Understanding the sub-lethal effects of ZNO nanoparticles and its implications to skin repair

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**UNDERSTANDING THE SUB-LETHAL EFFECTS OF ZNO
NANOPARTICLES AND ITS IMPLICATIONS TO SKIN REPAIR**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2018

UNDERSTANDING THE SUB-LETHAL EFFECTS OF ZNO NANOPARTICLES AND ITS IMPLICATIONS TO SKIN REPAIR

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Master of Engineering

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

18-May-2018

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Kenny

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WU ZHUORAN

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

..... 18-May-2018

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Assistant professor Dalton Tay Chor Yong

Authorship Attribution Statement

This thesis includes content where I received support from my supervisor and collaborators as follows:

- Assist. Professor Tay provided the initial project direction and edited the thesis.
- I prepared the thesis draft. Revisions and advice were taken from Assist. Professor Tay.
- I co-designed the study with Assist. Professor Tay.
- Miss Archana (PhD student in MSE) provided me with the gamma-H2AX antibody for the staining of DNA double strand breaks (DSBs).
- Dr. Wang Jun Kit provided me with the immortalized human keratinocytes which were used throughout the entire study.
- Mr. Yang Haibo (PhD student in MSE) provided me with guidance for the successful completion of real-time PCR analysis.

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Abstract

Understanding the cellular responses to nano zinc oxide particles (Nano-ZnO) (<100 nm) is key to its successful novel exploitation in nanomedicine and bio-nanotechnology. Unlike other conventional inorganic compounds, zinc is an essential co-factor for numerous enzymes that are known to regulate a wide spectrum of fundamental cellular activities such as redox homeostasis, metabolism and signal transductions. In human, as many as 3000 proteins have been revealed to contain Zn^{2+} . In this regard, Nano-ZnO can be considered as a unique class of biologically active nanomaterials that can either disrupt (at toxic concentration) or support (at non-toxic concentration) cellular functions. While studies have shown that high concentrations of Nano-ZnO can trigger oxidative stress-mediated cell death via the generation of reactive oxygen species (ROS), little is known about the effects of Nano-ZnO and its role in mediating ROS signalling in the low-dose range. Inspired by recent demonstrations of biological systems capable of reciprocating to low level of oxidative stress via the process of cell adaptation, this project seeks to examine whether (i) cells can adapt to low dose of Nano-ZnO, (ii) understand the underlying mechanism underpinning the adaption process and (iii) conduct proof-of-concept studies to unveil the biological consequences of Nano-ZnO mediated adaption.

Herein, using a “conditioning and challenge” (2Cs) experimental regime, it is showed that long term (48h – 144h) exposure of sub-lethal dose of Nano-ZnO (~ 100nm) (0.5 $\mu\text{g/ml}$) to human keratinocytes (HaCaTs), could induce adaptive response and dramatically improve the overall fitness of HaCaTs to cope with toxic level of oxidative stress. As Nano-ZnO is limiting in the low dose range, it was observed a generation of discrete ROS “hotspots” (~2%) in the HaCaTs cells, which could correspond to Nano-ZnO Loaded Keratinocytes (Nano-ZLKs). Interestingly, the occurrence of these ROS “hotspots” is sufficient to trigger a population wide activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) stress response pathway via a complex cross-talk between the Nano-ZLK and the “bystander” cells through paracrine signalling. Transcriptome analysis revealed that the cross-talk between these two sub-population of cells were mediated primarily via interleukins (1 α , 1 β , 6, 8, 15, 18, 23, 33). Further mechanistic studies revealed that it is the

Nrf-2 mediated enhancement in proteolytic activity of the Nano-ZnO conditioned cells, but not the anti-oxidant effects that accounts for the cyto-protective adaption process. Importantly, this study provides a first account depicting the capability of Nano-ZnO to induce a cross adaptive effect in the Nano-ZnO conditioned HaCaTs to alleviate the DNA damage and compromised cell viability induced by Ultraviolet-A (UVA) irradiation. Taken together, this study shows that the formation and cross talk of Nano-ZnO and the bystander cells via the ROS-Nrf2 stress response pathway is a pervasive feature in the low dose Nano-ZnO exposure range. These findings not only provide critical information regarding the nano-safety of Nano-ZnO in the context sunscreen products, but also shed light on the unique and exploitable bio-activity of Nano-ZnO for skin protection against oxidative damage.

