysis

Statistical significances were only assessed when at least three or more sample replicates were performed, using one-way ANOVA with post-hoc multiple variances and Tukey's equal variance assumed (IBM SPSS v20).

Chapter 4. Results and Discussions

4.1. Nanoparticle Characterization

4.1.1. Overview

Characterization is the basis of any toxicological study. Knowledge of the physical and chemical properties of a material are an imperative prerequisite for understanding the cellular responses and their underlying mechanisms. Size, surface area and shape are commonly cited as physical parameters which can affect the toxicity of nanomaterials. In addition, surface charge and coatings are also sometimes held responsible for key interactions with the cells and can determine the difference between biocompatibility and toxicity. Another key area of interest is the solubility dynamics. Nanomaterials are in some cases more soluble than their bulk counterparts. For metallic oxides, this can have significant ramifications as the metallic ions can cause a host of biological responses. The aim of this section is to present comprehensive characterization data of the nanoparticles used for toxicological studies. Special emphasis is given on testing their behaviors in cellular environments.

4.1.2. Specific Methods: Sunscreen Analysis

A sunscreen of high SPF was bought from the shelf for investigation. No ingredients could be found on the packaging. To extract the nanoparticle, the protocol proposed by Fontaine et al. was followed (La Fontaine et al. 2010).

Briefly, 5-6 g of the watery formulation was dissolved in 20 ml methanol and sonicated for 5 minutes using probe sonicator with 2 seconds pulse setting and amplitude of 40 W. Thereafter 20 ml of Millipore[®] water was added to the solution, vortexed thoroughly and sonicated for another 5 minutes. Finally, 10 ml of hexane was added to the solution, vortexed thoroughly and sonicated for the last time for 5 minutes. Thereafter the solution, 50 ml in total was transferred quickly to a separating funnel and left to reach equilibrium for 2 hours. The hexane separated to the top and with it was partitioned the organic part of the formulation while the aqueous part containing the nanoparticles phased out to the lower region and could be collected. Thereafter the solution was frozen in -70°C freezer and the solvent was efficiently removed by freeze drying leaving behind the nanoparticle powder.

After extraction, the powder was analyzed for the presence of nanostructures. The powders were prepared by making a very dilute suspension in ethanol and placing one or two drops on to a carbon tape attached on the sample holder. The ethanol was allowed to dry before placing the sample into the vacuum chamber.

4.1.3. Nanoparticles in Sunscreens

As discussed earlier, the usage of nanomaterials in sunscreens has gained a strong momentum. In particular, TNP and ZNP have found a niche in this application. In order to validate their presence, a commonly available consumer product which was thought to contain these nanomaterials was investigated. The

formulations were put through a series of characterizations to extract and subsequently characterize the nanomaterials, if any, in order to validate the need for their toxicological screening. In the context of this project, the major aim was to find out whether TNP and ZNP are used together in consumer products.

After washing off the formulation from the sunscreen, a series of characterizations were performed (Figure 4-1). Panel a, b and c show the high magnification images of the contents of the sunscreen. The morphology of the sunscreen particles seemed to consist of large micron sized perfect spheres (2-4 μm in radius) which were coated with smaller particles of size less than 60nm.

EDX was done coupled with the FESEM and revealed the presence of Zn, Ti, O and Si in the mixture. This was confirmed using XRF (panel d) and XRD (panel E) which revealed high levels of these elements. While doing EDX, we selected a few spheres which had their coating ripped off and they showed up as only Si on the micrograph. Thus it was hypothesized that the micron sized spheres were silica beads with ZnO and TiO₂ nanoparticles coated onto its surface. Studies have shown how silica beads/spheres can be used to adsorb/synthesize ZnO (Xia and Tang 2003) and TiO₂ (Ida et al. 2007) nanoparticles onto their surfaces and our results suggest that the sunscreen in question is in very close agreement with them. The XRD fingerprint gave further insights into the crystal structure of the nanostructures. It was found that the predominant crystal structure of TiO₂ and ZnO was rutile with anatase and wurtzite respectively.

4.1.4. Characterization of TNP and ZNP

The purchased nanoparticles were chosen in order to match closely with the properties of nanoparticles present in sunscreens. Many studies have suggested the effect of crystal structure on cellular response, therefore nanoparticles chosen were of the same crystal structure as the tested sunscreen. The Degussa P25 TiO₂ nanoparticles have a mixture of rutile and anatase phase in a 3:1 ratio. This composition was not only similar to the sunscreen tested, but also similar to other previously reported formulations (Faunce et al. 2008). Furthermore, a mixture of anatase and rutile has been reported as the most photoactive composition of TiO₂ (Nesseem 2011). In the case of ZnO, the purchased nanoparticles possessed the wurtzite crystal structure which was similar to the tested sunscreen and is the most commonly used crystal structure of ZnO.

TEM revealed that both ZNP and TNP possessed near-spherical shape and similar sizes of $\sim 20 \pm 5$ nm (Figure 4-2). DLS derived hydrodynamic sizes of individual NPs suggested substantial aggregation in all three media tested (Table 1). When both NPs were mixed together, the resulting suspension recorded an even larger hydrodynamic size, suggesting an even higher state of agglomeration. The zeta potential for the NPs was measured using electrophoretic light scattering, and both NPs displayed negative and comparable zeta potentials across all three media used.

Figure 4-2 – TEM images for commercially purchased (A) ZNP and (B) TNP.

4.1.5. Solubility profiles of TNP and ZNP

ICP-MS was used to measure NPs solubility by quantifying for their respective ions. TNP were found to be insoluble in water and cell medium, even under acidic conditions (down to pH 2). In neutral pH water, ZNP were found to be sparingly soluble, reaching a ZI concentration of less than $2 \mu\text{gml}^{-1}$. On the contrary, in cell medium their solubility reached substantial levels that increased with time. The solubility profiles of ZNP in the absence and presence of TNP are shown in Table 2. The concentration of ZI (in absence of TNP) reached in excess of $10 \mu\text{gml}^{-1}$ within 15 minutes, increasing continuously over two days. When measured in the presence of TNP, the solubility of ZNP was consistently lower at all times, though not significantly.

Table 1 - Characterization Data for ZNP and TNP. TEM was employed to get primary size of nanoparticles while DLS was used to measure the hydrodynamic size and zeta potential of nanoparticle aggregates in suspensions of water, PBS and Medium.

		SIZE (<u>nm</u>)											
		DLS						Zeta Potential (<u>mV</u>)					
NPs	TEM	Water		PBS		Medium		Water		PBS		Medium	
ZNP	20 ± 5	1610	±	1829	±	935	±	-8.0	±	-27.2	±	-9.8	±
		90		50		150		1.0		2.3		0.2	
TNP	21 ± 5	494	±	2049	±	1964	±	-13.7	±	-25.3	±	-9.8	±
		50		30		80		3.4		0.3		0.9	
BOTH	-	3653	±	3431	±	2095	±	-8.6	±	-22.0	±	-10.0	±
		250		250		60		1.7		4.4		0.5	

Table 2 – Solubility of ZNP in Cell Culture Medium after different time. Values are given in µgml⁻¹.

NPs	¼ hours	½ hours	1 hours	4 hours	24 hours	48 hours
ZNP	14.2 ± 0.1	13.5 ± 0.5	15.8 ± 0.8	16.9 ± 1.0	19.6 ± 0.1	23.2 ± 2.0
TNP	TNP are insoluble					
BOTH	12.9 ± 0.1	13.0 ± 0.4	14.9 ± 0.2	15.4 ± 2.9	19.0 ± 0.9	21.3 ± 0.9

4.1.6. Time-Weighted Average Dosage (TWAD)

Dosage is the most important parameter of consideration in toxicological assessments. Until recently, nanotoxicity studies had utilized a variety of different particle attributes to quantify dosage. There has been a lot of debate over which properties have the most relevance. Some of these parameters include absolute particle mass, volume, surface area and number. Others have tied this number with the cell target by using one of these parameters normalized to cell numbers or the cell growth area. However, one important aspect of *in vitro* nanotoxicity experiments has been largely ignored. This is the ability of nanoparticles to sediment over time onto the 2-D cell monolayers at the bottom of the well plate. Since sedimentation is a dynamic process involving competing forces like diffusion and deposition, the actual dosage experienced by the cell is likely to be a function of the particle sedimentation rate as well as the incubation time.

Recently, the *In Vitro* Sedimentation, Diffusion and Dosimetry (ISDD) model has gained popularity in order to simulate and predict the time-weighted average dosage experienced by the cells (Hinderliter et al. 2010). Diffusion and deposition are highly dependent on the particle size and density. However, in the case of nanoparticles another level of complexity is added due to their ability to agglomerate. This brings the size of agglomerates and their effective density into relevance. While size estimations are possible to carry out using techniques such as laser scattering, effective density remains elusive due to its dependence on

particle packing, aggregate porosity and the media density. By pelletizing a known mass of nanoparticle aggregates and measuring the pellet's volume, DeLoid et al. have developed a mathematical model to work out the effective density (DeLoid et al. 2014).