Lay Summary

While the application of zinc oxide nanoparticles (Nano-ZnO) in sunscreen products is widespread, questions pertaining to the safe usage of Nano-ZnO as well as the long-term bio-effects of Nano-ZnO in human skin remains open. Because zinc is one of the most important metallic ions and plays critical roles in many biological functions, it is hypothesized that chronic exposure of human keratinocytes (HaCaTs) to non-toxic dose of Nano-ZnO will elicit an adaptive response. In this study, it was found that pre-exposing HaCaTs to non-toxic dose (0.5 $\mu\text{g/ml}$) of Nano-ZnO could confer an advantage to the cells to counter the effects of oxidative damage that is induced either by toxic level of Nano-ZnO or by UVA irradiation. At the mechanistic level, it was showed that low dose of Nano-ZnO is sufficient to engage the nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) stress response pathway via direct, which is triggered via reactivity oxygen species (ROS) generation in the Nano-ZnO Loaded Keratinocytes (Nano-ZLK), or indirect (paracrine signalling) means with the rest of the “bystander” cells. The net effect is the enhancement in the cells capability to better remove any damaged or abnormal intracellular proteins and prevent its accumulation in the cells, thus leading to better overall cell fitness. Collectively, these findings offer a further clarification on the safe usage of Nano-ZnO in skin care products and point to an exploitable concept of Nano-ZLK for skin protection.

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Abbreviations

A549	Human epithelial cells
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ARE	Antioxidant response element
ASC-2P	L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate
D _H	Hydrodynamic diameter
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
ED50	Effective dose
ENMs	Engineering nanomaterials
HaCaTs	Human keratinocytes
Keap-1	Kelch like-ECH-associated protein 1
LPS	Lipopolysaccharide
MAFs	Musculoaponeurotic fibrosarcoma proteins
MAPK	Mitogen activated protein kinase
MEF	Murine embryonic fibroblasts
MMPs	Matrix metalloproteinases
Nano-ZLK	Nano-ZnO loaded keratinocytes
Nrf-2	Nuclear factor (erythroid-derived 2)-like
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SAR	Structure-activity relationship
SCD	Sickle cell disease
SD	Standard deviation
TAC	Total antioxidant capacity
TBHP	Tert-Butyl hydroperoxide
TEM	Transmission electron microscope
TGF- α	Tumour necrosis factor-alpha

Chapter 1

Introduction

Zinc oxide nanoparticle (Nano-ZnO), due to its unique physical property for UV prevention, has been extensively applied in many skincare products. Yet, understanding of its biological effects on human skin has not been complete, particularly in the low/ sublethal dose range. Inspired by the recent discoveries regarding the cellular adaptive response in reciprocating a low-grade oxidative stress generated by a wide range of oxidative stressors, it is postulated that Nano-ZnO, which is well capable of inducing oxidative stress, could lead to skin cells to progressively adapt to mitigate the oxidative stress-induced damages. To verify this hypothesis, one constraint that is needed to break through is the low signal-to-noise ratio that typically occurs in the low dose context. To achieve this, a well-tailored in vitro conditioning and challenge regime was incorporated in the study. Additionally, to find out the downstream effectors that are regulated by oxidative stress (ROS) for the Nano-ZnO induced cellular adaption, a combination of material characterization techniques and biological approaches was employed. This chapter will elaborate a brief background of the relevant recent findings, outline the problem statement, state the hypotheses and objectives, and give an overview of the content in the entire thesis.

Engineered nanomaterials (ENMs) are now broadly applied in different fields of products including sporting goods, tires, strain-resistant clothing, sunscreens, cosmetics, and electronics and have gradually infiltrated into emerging domains such as medicine for purpose of diagnosis, drug delivery, and imaging.¹ The unique physiochemical properties of ENMs can be attributed to their relatively small size, large surface area, chemical compositions (purity, crystallinity, electronic properties, etc.), surface structures (coatings, surface moieties), solubility, shape, and aggregation.¹⁻² Amongst these are zinc oxide nanoparticles (Nano-ZnO) which have been broadly applied to a variety of domains, such as food manufacturing, sunscreen, cosmetics, and antibacterial and antifungal products.³⁻⁴ Nano-ZnO is widely used in the formulation of skin care products, especially sunscreen, due to its excellent performance in UV protection. It prevents UV from penetrating human skins by both light scattering and light absorption.⁵ Due to its extremely small size and the inevitable human exposure to Nano-ZnO containing products, concerns have been raised on its potential toxicity to human bodies.¹⁻² Therefore, in recent years, it has been witnessed a surge in the number of publications that seek to elucidate the toxic effects of Nano-ZnO using different in vitro and in vivo models.^{2, 6-9}