The effective density of TNP agglomerates was calculated to be 1.255 gml⁻¹. This is substantially lower than the density of TiO₂ which is 4.23 gml⁻¹ and suggests that the agglomerates of TNP are substantially less dense than bulk TiO₂ and thus their sedimentation rate would be much slower. Simulations run to predict the deposition profile against incubation time confirmed this as TNP deposited completely onto the cell monolayer within 4 hours and 12 hours, when using 6-well and 96-well plates respectively. Based on this, two hours was chosen as the pre-exposure time when preparing TNP-exposed and TNP-loaded cells so that only a fraction of the TNP would have made contact with the cells until the cells were treated with ZNP. It was also interesting to note that the TWAD for 96-well plate format at 24 hours was 100 times greater than the TWAD at 2 hours incubation time. For 6-well plate the difference was 60 times.

In the case of ZNP, the ISDD model was not applicable. This was because the solubility of ZNP was higher than majority of the concentrations tested. Therefore the cells would be interacting with ZI rather than depositing ZNP.

4.2. Cytotoxicity of Single Nanoparticle

4.2.1. Overview

Evaluating the response of HPEKs to the two NPs in question, TNP and ZNP, was critical before delving into their combinatorial impacts. This section delves into measuring the cytotoxicity and cellular uptake profiles of these nanoparticles individually. HPEKs were chosen as the cellular model to represent the skin related toxicity because of their abundance in skin tissues.

4.2.2. Specific Methods: Transmission Electron Microscopy on HPEKs

To complement confocal microscopy and flow cytometry, biological TEM was performed on HPEKs exposed to NPs in order to study the cellular uptake. The imaging was performed by a co-worker, Dr. Zhao Yun, as part of a collaborative study on the effect of TNP on HPEKs (Zhao et al. 2013). Briefly, HPEKs (including controls) were fixed with 2.5% glutaraldehyde (SPI, USA) diluted in PBS overnight at 4°C and then treated with 1% osmium tetroxide (SPI, USA) for 1 h at RTP. The consequent pellets were dehydrated using ethanol gradient (25% - 100%) and pure acetone, for 20 min each at RTP. Resin infiltration was performed overnight in SPI-PonTM-Araldite[®] (SPI, USA), followed by embedding pure resin at 60°C for 72 h. Post-polymerization, sectioning was performed using an ultramicrotome (Leica Ultracut UCT) and collected on 200 mesh copper grids. Finally staining with uranyl acetate and lead citrate was

performed before the sections were viewed under a Philips EM 208 transmission electron microscope (accelerating voltage: 100 kV).

4.2.3. TNP induce minor cytotoxicity

Single particle cytotoxicity analyses serve as the basis of comparison for this study. In order to understand how the dual nanoparticle systems behave, it was imperative to establish first-hand an understanding of the cellular response to single nanoparticle exposure. Studies reported on TNP of the kind used in this study suggest that TNP exhibit low levels of toxicity except under UV irradiation (Kathawala et al. 2013). However, most studies use HaCaT cells which are not primary cells and therefore might behave differently. Furthermore using primary cells brings this *in vitro* model closer to mimicking real life scenarios.

TNP at various concentrations were exposed to HPEKs for 24 hours (Figure 4-3). In line with expectations, TNP do not cause a severe plummet in metabolic activity. Even at the highest concentration tested (AE: 400 μgml^{-1} , TWAD: 24 mgcm^{-2}), there was only about 20% reduction in metabolic activity. At lower concentrations the effect on cell viability was minimal. Others have shown similarly low toxicity of TNP on HaCaT cells (Fujita et al. 2009), bronchial epithelial cells (Gurr et al. 2005) and other cell lines (Jugan et al. 2012; Kang et al. 2009; Linnainmaa et al. 1997). The cellular response after different times of exposure was also tested (Figure 4-3). Until 4 hours, there was no change. There was a drop in metabolic activity in subsequent time points indicating that TNP

causes a direct response only after sufficient time for uptake is allowed. Previous studies have also shown a time dependent response to TNP exposure on various cell lines (Wang et al. 2007).

Figure 4-3 – Metabolic activity was measured (WST-8) (A) after 24 hours TNP exposure of various concentrations to HPEKs and (B) after TNP treatment (AE: 200 µgml⁻¹) for different exposure times to HPEKs. * indicates $p < 0.05$ significance w.r.t. the control.

To study the uptake of TNP, FITC-tagged TNP were utilized and measured using flow cytometry (Figure 4-4). Two parameters were measured: (a) the percentage of HPEKs which take up TNP and (b) the average amount of TNP which are taken by the HPEKs. After 4 hours of exposure, all cells showed a substantial level of TNP association. In order to discern the membrane bound TNP from the

fully internalized TNP, fluorescent quenching was performed. This revealed that about 40% of the signal came from membrane bound TNP while 60% came from completely endocytosed TNP. However, all (100%) cells had both fully internalized TNP and membrane bound TNP. Other groups have also observed TNP binding to the cell membrane before endocytosis. (Jaeger et al. 2012)

Figure 4-4 – Flow cytometry was performed on cells treated with FITC-tagged TNP at 50 μgml^{-1} for 4 hours (TWAD: 11.5 μgcm^{-2}). Quenching was performed to differentiate between TNP which were fully internalized and TNP which were bound to the cell surface. * indicates $p < 0.05$ significance w.r.t. the control.

Confocal microscopy was employed to track the FITC-tagged TNP (Figure 4-5). This was to map out up the uptake process. TNP resting on the cell membrane or possibly bound to it were observed almost immediately after exposure (solid arrows). Completely internalized TNP became visible from the one hour mark (broken arrows). These increased steadily in number up to 4 hours which is the point at which endocytosis generally reaches equilibrium. Many TNP were observed near and around the nucleus (dotted arrows). However, even after 24

hours of treatment they did not seem to penetrate the nuclear membrane and remained in the perinuclear region. Z-axis stacks were also performed and they confirmed no nuclear penetration. This is line with other studies who have shown a lack of nuclear penetration but possible accumulation of TNP around the nucleus (Janer et al. 2014; Shi et al. 2013).

To further confirm, TEM images which were obtained through a collaboration with a co-worker, Dr. Zhao Yun, were also analyzed (Figure 4-6). Endocytosis of TNP could be confirmed as numerous cells were observed with vacuoles containing dark spots which were believed to be metallic NPs, in this case TNP. Furthermore, it was also observed that TNP were crowding up near the nuclear region, again confirming the peri-nuclear localization.

A	Cell Membranes	TNP	Merged
0 hours
1/4 hour
1/2 hours
1 hours

Figure 4-5 – (A) Confocal images of HPEKs after exposure to 50 µgml⁻¹ TNP for increasing times (TWAD: 0, 0.05, 0.19, 0.74, 2.9, 11.5 and 115.7 µgcm⁻²) showing FITC-tagged TNP (green) and cell membranes (red). **(B)** Confocal image shows the nucleus (blue) of HPEKs after 24 hours incubation with FITC-tagged TNP (green). Solid arrows indicate TNP found on or near the cell membrane, broken arrows point towards completely internalized TNP while dotted arrows show TNP localized in the perinuclear region.

Figure 4-6 - Ultrastructural analysis using TEM. Primary human keratinocytes were treated with 4 μ M tamoxifen and 10 μ g/mL TiO₂ nanoparticles for 24 h and processed for TEM analysis. (A) Image showing peri-nuclear localization. (B) Higher magnification of “A”. (C-D) Both images show TNP taken up and residing in vacuoles post-endocytosis.

4.2.4. ZNP induce acute cell death

In contrast to TNP, ZNP are considered much more toxic (De Berardis et al. 2010; Jeong et al. 2013; Kao et al. 2011; Kasemets et al. 2009). Even without UV irradiation, ZNP can cause acute toxicity along with other unfavorable cellular responses. A similar approach was followed for ZNP using metabolic

activity as indicative of cell viability (Figure 4-7). A concentration dependent trend was observed as ZNP treatment incurred a plunge in metabolic activity. There was a 50% reduction in cell viability at 10 μgml^{-1} and almost complete loss in activity at 20 μgml^{-1} . These concentrations were much lower than TNP. Metabolic activity was also tested for different exposure times. Exposure times of 4 and 12 hours did not cause a drop in metabolic activity up to 10 μgml^{-1} . However, at subsequent concentrations the fall at lower exposure times was just as sharp as at 24 hours.

Other studies had proposed the role of ZI in ZNP induced toxicity. However, most studies still treat this solubility to be substantial inside the cell after particle uptake (Kao et al. 2011). In slight contrast, our solubility tests (section 4.1.5) had shown that ZNP solubility was substantial even prior to uptake. In fact, ZI were the key species present at the time of exposure. Therefore, it was hypothesized that ZNP-induced toxicity should be similar to any salt which can release the same concentration of ions. A parallel test using ZnSO₄ (a soluble Zn salt) was conducted (Figure 4-7). There was excellent correlation between similar stoichiometric ZI concentrations confirming the above hypothesis.

Due to the high solubility of ZNP in cell culture medium (>15 μgml^{-1} within 1 hour), ZNP in most of the concentrations tested should be in a completely solubilized state. In line with this expectation, particle uptake studies using FITC-tagged ZNP failed to show any uptake through flow cytometric or confocal analyses. Only when the concentration of ZNP was increased to very high levels

(50 μgml^{-1} and above) did the fluorescent particles appear. However, such concentrations were irrelevant to the study as not many cells would survive such elevated levels of ZNP.

Figure 4-7 – Metabolic activity was measured (WST-8) after (A) ZNP treatment at various concentration and for different exposure times to HPEKs and (B) after ZNP and ZI concentrations. Data are expressed as mean $\pm$ SD. * indicates $p < 0.05$ significance w.r.t. the negative control which contains no nanoparticles.