Similar to other types of ENMs, the toxicity induced by Nano-ZnO has been linked to excessive oxidative stress, which could be partially attributed to the dissolved Zn^{2+} in physiological microenvironments. Oxidative stress stems from the over-production of reactive oxygen species (ROS), which generally refers to a group of chemically reactive oxygen containing radicals and non-radicals including O_2^- , $\bullet\text{OH}$ and $\text{NO}\bullet$.¹⁰⁻¹¹ ROS can disrupt redox homeostasis by oxidizing cellular components such as lipids, proteins, carbohydrate, and DNA.¹²⁻¹⁴ To restore oxidative balance, a battery of cyto-protective responses (e.g. production of multiple detoxifying enzymes and antioxidants) are known to be activated to alleviate the adverse effects induced by ROS.^{13, 15} However, cells would undergo programmed cell death (apoptosis) when the stress is beyond the cells capacity to cope with. Recent mechanistic insights with regards to the toxicity of Nano-ZnO have also allowed scientists to adopt a “safer by design” of Nano-ZnO.¹⁶

While the destructive effects of ROS are widely documented, evidences are accumulating to demonstrate that ROS is equally essential for fundamental signal transductions events.¹⁷⁻²⁰ Biological systems have also been shown to adapt to mild levels of oxidative stress by maximizing its antioxidant capability at the molecular level. For instance, it was previously shown that cells exposed to moderate levels of oxidants (e.g. H₂O₂, peroxynitrite, menadione) can display enhanced tolerance towards harmful levels of oxidative stress induced by the same oxidant (self-adaption).²¹⁻²² Oxidative stress adaption is therefore referred to an innate reciprocal response that is exhibited by the cells to cope with the environmental and physiological shifts in the level of oxidative stress.²³⁻²⁴ It is, however, unknown whether Nano-ZnO mediated oxidative stress could similarly induce an adaptive response in human keratinocytes at low dose or non-toxic concentrations.

1.1 Hypothesis

- Non-cytotoxic dose of Nano-ZnO could induce an adaptive cellular response in human keratinocytes that will enhance the overall fitness of the cells against oxidative stress via induction of oxidative stress
- The enhanced level of oxidative stress in the cells entails activation of Nrf-2, antioxidants, and proteases to mediate the adaption process.
- Nano-ZnO can induce cross-adaptation in human keratinocytes against other harmful environmental stressors.

1.2 Objectives and scope

- To design and employ a ‘conditioning and challenging’ (2Cs) regime to examine the Nano-ZnO induced adaptive response.
- Quantify the intracellular oxidative stress in the cells that are exposed to low dose of Nano-ZnO.

- Identify the downstream effectors (e.g. Nrf-2, antioxidants, proteases) that respond to the change of oxidative stress and are responsible for the cellular adaptive response that is potentially caused by low dose of Nano-ZnO.
- To examine whether the Nano-ZnO-induced adaptation could be applied to remediate UV-incurred skin damage.

1.3 Dissertation Overview

The thesis firstly addresses the assumption and objectives made according to the current understanding with respect to Nano-ZnO. Secondly, it provides an up-to-date review on relevant published works pertaining to this thesis and highlights the critical knowledge gaps. Thirdly, the required equipment, techniques as well as methods are introduced. Following which is the results analysis as well as an elaborate discussion about the findings in this study. Finally, reconnaissance work that are not included in the main chapters and outstanding questions that are beyond the scope of this study will be addressed at the end of the thesis. The overall arrangement of the content of the thesis is as follow:

Chapter 1 provides a rationale for the research and outlines the goals and scope, together with the findings that are discovered in the study.

Chapter 2 reviews the literature concerning the application of Nano-ZnO, oxidative stress and its relation to Nano-ZnO induced cytotoxicity, cellular adaption and its correlation to oxidative stress, and paracrine signalling. Next, knowledge gaps that have not been bridged according to the literature reviews will be summarized. At last, aims of this study will be set in the context of literature review.

Chapter 3 discusses the principles underlying the characterization techniques employed and the methods of data analysis, including errors.

Chapter 4 elaborates the materials and methods used in the study. Following which is illustration of the primary results.

Chapter 5 gives a general discussion on the results that have been mentioned in *Chapter 4*, the reconnaissance work that aided to discover the major outcomes, and a short summary of the major findings in this study with some outstanding questions that are worth putting future endeavour to explore.

Supplementary Information presents the additional information that is not included in the main text of the thesis yet is an essential component that constitutes the entire study.

1.4 Findings and Outcomes/Originality

This research has led to several novel outcomes:

1. Design and implementation an *in vitro* “conditioning and challenge” 2Cs regime that has proven to be useful to examine Nano-ZnO induced adaptive response.
2. Identify that the Nrf-2 stress response pathway is a key regulator in Nano-ZnO induced adaptive response in the context of low dose of Nano-ZnO conditioning, which is downstream of the oxidative stress and subsequently recruits interleukins and proteasomes to improve the overall fitness of HaCaTs.
3. Demonstrate the cross-adaptability of HaCaTs to Ultraviolet-A induced by a low dose of Nano-ZnO.

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Chapter 2

Literature Review

Nano-ZnO due to its excellent physical properties has been widely used in the field of skincare product for skin protection. However, while the toxicology of Nano-ZnO has been well understood in the high dose range, the biological effects that it exerts on biological systems in the low/sublethal range has not been determined. A detailed review of the important recent findings that are fundamental to this study will be presented herein. This chapter will start with a brief introduction regarding the key applications of Nano-ZnO within which its application for skin protection will be discussed in detail. Next, a detailed elaboration of the toxicology of Nano-ZnO will be given in which the core of the toxicology (ROS) and the well-acknowledged hierarchical oxidative stress model will be highlighted. Building on the knowledge of ROS in mediating cellular damage/ death, elaboration on the role of ROS in modulating cellular adaption is provided with the connection to the paracrine signalling. Finally, outstanding questions to answer in the literature and specific aims of this study will be stated.

2.1 Overview

The following sections will briefly conclude the main findings concerning the application of Nano-ZnO, oxidative stress and its relation to Nano-ZnO induced cytotoxicity, cellular adaption and its correlation to oxidative stress, paracrine signalling. All these will lay a groundwork for the study and assist to explain the major findings discussed later in this thesis.

2.1.1 Applications of Nano-ZnO

The rapid advancement in the field of ENMs fabrication techniques have opened up many exciting novel opportunities that could benefit a wide range of industries. Nanomaterials refer to any structures in which at least one dimension (length, width, or thickness) is in the nano-sized range (1-100nm).¹ Due to the extremely small size, nanomaterials exert markedly distinct chemical and physical properties (e.g. optical, electrical, thermal, magnetic, catalytic characteristics, etc.) from their bulkier counterparts.²

Amongst all these ENMs is Nano-ZnO. Recent advances in the synthesis of Nano-ZnO has enabled researchers to synthesize Nano-ZnO with different morphologies, such as nanocombs, nanorings, nanohelices, nanobelts, nanowires, and nanocages under specific growth conditions.³ This structural diversity makes Nano-ZnO an ideal material for optoelectronic as well as piezoelectric applications. Also, due to its excellent electric property, high surface area, chemical stability, biocompatibility, and high absorption of biomolecules, Nano-ZnO is also well-suited for biosensor applications. One established biosensor model discovered by Khan et al. incorporated Nano-ZnO and chitosan, which is well-known for its efficient immobilisation of biomolecules, to detect cholesterol for the diagnosis purpose.⁴ When it comes to food manufacturing, Nano-ZnO excels many other antibacterial additives due to its ultra-low effective dose (ED50) and capacity to extend the shelf-life of food products.⁵ Other applications including gas sensors, semiconductors, pharmaceutical manufacturing, catalyst for pollutant degradation, and even drug carrier

have also been elaborately discussed in many publications. (**Table 2.1** summarises the potential applications of Nano-ZnO)

Table 2.1. List of different Nano-ZnO morphologies and the corresponding applications

Size	Morphology	Application	Reference
-	Nanobelt	Nano-resonator	6
-	Nanoparticles	Catalyst for pollutant degradation	7
50-70nm >1 μ m	Nanoparticles	Antibacterial agent	8
15-90nm	Nanowires	Semiconductor	9
50-60nm	Nanoparticles	Gas sensor	10
38nm	Nanofilm	Biosensor	11
<100nm	Nanoparticles	UV absorbers	12

Figure 2.1. Comparison of the scattering efficiencies of microfine zinc oxide and microfine titanium dioxide for white light (visible spectrum).¹⁷

Figure 2.2. Diffuse reflectance spectroscopy of microfine zinc oxide and microfine titanium dioxide at 2% and 6% concentrations. Zinc oxide is more protective than titanium dioxide in the area from 340 to 380nm.¹⁷