Instead, the focus was shifted to measuring intracellular zinc ions (ZI) levels. ICP-MS was performed on the cell lysates after and an increase in the amount of zinc was observed in HPEK samples which were treated with ZNP (Figure 4-8). A fluorescent zinc ion chelator was used to confirm this increase and visualize the localization of the free ZI. Exposure to 5 μgml^{-1} ZNP caused a small increase in fluorescence, which increased dramatically at exposure of 10 and 20 μgml^{-1} ZNP (Figure 4-8). On closer observation, it was observed that ZI localized in the

form of specks in the cytoplasm. This suggested that they were packetized in vesicles in the cells. Other studies have suggested similarly as well (Kao et al. 2011; Xia et al. 2008). Furthermore it was observed that unlike TNP, ZI do enter the nucleus and sometimes the nucleolus. A possible route for nuclear uptake of ZI is through association with metallothioneins (Nejdl et al. 2014). Increased cytosolic ZI can trigger expression of apo-metallothionein (apo-MT) through metal regulatory transcription factor 1. Apo-MT can bind up to seven atoms of ZI and subsequently gain entry into the nucleus through nuclear pores. It is possible that the ZI are released into the nucleoplasm.

C	Semi-quantitative amount of ZI normalized to NC (0 μgml^{-1}) ¹⁾			
	NC (0 μgml^{-1})	5 μgml^{-1}	10 μgml^{-1}	15 μgml^{-1}
	1.0 $\pm$ 0.2	3.6 $\pm$ 0.11	17.9 $\pm$ 1.2	29.1 $\pm$ 2.4

Figure 4-8 – (A) Intracellular Zn concentration was measured by ICP-MS of the cell lysates after exposure to ZNP. Data are expressed as mean $\pm$ SD. * indicates $p < 0.05$ significance w.r.t. the negative control which contains no nanoparticles. (B) Confocal images of HPEKs exposed to different concentrations of ZNP with nuclei stained using Hoechst dye (blue) and free ZI stained using Newport Green DCF. (C) Semi-quantitative analysis performed using ImageJ. The arbitrary values shown are normalized to the NC group and indicate the amount of green fluorescent specks per cell.

Taken together with the heightened solubility of ZNP, these results coherently conclude that ZI are chiefly responsible for the cytotoxic effects on HPEKs. Furthermore, even if some particulate ZNP is taken up by the cells, there is strong reason to believe that it would rapidly dissolve in acidic lysosomal vesicles (Kao et al. 2011; Xia et al. 2008).

4.2.5. Effect of ZNP solubility and cell type

To further probe into the role of ZI, different cellular environment, where the solubility of ZNP was considerably lower, were investigated. HLFCs cells show a similar vulnerability to ZnSO₄ salt (Figure 4-9) as compared to HPEKs, but they show a much lower toxicity to ZNP. The reason behind this was the medium used for the two cell types. DMEM, in which HLFCs were cultured, was less conducive to ZNP solubility than EpiGRO, in which HPEKs were maintained (Figure 4-9). This translated into lower ZI availability for HLFCs and thus a

lower toxic response. Other studies have also shown a difference in ZNP solubility depending on the culture media (Xia et al. 2008).

Figure 4-9 – (A) HLFCs were exposed to ZNP and ZnSO₄ at various stoichiometric concentrations. * indicates $p < 0.05$ significance between ZNP and ZnSO₄ at the same ZI concentration. **(B)** Solubility of ZNP in EpiGro and DMEM. * indicates $p < 0.05$ significance between DMEM and EpiGRO at the same time.

4.2.6. DNA Quantitation and PI staining

The major assay used in this study to measure cell viability was the WST-8 assay which tests metabolic activity. However, it was important to run other assays concurrently which also measured cell viability in order to confirm the robustness of the WST-8 assay for the *in vitro* model in question. DNA quantitation was one such assay which was run and it showed excellent correlation with the metabolic assay (Figure 4-10). Similarly, PI staining is another assay which quantifies cell death and was one more route to measuring cell viability and good correlation was observed (DATA not shown).

Figure 4-10 – Metabolic activity against DNA quantitation to test cytotoxicity of HPEKs in response to ZNP and TNP exposure.

4.3. Cytotoxicity of Dual Nanoparticle

4.3.1. Overview

Both ZNP and TNP were put through extensive toxicity screening. A deep understanding into their cellular responses as well as an insight into the possible mechanisms behind them have been the subject of research for the past decade or more. Recent literature as well as the results reported in the previous section, both indicate that ZNP are more toxic than TNP, owing to the enhanced solubility of ZNP which produces excessive zinc ions (ZI). A spike in the intracellular ZI level has a series of repercussions upsetting the homeostasis of the cell causing severe compromise of cell viability. On the other end, TNP do not become toxic until at very high doses. Even so they are taken up generously by HPEKs but importantly do not enter the nuclei.

This section discusses the core of this study: the effect when these two contrasting nanoparticles, TNP and ZNP, are treated to HPEKs together. There are a number of ways that this exposure could be tuned. For instance both particles may be mixed together and exposed simultaneously to the cells or exposed one after the other. It is these protocols which were exploited in order to identify the combinatorial effects of ZNP and TNP as well as singling out the relevant mechanisms at play.

4.3.2. Specific Methods: Dual Nanoparticle Treatments

In a usual toxicity test, cells were exposed to nanoparticles for a set amount of time after which a cellular response(s) is measured. In dual nanoparticle studies, the protocol in which the two nanoparticles are exposed to the cells is an added parameter. For example, exposing ZNP to cells before TNP may have a different response from cells compared to exposing TNP to cells before ZNP. This relative time of exposure was a useful parameter which was exploited in order to single out certain inter-nanoparticle responses. Based on this, different terminologies were used to categorize HPEKs which had undergone certain treatments. The following is a description of the protocols which were used in this study:

a. Control Cells

This category was HPEKs exposed to only one type of nanoparticles. These are called so because the responses from these control cells were compared to dual nanoparticle treated cells in order to elucidate any interesting differences.

b. TNP-exposed Cells

These cells were referred to as TNP-exposed cells as they had been allowed to interact with TNP first. The consequence of such a treatment was that both intra and extra-cellular TNP were present at the time when ZNP was introduced. TNP-exposed cells were prepared by initially seeding HPEKs in appropriate cell culture plates as per normal. After overnight incubation, HPEKs were exposed to TNP for 2 hours. After 2 hours, the medium *was not* aspirated and cells were not washed. At this stage, the resulting HPEKs contained both extra- and intra-

cellular TNP and were referred to as TNP-exposed cells. ZNP was then treated to TNP-exposed cells for 24 hours (unless otherwise stated) to study the effect extra- and intra- cellular TNP could have on the toxicity of ZNP. The TWAD of TNP was based on 24 hours incubation because the TNP were never removed and remained in the cellular environment for 24 hours.

c. TNP-loaded Cells

TNP-loaded cells were prepared by seeding HPEKs in the appropriate cell culture plates and equilibrating them overnight. The next day the HPEKs were treated with TNP for 2 hours after which the HPEKs were washed to remove any extracellular TNP. At this stage, there were only membrane bound and intracellular TNP left with the HPEKs which were now referred to as TNP-loaded cells. ZNP exposure was then carried out to study the effect of intracellular TNP on ZNP induced toxicity. The TWAD of TNP was based on 2 hours incubation because no external TNP remained in the cell culture plates beyond the initial 2 hours.

d. ZNP-exposed Cells

This group was the exact opposite of TNP-exposed cells. In this case HPEKs were seeded as per normal and after overnight incubation were exposed to ZNP for 2 hours. After 2 hours, the medium *was not* aspirated so that the HPEKs contained extra- and intra-cellular ZNP and were called ZNP-exposed cells. These cells were treated with TNP for 24 hours to see the effect pretreatment of ZNP could have. The TWAD of TNP was therefore based on 24 hours incubation.

e. SIM-exposed Cells

To prepare this group of cells, HPEKs were seeded as per normal and equilibrated overnight. Both nanoparticles, TNP and ZNP, were added to the cells together and treated for up to 24 hours. In this case there was no pre-treatment of any nanoparticle therefore at the time of nanoparticle exposure, there were no intra-cellular or membrane bound nanoparticles. The group is called “SIM (*simultaneously*)-exposed cells”. The TWAD of TNP was calculated based on 24 hours exposure.

4.3.3. TNP adsorb Zinc Ions in cell medium

There were two major findings from the single nanoparticle screening described in the previous section. First, it was evident that out of the two, ZNP was much more toxic nanoparticle. This was shown to be due to superior solubility of ZNP which can release zinc ions (ZI). This elucidated the second important conclusion which was identifying the ZI to be the source toxicity. Hence, it was hypothesized that when exposed to both TNP and ZNP, the combined toxicity of the two nanoparticles would hinge on the effect of TNP on ZI concentration. Any changes in the concentration of ZI could potentially alter the extent of cellular response.

One potentially critical characteristic of TNP identified was its zeta potential; TNP exhibits a negative surface charge. Since ZI are positively charged, the TNP surface could potentially adsorb the ions onto its surface by electrostatic

attraction. Such an interaction could immobilize the ZI effectively reducing the free ZI concentration.

In line with the abovementioned, tests revealed that free ZI were reduced depending on the TNP concentration present (Figure 4-11 A). Even at the lowest concentration tested (25 µgml⁻¹) there was a significant reduction in free ZI. The same phenomenon was observed when ZnSO₄ salt was used as the source of ZI. This confirmed that TNP could scavenge ZI.

Two other bivalent cations, magnesium and calcium were also tested in a similar way to see whether the ion adsorption was exclusive or preferential to ZI. In water, all cations were adsorbed and scavenged by TNP (data not shown). Interestingly, in medium, when tested individually, Mg²⁺ and Ca²⁺ were scavenged by TNP but the effect was much lower as compared to ZI (Figure 4-11 B). Mg²⁺ ions showed the least absorption followed by Ca²⁺ and Zn²⁺ respectively, and higher concentrations of TNP adsorbed more ions which reinforces the observation. More interesting, when TNP were tested with all three cations together, only Zn²⁺ were adsorbed (Figure 4-11 C). This clearly shows that TNP not only adsorb positive species of ions, but their adsorptive capabilities are preferential in the case of ZI. Since the adsorption was observed in water as well as medium, the role of electrostatic forces can be established. However, the selectivity towards ZI in medium points towards a role of the protein corona as well which could be facilitating the adsorption preferentially for ZI. An analysis of the kind of proteins which TNP adsorb may confirm the role of proteins.

However, this was beyond the scope of this study. Nonetheless, the ion adsorptive effect of TNP was conclusively evident.

Figure 4-11 – (A) Effect of TNP on the concentration of free solubilized ZI measured using ICP-MS. * indicates $p < 0.05$ significance w.r.t. to control which has no TNP nanoparticles. Ion adsorptive effect of TNP (100 and 400 µgml⁻¹) on Mg, Ca and Zn ions (B) individually and (C) together.

4.3.4. TNP alleviate ZNP-induced toxicity in HPEKs

Once the adsorption of ZI onto TNP surface was confirmed, it was investigated whether this phenomenon could protect the cells from ZNP-induced toxicity. TNP-exposed cells¹ were utilized for this purpose and the effect of subsequent ZNP treatment was studied. The results were particularly revealing (Figure 4-12). TNP-exposed cells experienced no drop in metabolic activity up to 5 µgml⁻¹ of ZNP treatment. At 10 µgml⁻¹ of ZNP, there was a drop in metabolic activity only in HPEKs which had been pre-exposed to no or low concentration of TNP. In contrast, cells pre-exposed to higher concentrations exhibited no reduction in

¹ TNP-exposed cells contain intra- and extra- cellular TNPs (see Methods for details).

metabolic activity compared to the negative control. The same was observed at 15 μgml^{-1} of ZNP; cells pre-exposed to highest concentration of TNP (AE: 400 μgml^{-1} , TWAD: 24 mgcm^{-2}) still exhibited uncompromised metabolic activity. At 20 μgml^{-1} of ZNP, presence of TNP did not protect HPEKs from being severely compromised.

Figure 4-12 – Metabolic activity of TNP-exposed HPEKs was measured (WST-8) after exposure to ZNP at different concentrations. TNP-exposed cells have intra- and extra- cellular TNP. Dosage of TNP used to prepare the TNP-exposed cells were 25, 100 and 400 μgml^{-1} (TWA dosage: 1.5, 6.0 and 24.0 mgcm^{-2} respectively). Control cells are only treated with ZNP. Data is expressed as mean $\pm$ SD. * indicates $p < 0.05$ significance w.r.t. to control which has no TNP.

4.3.5. Intracellular TNP play “vigilante”

It was evident that TNP-exposed cells were more resilient to ZNP-induced toxicity. Results suggested that TNP were playing the role of a “vigilante” in protecting the cells from ZNP. The mechanism hypothesized for this protection rested on the ability of TNP to scavenge and immobilize ZI. TNP-exposed cells contain both intra- and extra- cellular TNP. However, a more intimate protection would be afforded if intracellular TNP could also scavenge ZI. For this inquiry, the same study was repeated on TNP-loaded cells² because they only contain intracellular TNP. Interestingly, TNP-loaded cells also exhibited strong resilience to ZNP-induced toxicity. There was a significant recovery in metabolic activity even at the highest concentration of ZNP (Figure 4-13). This was a clear indication that intracellular TNP were the species which could shield HPEKs against ZNP.

² TNP-loaded cells do not contain any extracellular TNPs. Cells are washed before ZNPs treatment so all but intra-cellular and membrane bound TNPs are removed (see Methods for details).

Figure 4-13 – Metabolic activity measured (WST-8) after exposure of ZNP to control and TNP-loaded cells. TNP-loaded cells have only intracellular and membrane bound TNP (AE – 100 µgml⁻¹, TWAD – 60 µgcm⁻²). * indicates $p < 0.05$ significance w.r.t. the sample group without any TNP.

4.3.6. Extracellular TNP do not contribute to protective effect

To explore the effect of extracellular TNP, HPEKs were treated with both nanoparticles simultaneously so that only extracellular TNP were present at the onset of ZNP exposure. This treatment did not show any significant difference from control cells. There was no protection afforded to HPEKs. In effect, this further proved that intracellular TNP were needed to trigger the protective effect.

4.3.7. TNP modulate intracellular zinc ion (ZI) concentration

That TNP plays “vigilante” and alleviates ZNP-induced toxicity was proven convincingly. To prove whether this happened by immobilizing ZI in the cell, the cellular environment was tested (Figure 4-14). NPG staining showed that both

TNP-exposed and TNP-loaded cells had considerably lower amount of free ZI compared to the control at all doses of ZNP. Cells with only intracellular TNP (TNP-loaded cells) showed the most amount of reduction again emphasizing their *vigilante* behavior. Confocal microscopy was also performed to visualize the phenomenon (Figure 4-15). Cells with intracellular TNP showed substantially lower fluorescence from ZI as TNP-exposed and TNP-loaded cells showed a 44% and 36% reduction in ZI signal respectively.

Figure 4-14 – Newport Green DCFTM fluorescence is measured after control, TNP-exposed and TNP-loaded cells are exposed to ZNP for 24 hours. TNP-exposed cells have both intra- and extra- cellular TNP (AE – 100 µgml⁻¹, TWAD – 6.0 mgcm⁻²). TNP-loaded cells have only intracellular and membrane bound TNP (AE – 100 µgml⁻¹, TWAD – 60 µgcm⁻²). Data is expressed as mean ± SD. * indicates $p < 0.05$ significance w.r.t. the group without any TNP.

Figure 4-15 – (A) Confocal images of HPEKs with nuclei stained using Hoechst dye (blue) and free ZI stained using Newport Green DCF™ (green). TNP-exposed cells have both intra- and extra- cellular TNP while TNP-loaded cells have only intracellular and membrane bound TNP (AE – 100 μgml⁻¹, TWAD – 6 μgcm⁻²). **(B)** Semi-quantitative analysis performed using ImageJ. Values shown are normalized to “Only ZNP” group and indicate the amount of green fluorescent specks per cell.

An alternate method to confirm the modulation of free ZI concentration due to intracellular TNP was using ICP-MS (Figure 4-16). Concurrently metabolic activity was also tested to really see translation of this modulation into cell viability. For control cells, at ZNP dosage of 2.5 μgml^{-1} , there is no significant increase in ZI concentration and comprehensibly no decrease in metabolic activity either. Things get interesting at 10 μgml^{-1} , where there is a sharp increase in ZI concentration marked by a steep fall in metabolic activity as well. For TNP-exposed cells, there is not much difference at lower ZNP dosage. However, at 10 μgml^{-1} ZNP treatment, TNP-exposed cells exhibit a significant resilience to ZI concentration spike accompanied by a significant alleviation in toxicity as well. This really ties together the connection between ZI and ZNP-induced toxicity as well as the *vigilante* effect TNP can impart.

Figure 4-16 – Metabolic activity measured (WST-8) after exposure of ZNP to control and TNP-exposed cells and the corresponding intracellular ZI concentration in the cell lysates. TNP-exposed cells have both intra- and extra- cellular TNP (AE – 100 μgml^{-1} , TWAD – 6.0 mgcm-2). * indicates $p < 0.05$ significance w.r.t. the sample group without any TNP.

4.3.8. “Vigilante” Effect in Human Primary Dermal Fibroblasts

In order to extend the “vigilante” effect of TNP to other cell types, human primary dermal fibroblasts (HPDFs) were chosen as a suitable comparative model. Given their abundance in skin tissue and their importance in various functions of the skin, their relevance to nanotoxicity from the dermal route of exposure is highly relevant. In fact many studies have previously investigated the effects of ZNP and TNP on HPDFs (Setyawati et al. 2013a).

Although HPDFs also showed sharp ZNP-induced cytotoxicity, the onset of cell death was at much higher concentrations ($> 50 \mu\text{gml}^{-1}$ for HPDF: $> 5 \mu\text{gml}^{-1}$ for HPEKs). It must be noted that the growth medium used was different for HPDFs and interestingly, ZNP were less soluble in DMEM compared to the medium used for HPEKs ($<15 \mu\text{gml}^{-1}$ in DMEM: $>20 \mu\text{gml}^{-1}$ in HPEKs medium after 24 hours). As a consequence, HPDFs encounter a lower concentration of ZI which can explain the lack of cell death at lower concentrations. In effect, this again reinstates the central role which ZI (or ZNP solubility) play in ZNP-induced toxicity.