Of note is the prevailing usage of Nano-ZnO in skincare products. Prolonged exposure to the sun has been demonstrated to be a potential cause of inflammation, immune system dysfunction, genetic mutations, and even photo-carcinogenesis.¹³ And these damages have become even more pronounced in recent years due to ozone depletion. In contrast with

traditional skin protection strategies, which employed chemicals (e.g. oxybenzone) as UV absorbers, the alternative use of Nano-ZnO dramatically reduces the usage of chemicals, thus reducing the potential harm (e.g. allergic responses) caused by them.¹⁴ Compared to the micron-sized counter parts, the use of Nano-ZnO in sunscreen products is favoured as it appears transparent when applied onto the human skin and thus fulfils the aesthetic requirement of skin-care products.¹⁵ Previously, it was believed that inorganic particles such as Nano-ZnO and TiO_2 exert their protection by scattering the UV light. Due to this traditional belief, TiO_2 was once thought to be a better candidate for UV protection in the sunscreen formulations (**Figure 2.1**). However, recent investigations have rejected this idea and proposed that the UV protection in fact results primarily from physical absorption

Figure 2.3. Mechanism by which Nano-ZnO protects skin from UV-induced damage. Sun light not only has visible light but also light in the invisible range such as UV. When UV reaches the ozone layers, UVC will be mostly filtered out, leaving only UVA and a small amount of UVB that can approach living organisms along with visible portion. Diagram drawn based on the findings in articles.¹⁶⁻¹⁷

via the band gap which varies between materials.¹⁶ Because of the unique band gap of Nano-ZnO, it outperforms TiO₂ at the range of 340-380nm, which belongs to UVA (320-400nm) (**Figure 2.2**).¹⁷ This clearly indicates ZnO is a better skin protector as UVA accounts for more than 90% of the UV light that can reach human skins and it is more penetrable thus more detrimental than UVB (290-320nm). (**Figure 2.3** illustrates the way in which Nano-ZnO protects skins against UV)

2.1.2 Reactive oxygen species (ROS)-mediated cytotoxicity induced by Nano-ZnO

Induction of oxidative stress is deemed as the primary mechanism in which ENMs induce toxicity to various kinds of organisms. Oxidative stress reflects an imbalance between the systemic retention of reactive oxygen species (ROS) (e.g. O₂^{·-}) and the systemic capacity to readily neutralize the ROS or to repair the consequential damage. Under normal conditions, ROS are generated slowly and with a small amount thus can be easily removed by innate cellular defence mechanisms, such as the upregulation of antioxidants, glutathione, NADPH, etc. However, when the ROS level exceeds the organism's capacity to cope with, the oxidative stress can induce detrimental and irreversible damages to the cells, tissue and even organs, leading to cellular injuries, functional impairment and even death.¹⁹ **Figure 2.4** shows the well-established hierarchical oxidative stress model that summarises the oxidative stress-triggered biological responses at the cellular level according to the extent of the oxidative injury. Based on this model, when the level of ROS is slightly upregulated, this will invoke a tier 1 response by the cells, whereby the Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) stress response pathway will be activated to restore redox-homeostasis. Conversely, with increasing level of ROS, this will elicit a tier 2 response by the cells to engage several proinflammatory pathways (e.g. MAPK, NF-κB) and cytokines productions. Finally, excessive level of ROS will inevitably induce cytotoxic effects (Tier 3 response) to the cells. More details regarding Nrf-2, proinflammatory cytokines, and paracrine effect will be elaborated in the following sections.

Due to its extremely small size and heightened surface chemical reactivity, concerns have

long been cast on the safety usage of Nano-ZnO.²⁰ Therefore, many investigations have been conducted in order to understand the toxicology of Nano-ZnO.^{19, 21-22} Assessments of its toxicity with various cells lines, bacteria, algae, plants, zebra fish and mice have consistently demonstrated that Nano-ZnO induced ROS to be a critical upstream molecular event leading to various cytotoxic outcomes.²³ With regards to the mode of Nano-ZnO