TNP on the other hand induced significant but low cell death even at high concentrations similar to the other cell type. Even at $400 \mu\text{gml}^{-1}$, viability was up to 80% after 24 hour treatment. When TNP-treated HPDFs were exposed to high concentration ZNP, there was significantly less reduction in viability compared to the treatment of ZNP alone. This was in line with earlier observations on

HPEKs and therefore it can be concluded that TNP' vigilante effect is not cell type specific.

Due to lower solubility of ZNP in DMEM, HPDFs experience a lower dosage of ZI at the onset of ZNP exposure. Since it is the ions which really cause the acute toxic effects, the HPDFs withstand ZNP-induced toxicity at lower concentrations. Apart from solubility in media, ZNP also dissolve copiously inside the acidic lysosomes into which they are packetized following endocytosis (Xia et al. 2008). As this is a more roundabout way of overloading the intracellular environment with ZI, it is logical that the toxicity is delayed as well as sharp only at high concentrations of ZNP. In the case of HPEKs, loading the cells with intracellular TNP was the most effective strategy to protect against ZI as it ensured an ion adsorbing species in the cells at the time of Zn²⁺ ion uptake. In the case HPDFs, however, there is a delay in the uptake of cell death inducing concentrations of ZI. To test this possibility, ZNP-exposed and SIM-exposed HPDFs were also compared. The vigilante effect was still profound even though TNP did not have a head start in terms of cellular exposure.

4.4. Oxidative and Genotoxic Potential of Nanoparticles

4.4.1. Overview

The ability of NPs to induce ROS has been a major focus of nanotoxicology. There are two ways in which NPs are believed to produce oxidative stress. The

first mechanism is facilitated by the way NPs are taken up; cells take up NPs via endocytosis. This process packets the NPs into vesicles by engulfing them with the cell membrane. These packets have an acidic environment which allows NPs to produce ROS (Nohl and Gille 2005). The second mechanism rests on the ability of NPs to gain entry into the oxidative organelles e.g. mitochondria (Zhang and Gutterman 2007). This interaction can be either by a direct penetration or indirectly through leached out ions which enter these organelles and trigger oxidative stress.

Much of the attention to ROS generation is because it can trigger DNA damage. ROS is a by-product of aerobic metabolism and thus some level of it is always present and indeed necessary for normal function. The cell is well equipped to counter this oxidative challenge. The problem comes in, however, when there is an imbalance between the oxidative and anti-oxidative processes. This is where ROS induced by NPs play a damaging role by overloading the cells' anti-oxidant mechanisms. This perturbation can result in the oxidation of biomolecules like lipids, proteins, RNA and DNA, as well as the oxidative inactivation of enzymes through oxidation of their co-factors.

Therefore, ROS is one of the focal point in the chain of events which lead to nanotoxicity. In fact, oxidative stress is unique as it can lead to both cytotoxicity including apoptosis, cell death, mitochondrial depolarization, etc. as well as genotoxicity by causing ROS-mediated DNA damage. Therefore it was

important to study ROS from both standpoints: as a toxicity marker in itself and as DNA damage mediator.

Earlier parts of this thesis have dealt with the cytotoxic repercussions of the nanoparticles in question. However, as discussed above, one of the more subtle yet consequentially extremely relevant threats is DNA damage. There are a number of reasons why nanoparticles pose a realistic threat as far as genotoxicity is concerned. The more direct mechanism in which nanomaterials can cause DNA damage is by gaining entry into the nucleus of the cells. Being of such small sizes, this remains a plausible reality for nanomaterials if their physicochemical properties permit (Ng et al. 2014). The other more indirect mechanism is through the induction of reactive oxidative species (ROS) and depletion of the antioxidants in the cellular environment. The ROS generated can in turn cause DNA damage (Li et al. 2009a; Wang et al. 2009).

DNA strand breaks or DSBs are the most lethal kinds of DNA damage and can trigger genomic instability and ultimately cancer (Jeggo and Lobrich 2007; Singh et al. 2009). The cell is equipped to deal with such instances by arresting damaged cells until repair is carried out before returning them to the normal cell cycle. Such a strategy guards against mutations. Histone variant H2AX is one of the five histones that enclose DNA into chromatin. During cell cycle-arrest and DNA repair in the advent of DSBs, it is rapidly phosphorylated by PI3K-like kinases on Ser139 from carboxyl terminus to form γ -H2AX at DSB sites (Bonner et al. 2008; Jeggo and Lobrich 2007). This leads to recruitment of DNA repair proteins

and apoptotic proteins which form DNA damage-induced foci resulting in signal amplification. Therefore using fluorescent antibodies specific to γ -H2AX, DNA damage can be detected. This technique is called Immunofluorescence staining. Furthermore γ -H2AX detection via immunocytochemistry was demonstrated to be 10 – 100 times more sensitive than the comet assay (Ismail et al. 2007; Leopardi et al. 2010; Trouiller et al. 2009).

This section focuses on the combined oxidative capabilities and genotoxic potentials of TNP and ZNP. Further, the link between these two toxic endpoints is discussed and the underlying mechanism behind the contrast between dual nanoparticle and single nanoparticle treatment is investigated.

4.4.2. Specific Methods – DNA Damage and ROS analysis

4.4.2.1. Immunofluorescence staining (γ -H2AX assay)

To study DNA damage, HPEKs were grown on 15mm square no. 1 cover slips (thickness ~ 160 μ m) in 6-well plates to confluence. They were then exposed to nanoparticles for 24 hours. For dual nanoparticle treatments, similar protocols as mentioned in the previous sections were used. As a positive control, ultraviolet irradiation of HPEKs for 5-20 mins was performed to induce DNA damage. A filtered VL-115.C UV lamp (Viber Lourmat, Germany) at 254nm was used. After the exposure, the HPEKs were incubated in fresh medium for 24 hours (Hanasoge and Ljungman 2007; Lu and Lane 1993).

After the 24 hours incubation, HPEKs were fixed using cold 4% paraformaldehyde in PBS (Sigma-Aldrich, USA) at room temperature for 10mins. HPEKs were then washed thoroughly with PBS to remove any leftover NPs and the cell membrane was permeabilized with 0.5% v/v Triton X-100 (Sigma-Aldrich, USA) in distilled water for 10 mins at room temperature. After further washing, unspecific sites were blocked by treating them with a blocking buffer made up of 5% w/v bovine serum albumin (BSA; Dako, Denmark) in PBS for 30 mins at room temperature. Damaged DNA was then tagged using the primary antibody, anti-phospho-histone γ -H2AX (Ser139) rabbit monoclonal antibody (9718; Cell Signaling Technology, USA), diluted with blocking buffer to a ratio of 1:400 and incubated overnight at 4°C. On the following day, HPEKs were washed thoroughly with PBS to remove an unbound antibody. HPEKs are then incubated with FITC-conjugated secondary antibody, Alexa Fluor® 568 goat anti-rabbit IgG antibody (A-11034; Invitrogen, USA) diluted to a concentration of 1:200 in blocking buffer for 30mins in the dark at room temperature. After washing, the nuclei are stained with Hoechst 33342 (Molecular Probes, Catalogue number H3570).

4.4.2.2. Oxidative Stress Quantification

HPEKs were grown on 15mm square no. 1 cover slips (thickness ~ 160 μ m) in 6-well plates to confluence. They were then exposed to nanoparticles for 4, 8 or 24 hours. For dual nanoparticle treatments, similar protocols as mentioned in the

previous sections were used. As a positive control, ultraviolet irradiation of HPEKs for 20 mins was performed to induce oxidative stress. A filtered VL-115.C UV lamp (Viber Lourmat, Germany) at 254nm was used. Before irradiation, the medium in the wells were removed with the lids off. Immediately after fresh medium was added and the HPEKs were incubated for 2 hours (Chan and Yu 2000).

Treated cells were incubated with CellROX[®] reagent (Life technologies, US; C10444) according to the manufacturer's suggested protocol. Briefly, CellROX[®] reagent at a final concentration of 5 μ M was added to the cells and incubated for 30 mins. Cells were then washed thoroughly with PBS and fixed with cold 4% paraformaldehyde in PBS (Sigma-Aldrich, USA) at room temperature for 10mins. The nuclei of cells were stained using Hoechst 33342 (Molecular Probes, Catalogue number H3570).

4.4.2.3. Confocal Microscopy of DNA Damage and ROS

Once the cells were stained with the relevant dyes, the cover slips were flipped and mounted onto glass microscope slides assisted by cold mounting medium and sealed using transparent nail polish. For γ -H2AX assay, the cells were either viewed instantly or stored in the dark at 4°C for up to a week before being viewed. For the ROS assay, the cells were viewed within 24 hours according to the manufacturer's instruction. Microscopy was performed either on Leica TCS SP5 Broadband Confocal microscope using an oil lens at magnifications up to 60x or

Nikon A1-Rsi Confocal Microscope using an oil lens up to 100x. Post-image processing was performed using ImageJ 1.47v.

4.4.3. Inducing Oxidative Stress by UV-irradiation

UV-irradiation has been shown to induce ROS as well as DNA damage mediated by ROS (Rastogi et al. 2010). Therefore it was used as the positive control since it could not only be a standard of comparison for ROS and DNA damage individually, but also for ROS-mediated DNA damage specifically. Using image analysis, the intensity of oxidative stress induced was categorized into 4 groups: no, low, medium and high ROS (Figure 4-17).

As expected, the negative control did not exhibit much ROS (97% cells showed no detectable ROS). The amount being exhibited was the endogenous residual amount which is essential for normal metabolic activity. UV-irradiated HPEKs, in contrast, showed severe oxidative stress. Only 6% cells showed no increase in ROS while 45% and 42% HPEKs showed high and medium levels of ROS respectively. This validated the positive control for subsequent experiments and comparisons.

4.4.4. TNP induce substantial ROS generation in HPEKs

TNP did not generate ROS in HPEKs at concentrations below 50 µgml⁻¹ regardless of incubation time (data not shown). For higher concentrations, there

was a notable increase in ROS (Figure 4-17). At 4 hours of TNP exposure, the percentage of cells demonstrating no detectable ROS dropped to 93%, 85% and 71% for 50, 100 and 200 μgml^{-1} . Furthermore, at increasing concentration of TNP, the percentage of HPEKs showing low, medium and high levels of oxidative stress all increased. This meant that there was a strong concentration dependent trend. At higher concentrations, TNP induced ROS in more cells and with increasing intensity.

After 8 hours of TNP exposure, there was a sharp rise in the ROS induced at all concentrations. At 50 μgml^{-1} , 40% HPEKs were now experiencing an increase in oxidative stress while at 100 and 200 μgml^{-1} , only a third of the cells or less were now ROS free. Again the intensity of ROS increased with the increase in concentration confirming the strong concentration dependency. To bridge the two parameters of increasing concentration and exposure time, the time weighted average dosage (TWAD) was tabulated for each treatment. Interestingly, the TWAD and ROS increase correlated perfectly.

Previous studies have shown low or no oxidative stress in the absence of illumination in epithelial cells (Gurr et al. 2005) and macrophages (Xia et al. 2006). Interestingly in human keratinocytes cell lines HaCaT, a significant increase in oxidative stress was induced at concentrations as low as 5 and 50 μgml^{-1} (Jaeger et al. 2012). Another study, however, reported the contrary and substantiated the lack of ROS generation by showing no significant effect on the expression of genes related to oxidative stress (Fujita et al. 2009). However, both

studies used different types of TNP. While Jaeger et al. used P25 Degussa TNP which are a mixture of anatase and rutile, Fujita et al used 100% anatase TNP. In this study, P25 Degussa TNP were used and substantial ROS generation was observed in line with previous reports studying similar parameters.

A	4 HOURS		
	Hoechst	CellROX [®]	Merged
50 μgml^{-1} 11.5 μgcm^{-2}
100 μgml^{-1} 23 μgcm^{-2}
200 μgml^{-1} 46 μgcm^{-2}

B	8 HOURS		
	Hoechst	CellROX [®]	Merged
50 μgml^{-1} 46 μgcm^{-2}
100 μgml^{-1} 92 μgcm^{-2}
200 μgml^{-1} 184 μgcm^{-2}

Figure 4-17 – (A, B) Confocal images of HPEKs exposed to TNP for 4 hours and 8 hours showing ROS. (C) Quantitative analysis of ROS generated by TNP. NC is the negative control HPEKs which are not treated with NPs. PC is the positive control which is UV-irradiated HPEKs. * indicates $p < 0.05$ w.r.t. NC.

TNP are insoluble in cell medium (and water) even at low pH. Thus there is no chance of ions leaching out from these NPs and attacking the cellular organelles. Nonetheless, there was a substantial and immediate uptake of TNP into the HPEKs. This was a two steps process: first TNP attach onto the cell membrane and second they are taken up by the cells through endocytosis. As discussed earlier, this endocytosis packetizes NPs into vesicles which have an acidic environment causing the NPs to produce ROS (Nohl and Gille 2005). The ROS signals come from both the mitochondrion (mitochondrial ROS) as well as the nucleus (nuclear ROS). This is because the ROS-dye used binds strongly to DNA after being oxidized by free radicals. Thus it localizes in the nuclei and mitochondria, the two DNA carrying organelles. Nuclear ROS increase followed closely with the overall oxidative stress because all cells exhibiting ROS invariably also exhibited nuclear ROS. There were several bi-nucleated, multinucleated and mitotic cells observed all demonstrating ROS and possibly some form of genotoxicity. The signal was strongest from the nucleoli.

Mitochondria is one of the major oxidative organelles. Based on this distinction, there was a trend observed. The mitochondrial level of ROS increased initially with increasing TWAD, but then dropped at higher concentration and exposure time (Figure 4-18). This implied that TNP-induced ROS was shifting from the mitochondria to the nuclei of the cells. The TNP uptake profile could explain such a shift. As earlier discussed, after being taken up, the TNP tend to move deeper within the cells and end up localizing in the perinuclear region. Therefore any ROS generated from TNP was more likely to be localized in the nucleus as

the incubation time was increased. This could have a marked effect on TNP's DNA damage potential which will be discussed in the next section.

Figure 4-18 – Mitochondrial ROS induced in HPEKs by TNP at different concentrations and exposure times. * indicates $p < 0.05$ w.r.t. NC.

4.4.5. TNP do not induce heavy DNA damage in HPEKs

The genotoxic potential of TNP, like its cytotoxic potential, was quite sedate (Figure 4-19). At 1 µgml⁻¹, there was no observable increase in DSBs. However at 100 µgml⁻¹, there was a significant induction of DSBs. The uptake passage was previously studied and discussed in earlier sections. It was observed that although there is substantial and time dependent uptake of TNP in HPEKs, TNP failed to gain any entry into the nuclei of cells (Figure 4-5). They did seem to crowd up in the perinuclear region but could not penetrate the nuclear membrane. This could

explain their low genotoxic potential as they were unable to interact physically with DNA and induce primary direct genotoxicity.

Instead, there was strong indication that TNP induced ROS-mediated DNA damage which is a type of primary indirect genotoxicity (Ng et al. 2014). The oxidative potential of TNP was examined earlier in detail. The positive control in that experiment was UV-irradiated HPEKs too. It was observed that although TNP induced ROS in a concentration dependent way, the level and intensity was still much lower than UV-irradiated HPEKs. DNA damage followed the same trend suggesting that causality stemmed from ROS. One observation made earlier was that the ROS localization increased in the nuclei when the time-weighted average dosage (TWAD) was increased. This was supported by confocal images which suggested that TNP travel deeper into the cell and accumulate in the perinuclear region. From here any ROS generated would have a strong likelihood to cause oxidative damage to critical molecules in and around the nuclei. Apart from DNA itself, molecules which play a critical role in DNA repair and replication like RNA, lipids and enzymes may also be damaged and indirectly affect DNA health.

A	Nuclei	γ -H2AX foci	Merged	
Negative Control
TNP 1 μgml^{-1}
TNP 100 μgml^{-1}
UV-irradiation
B	Percentage of cells showing DNA damage (100%)			
	NC	TNP 1 μgml^{-1}	TNP 100 μgml^{-1}	UV (PC)
	3.6 $\pm$ 1.2	8.2 $\pm$ 2.7	18.3 $\pm$ 3.1	68.1 $\pm$ 4.3

Figure 4-19 – (A) Confocal images of HPEKs exposed to TNP for 24 hours with nuclei stained using Hoechst dye (blue) and γ -H2AX foci stained green. (B) Semi-quantitative analysis shown below each image was performed using ImageJ. The data labels show the percentage of cells showing DNA damage.

4.4.6. ZNP-caused ROS generation is concentration dependent

ZNP were found to be soluble in cell medium. This was especially true in slightly acidic pH. Therefore, in their case the HPEKs experienced an increased level of intracellular ZI. After 4 hours of ZNP treatment, there was a concentration dependent increase in the oxidative stress encountered by HPEKs (Figure 4-20). However, only cells exhibiting low ROS showed significant increase from 3% at 2.5 μgml^{-1} to more than 10% at 5 and 10 μgml^{-1} . The fraction of cells exhibiting medium to high levels of oxidative stress did not increase much.

This dynamic evolved after 8 hours of incubation but did not change drastically. The concentration dependent trend was still there but the effect of incubation time was not comparable over all concentrations. At 2.5 μgml^{-1} , there was no increase in ROS production. At 5 μgml^{-1} , the fraction of cells demonstrating oxidative stress increased from a quarter at 4 hours exposure to a third of the population at 8 hours. It was mostly the medium to high ROS categories that showed an increase.

At 10 μgml^{-1} also, the fraction of cells exhibiting ROS increased from 21% to 33% after 4 and 8 hours exposure respectively. However, this increase was only limited to the low and medium levels of ROS category. Interestingly, there were more cells exhibiting ROS at 5 μgml^{-1} than 10 μgml^{-1} after 8 hours of exposure. This could be due to a drop in cell viability caused by the overloading of ZI at higher concentration. The same could also explain why there was no increase in cells exhibiting high level of ROS at 10 μgml^{-1} after the additional exposure time.

Previous studies have also shown a concentration dependent oxidative stress of ZNP on keratinocytes cell lines, HaCaT (Bae et al. 2012; Jeong et al. 2013; Ogiso et al. 2002). The concentrations used in most studies was 20 µgml⁻¹ which is similar to the doses used in this study. The difference across the levels of ROS induced could be due to different cell culture media which affects solubility of ZNP and different surface properties of ZNP. Nevertheless ROS has been linked strongly with the cyto- and geno- toxic effects induced by ZNP.

As far as localization of the signals was concerned, ZNP-induced ROS was more strongly localized in the nuclei and sometimes in the nucleoli. Again there were numerous bi-nucleated, multi-nucleated and mitotic cells and the signal was strongest from the nucleoli. The mitochondrial ROS did not elevate strongly and remained at the levels similar to the negative control (Figure 4-21). This may be due to the ability of ZI to be picked up by zinc chelating proteins which can transport them into the nuclei (Nejdl et al. 2014).

A	4 HOURS		
	Hoechst	CellROX [®]	Merged
2.5 µgml ⁻¹
5.0 µgml ⁻¹
10.0 µgml ⁻¹

B	8 HOURS		
	Hoechst	CellROX [®] [®]	Merged
2.5 µgml ⁻¹
5.0 µgml ⁻¹
10.0 µgml ⁻¹

Figure 4-20 – Detailed analysis of ROS generated by ZNP. NC is the negative control HPEKs which are not treated with NPs. PC is the positive control which is UV-irradiated HPEKs. * indicates $p < 0.05$ w.r.t. NC.

Figure 4-21 – Mitochondrial ROS induced in HPEKs by ZNP at different concentrations and exposure times. * indicates $p < 0.05$ w.r.t. NC.

4.4.7. ZNP cause heavy DNA damage

After 24 hour treatment with single nanoparticles, the γ -H2AX foci signifying DSBs were analyzed (Figure 4-22). ZNP induced a strong genotoxic response. Even at 1 μgml^{-1} there was a significant rise in the percentage of cells displaying DSBs. This increased 3-fold when the ZNP concentration was increased to 10 μgml^{-1} , almost as high UV-irradiation treatment for 15 mins. This was in line with previous reported studies which suggest that ZNP can induce p53-mediated, oxidative stress induced (Ng et al. 2014).

Figure 4-22 – (A) Confocal images of HPEKs exposed to ZNP for 24 hours with nuclei stained using Hoechst dye (blue) and γ -H2AX foci stained green. (B) Semi-quantitative analysis shown below each image was performed using ImageJ. The data labels show the percentage of cells showing DNA damage.

The oxidative potential of ZNP was investigated previously (Figure 4-20). Even at 10 μgml^{-1} , ROS generated was not very high. When exposed for a longer time,

there was a relative increase in the ROS. Still this was much lower than the positive control, UV-irradiation. Therefore ROS-mediated DNA could not be the major pathway to the genotoxicity of ZNP.

Instead, the role of zinc ions (ZI) was suspected. As previously discussed, ZNP can cause a spike in the intracellular ZI level. The coping mechanism of cells towards excessive ZI is expression of apo-metallothionein (apo-MT) through metal regulatory transcription factor 1 (Nejdl et al. 2014). Apo-MT can bind up to seven atoms of ZI and enter into the nucleus through nuclear pores and it is possible that the ZI are released into the nucleoplasm (Nejdl et al. 2014). In line with theoretical expectations, it was observed that nuclear ZI levels increased with increasing concentration of ZNP exposure (Figure 4-23). Although at 2.5 μgml^{-1} , there was no significant increase in nuclear ZI levels, at 5 and 10 μgml^{-1} , the increase was drastic. This corresponded well with the observed DSBs in HPEKs. Free ZI in the nucleoplasm can exert a direct primary genotoxic effect by physically interacting with the DNA (Ng et al. 2014).

Figure 4-23 – Nuclear ZI after ZNP exposure to HPEKs. Values are normalized to ZNP 10 and NC is subtracted out. * indicates $p < 0.05$ w.r.t. NC.

4.4.8. Oxidative potential of Dual Nanoparticles is additive

The mechanism by which TNP and ZNP induce toxicity is quite distinct. The source of this stark difference was the solubility of the two NPs. This is one of the themes that dominated the combinatorial cytotoxicity of the two NPs. While TNP remains insoluble and is therefore endocytosed in particulate form, ZNP dissolve and eventually exist in the form of ZI. ROS generation is a surface mediated process in the case of TNP and it is the acidic environment of endosomal lysosomes which facilitates the process. While ZI were also able to induce ROS by possibly interacting directly with oxidative organelles and DNA, their effect was marginal when compared to the oxidative potential exhibited by TNP.

Since TNP was the more potent ROS agent the approach of evaluating oxidative stress in dual nanoparticles was aligned accordingly and the effect of ZI on TNP-induced ROS was investigated³. Keeping in mind the same interaction observed earlier whereby ZI can adsorb onto the surface of TNP, the ROS generation in dual nanoparticle treatment could be irregular. One possibility was that the adsorption of ZI onto the TNP surface could passivate the TNP surface and quench the ROS generation. The converse could also be possible, where the surface adsorption enhances the TNP surface activity and by facilitating electron transfer, increases the oxidative potential of TNP. However, considering the relative particle concentrations of the two NPs, the effect might probably be minimal. This is because there is ≈ 2.4 million square centimeters of surface for every 1 μg of TNP considering hydrodynamic diameter of TNP aggregate of approximately $\approx 2\mu\text{m}$, effective aggregate density of 1.255 gml^{-1} and assuming a circular non-porous aggregate. On the other hand, even at the highest dosage of ZNP tested (i.e. 20 μg), the maximum surface that the ZI can cover considering they are packed next to each other is at best 0.8 square centimeter. Of course the ZI do not need to be tightly packed to cause an influence on the TNP surface but the numbers are so disproportionately tilted towards the TNP surface that it

³ This statement is diametrically opposed to how the dual nanoparticles cytotoxicity was investigated. Since, ZI were the more toxic species in that case, the study approach was to study the effect of TNPs on the ZI induced toxicity.

would be hard to imagine there would be an effect. The coverage would be so minuscule that even if all the ions are adsorbed onto the TNP surface, which they are not, it would leave majority of TNP surface still ZI free and interacting with the cellular environment as per normal. Therefore, it was hypothesized that dual nanoparticles would show an additive effect as compared to single nanoparticle. ZNP-exposed cells were prepared using different dosages of ZNP and then treated with TNP. All ZNP-exposed cells showed an increase in ROS generation after TNP treatment which was similar to the amount of ROS generated by TNP individually (Figure 4-24A). Therefore it can be said that the ROS generation was additive in nature. Two particles produced the sum amount of ROS that they produced individually. Similarly, there was no significant change in the ROS generated when TNP-exposed cells were treated with ZNP (Figure 4-24B). This again confirmed the additivity of oxidative stress.

Figure 4-24 – (A) ROS generation of ZNP-exposed cells after 0 and 200 μgml^{-1} TNP treatment. The insert shows ROS generation after 200 μgml^{-1} (TWAD 46 μgcm^{-2}) TNP treatment of control (non ZNP-exposed) HPEKs. (B) ROS generation of TNP-exposed cells after 0 and 10 μgml^{-1} ZNP treatment. The insert shows ROS generation after 10 μgml^{-1} ZNP treatment of control (non TNP-exposed) HPEKs. * indicates $p < 0.05$ w.r.t. the “0 μgml^{-1} ” group.

4.4.9. TNP reduce Nuclear ZI concentration

It was clear that ZNP exhibit a substantial genotoxic potential while TNP was much milder. Since ZI were again found to play a central role, it was again hypothesized that TNP could reduce the DNA damage induced on HPEKs by ZNP. HPEKs were, therefore, treated with TNP and ZNP together to understand the combinatorial response (Figure 4-25). In line with the hypothesis, it was observed that both TNP-exposed and TNP-loaded cells were significantly resilient to DNA damage induced by ZNP. There was no increase in γ -H2AX foci when intracellular TNP was present. The same was not observed for SIM-exposed cells because they do not have intracellular TNP. This symptom was complemented closely by the nuclear ZI after these treatments. Both TNP-exposed and TNP-loaded cells showed significantly lower nuclear ZI than the control. Again, SIM-exposed cells did not have the same magnitude of effect in lowering ZI. Taken together, presence of intra-cellular TNP, but not extracellular TNP, seemed to hinder the buildup of nuclear ZI and thereby negate the genotoxic potential of ZI.

Figure 4-25 – (A) Double stranded breaks (DSBs) occurrences indicating γ -H2AX foci in HPEKs exposed to ZNP for 24 hours. Quantitative analysis was performed using ImageJ showing the percentage of cells showing DNA damage with respect to NC. (B) Nuclear ZI after ZNP exposure to HPEKs. Values are normalized ZNP 10 and NC is subtracted out. For both, * indicates $p < 0.05$ w.r.t. NC. Ψ indicates $p < 0.05$ w.r.t. ZNP 10.

4.5. Conclusions

It was established that ZNP and TNP interact with the cell in different ways. Being soluble, ZNP's toxicity relies on the uptake of zinc ions (ZI). In effect, ZNP exposure can overload the cells with excessive ZI causing an imbalance in the homeostasis of the cell. The ZI can trigger oxidative stress by gaining entry into oxidative organelles of the cells but more importantly ZI can cause severe DNA damage by interacting directly with the DNA. All of this allows ZI to induce acute cytotoxicity impairing the cell viability even at low concentrations. TNP on the other hand is taken up by the cells in large amounts through endocytosis and travels deeper into the cell until the perinuclear region. Since endocytosis packetizes NPs in acidic lysosomes which allow NPs to produce ROS, TNP induces oxidative stress on the cells which is localized in the mitochondria as well as the nuclei. However, TNP has low genotoxicity because it is unable to penetrate the nucleus of the cell and causes only primary indirect genotoxicity through ROS-mediated DNA damage at very high concentrations. TNP also remains relatively non-cytotoxic until very high concentrations.

The prerequisite of studying dual nanoparticle toxicity was to develop an understanding of how they the two nanoparticles can interact. It was observed that the negative surface of TNP was able to chelate ZI. This interaction had a profound effect on their combined impact on cells because the adsorption onto the TNP surface immobilizes the ZI and restricts them from causing toxicity. In support of this mechanism, it was observed that in the presence of intracellular

TNP, the concentration of free ZI in the cells reduced. Consequently, the ZI induced toxicity was alleviated and cell viability was restored. Similarly, the amount of ZI that passed into the nucleus was also reduced significantly causing a considerable reduction in the DNA damage afforded to cells. Conclusively, intracellular TNP could play a role akin to a *vigilante* and protect the cells from ZNP-induced cyto- and geno- toxicity.

Chapter 5. Recommendations

Since the inception of toxicology, selecting meaningful models has been a major challenge. While ethics, cost and technology limits the usage of complex models, the alternatives, one may argue, are an over-simplification of the actual scenario. However, simpler models allow the delineation of more fundamental processes which are otherwise clouded by the intricacies of complex models. For instance, 2-D models can allow high repeatability and therefore isolate the effects dwelling from particle exposure with high parametric control. On the other hand, animal models need a lot more precise control of the environment in order to ensure repeatable results. Even then external factors such as building vibrations or trauma induced in animal during toxin administration can generate a host of effects as well which can muddle up the particle toxicity.

This dilemma is also at the heart of the current study. In order to bridge the gap between testing and real life exposure conditions, dual nanoparticle systems were considered distinctly from single nanoparticle systems and primary keratinocytes were used to represent the exposure environment. However, in its current form, this study still lacks the complete complexity of human skin and a truly realistic exposure profile. Nonetheless, by simplifying the system it was possible to delineate some core fundamental particle interactions which would have been shrewdly impossible to identify had a more complex system been used instead.

Practically, toxicological assessment for any new toxin has to have a multi-pronged approach. While simple and high throughput systems such as the one

employed in this study can provide clues to some basic interactions, the next step in unfolding the toxicity pathway has to involve progressing to a more functional and relevant model. This chapter suggests some future steps which can complete the jigsaw puzzle which this dual nanoparticle system presents. The findings in this study serve as a road map; much like the border pieces of the jigsaw which though lack multiple joining faces, can serve as critical starting points.

5.1. Downstream effects

The key interaction between TNP and ZNP was the ion adsorption effect. This was a direct interaction and had strong ramifications on the toxicity outcomes of the combination. There are also plenty of indirect effects that TNP and ZNP can have on each other which are theoretically possible. A taste of some of them were discussed in the build up to this thesis. Future studies should look to delve deeper into some of the more downstream effects which include looking at protein, RNA and gene expressions.

TiO₂ NPs have the ability to affect gene expression and have been shown to modulate genes involved in metal ion uptake.(Fujita et al. 2009) An examination of a report on the effects of ultrafine TiO₂ particles on gene expression profile in human keratinocytes (Fujita et al. 2009) revealed that key genes involved in zinc uptake and regulation were affected. Specifically members from the SLC30 family, SLC39 family and metal response-element transcriptional factor (MTF-1) are all significantly affected. It has been shown that modulation of these genes

can in turn modulate intra-cellular ZI concentrations (Devergnas et al. 2004; Franklin et al. 2003; Gaither and Eide 2001; Myers et al. 2012), suggesting that [TiO₂ NPs]_i could have done that. Besides curbing the ZnO/Zn²⁺, [TiO₂ NPs]_i may also immobilize a portion of the intracellular ZI by adsorbing the ions onto their negative surface. This could reduce the free Zn concentration in the cells and therefore alleviate some of the ZI dependent stress on the cell.

5.2. Other cell types

The *in vitro* model chosen in this study was human primary epidermal keratinocytes (HPEKs). This was due to their strong relevance to skin, which in turn is a major organ facing exposure to nanoparticle exposure. Each cell type has a unique set of intracellular make up and functionality varies greatly. An indication to this was addressed in this study as well. Human primary dermal fibroblasts (HPDF) showed lower vulnerability against a soluble Zn²⁺ salt compared to HPEKs. In addition to the inherent cell response, the cell culture media can have a strong impact on particle characteristics like protein corona, aggregation, solubility and effective density. This, too, was briefly discussed in this study as ZNP solubility was shown to be much lower in Dulbecco's modified eagle's medium as compared to the medium used to culture HPEKs.

Taken together, different cell types may respond differently to nanoparticle exposure and therefore other cell types should also be rigorously analyzed. These

include other skin cells, e.g. fibroblast, melanocytes, sweat gland cells, etc., which can be subjected to nanoparticle exposure.

5.3. Co-cultures, 3-D cultures, etc.

While the aim of *in vitro* models is to make toxicity studies cheap, quick and ethically responsible, one of their major drawback is their ability to mimic real life scenarios. In other words, they are not completely translatable into human toxicity because how cells behave individually in cell cultures may be very different from how they behave when part of an organism. However, in the toxicity screening paradigm, *in vitro* cultures still remain indispensably important because they can serve as a starting point of toxicological analysis.

It is important that the progression is made to models which are closer to the human skin. In recent times there have been a number of *in vitro* models which take a step closer to mimicking real life (Kathawala et al. 2013). These include 3-D spheroids, cell sheets, scaffold-based cultures and others. A new group of models is coming up called *ex vivo*. Therefore the next step in understanding the dual nanoparticle system, or any other nanoparticle system, is to progress to such co-cultures. In the context of this nanoparticle pair, a 3D skin co-culture would be extremely relevant and enlightening. Such co-cultures have been developed (Cantòn et al. 2010; Dai et al. 2005; Huang et al. 2010; Li et al. 2009b; Sawicki et al. 2005) but their usage in toxicology analysis is absent and could be a novel direction for future work.

5.4. Other nanoparticle pairs

As shown in this study, the toxicity rendered by dual nanoparticles could be unexpectedly different from the participating individual nanoparticles. While this study focused on the TNP and ZNP pair due to their usage in sunscreens, there are numerous other examples of nanoparticles which are used in combinations. An analysis of 500 products on The Project on Emerging Nanotechnologies (PEN) database of consumer products showed more than 20 products which contained more than one nanomaterial. Even in cosmetics there were other combinations including TiO₂ paired with silver in one and carbon in another product.

Future work should analyze these combinations separately in order to investigate the kind of combinatorial responses they produce. Such an understanding is critical in order to make applications using nanomaterials safer.

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Supplementary Materials

Figure S 1 – UV-Vis absorbance of TNP and ZNP alone and combined showing a synergy of the dual nanoparticle system.

Table S 1 – (A) Sunscreen application data showing the mass per surface area of nanoparticles exposed to skin. (B) Cell culture experimentation data showing the mass per surface area of nanoparticles exposed to skin cells.

A		Mass per surface area
Average Sunscreen Application		4-5 mgcm ⁻²
FDA approves 25% NPs		~1 mgcm ⁻²
B	6-well plate	96-well plate
Surface area	9.5 cm ²	0.32 cm ²
Media Volume	1 mL	0.1 mL
For 100 µgml⁻¹	~ 0.011 mgcm ⁻²	~ 0.031 mgcm ⁻²

Figure S 2 – (A) ISDD Model simulations for TNP in 6-well and 96-well plates. (B-C) Corresponding time-weighted average (TWA) factor for various incubation times. (D,E) Time-weighted average

dosage (TWAD) for 2 and 24 hours against the absolute exposure for 96-well and 6-well plate format (different units are used for TWAD to fit into plot area).

Figure S 3 – Newport Green DCF (NPG) Fluorescence from TNPs of different concentrations after they are washed with Zn²⁺ ion solution. To obtain the Zn²⁺ ion solution, ZNPs of various concentration (x-axis) are allowed to dissolve in cell culture medium. The undissolved NPs are removed by centrifugation. The ion solution is then added to 96-well plates containing TNPs (control contains no NPs) of 50 and 200 µgml⁻¹. After 5 mins, the solution is aspirated from the wells and the NPs are treated with NPG for 30 mins. The NPs are washed with clean buffer and the fluorescence is recorded using a plate reader. Data is expressed as mean ± SD. * indicates p < 0.05 significance w.r.t. to the control which has “No NPs”.

Table S 2 – Some examples of the TWA factor and TWAD values used.

Format	6-well plate		96-well plate	
Time	TWA Factor	TWAD (AE: 50 ugcm ⁻²)	TWA Factor	TWAD (AE: 100 ugcm ⁻²)
0.25	0.009	0.049	0.003	1.00
0.5	0.036	0.189	0.012	3.78
1	0.141	0.742	0.048	14.84
2	0.557	2.932	0.188	58.66
4	2.183	11.491	0.747	233.28
8	6.181	32.529	2.977	930.28
12	10.177	53.564	6.609	2065.19
16	14.172	74.592	10.608	3315.06
20	18.166	95.612	14.608	4564.91
24	22.157	116.616	18.601	5812.84