Figure 2.4. The hierarchical oxidative stress model.¹⁹

induced cell death, majority of the research report apoptosis (programmed cell death) to be the primary mechanism.²⁴⁻²⁵ It has an exception however, as Wihelmi et al. found that Nano-ZnO induced Nrf-2-independent necrosis to macrophages in their *in vitro* study.²² They termed the necrotic cells as ghost cells, and they explained that these cells were considered undergoing necrosis because their morphology were completely dismantled. Nano-ZnO was also documented to induce a wide range of proteomic and transcriptional changes. A common reported finding is that Nano-ZnO activates both pro-survival and

anti-apoptotic genes expression. On one hand, to battle against the oxidative stress, detoxification-related genes including HO-1, SOD-2, and GSP are activated to ameliorate the oxidative stress to restore redox homeostasis. These genetic responses are regulated by Nrf-2. Under normal condition, Nrf-2 is bound to the Kelch like-ECH-associated protein 1 (Keap-1) to form a Keap1-Nrf-2 complex in the cytosolic compartment. Upon exposure to electrophile or oxidative stress, the cysteine residue of Keap-1 becomes oxidized and dissociates from Nrf-2. Following which, the Nrf-2 will translocate into the cells' nucleus to heterodimerize with small MAFs proteins and bind to the antioxidant response element (ARE) to trigger a battery of cytoprotective genes. Supporting this model, antioxidants such as GSH and SOD was indeed shown to be upregulated as the product of the activation of GSH and SOD respectively to suppress Nano-ZnO induced oxidative stress.²⁶⁻²⁷ In contrast, when the oxidative stress overwhelms the Nrf-2-Keap-1 regulatory pathway, mitochondria membrane potential will be perturbed leading to the impairment of normal metabolic activities.²⁴ These actions further lead to the excretion of cytochrome, which in turn can induce several pro-apoptotic effectors such as caspases (8, 9), resulting in programmed cell death ultimately (**Figure 2.5**). Interestingly, cells undergoing apoptosis can also function as a pro-survival signal to its neighbouring healthy cells.²⁸ It is generally believed that the toxicity of Nano-ZnO could be attributed to a simultaneous ROS induction and antioxidants depletion, one example of which is the study of Lee et al. in which they observed a significant decline of glutathione retention in human keratinocytes (HaCaTs) after 24h of Nano-ZnO treatment.²³

However, a debatable perspective regarding Nano-ZnO induced toxicity is that the dissolved form of Nano-ZnO (Zn^{2+}) is a primary cause of the toxicity. Proponents supported this paradigm by showing that equivalent concentration of Zn^{2+} (ZnCl_2) as compared to the nano-counterpart (Nano-ZnO) could exert similar cytotoxic effects.²⁴⁻²⁵ In contrast, there is another group of researchers that showed that Zn^{2+} that is dissolved (~4%) from the Nano-ZnO is negligible in the culture medium.²⁹⁻³⁰ Based on this discovery, they

Official symbol	Gene name	Fold change
HMOX1	Heme oxygenase(decycling) 1	22.9
SOD2	Superoxide dismutase 2	3.2
DDIT4	DNA-damage-inducible transcript 4	2.3
GZMB	Granzyme B	9.0
CLU	Clusterin	6.8
GSR	Glutathione reductase	2.5
CYP4F3	Cytochrome P450	2.1
GPX	Glutathione peroxidase	2.5
CASP4	Caspase 4	2.1
CASP8	Caspase 8	2.0
CASP9	Caspase 9	2.0
NGFRAP1	Nerve growth factor receptor	2.2
HSP70	Heat shock 70Kda protein	8.7
PDCD2	Programmed cell death 2	2.1
CASP1	Caspase 1	2.9
PECR	Peroxisomal trans-2-enoyl-CoA reductase	2.3
GADD45G	Growth arrest and DNA-damage-inducible	5.4
PRDX2	Peroxiredoxin 2	2.1
INCA	Inhibitory caspase recruitment domain protein	2.9
CDKN1A	Cyclin-dependent kinase inhibitor 1A	2.7
MT1M	Metallothionein 1M	8.7
RGS16	Regulator of G-protein signaling 16	9.7
NAIP	NLR family, apoptosis-inducing protein	-3.0
BNIP3L	BCL2	-2.7
P53AIP1	P53-regulated apoptosis-inducing protein 1	-3.5
XAF1	XIAP associated factor 1	-2.4
HDAC4	Histone deacetylase 4	-2.1
GCLM	Glutamate-cysteine ligase	-2.6
MAPK8IP2	Mitogen-activated protein kinase 8 interacting protein 2	-2.1

Figure 2.5. Redox-/apoptosis-related genes expression profile in human keratinocytes after Nano-ZnO treatment.¹⁸

argued that Nano-ZnO executed a particle-specific cytotoxicity to organisms. This is

conceivable since Nano-ZnO is expected to dissolve with an extremely low rate ($K_{sp} \sim 3.0 \times 10^{-16}$) under physiological pH.²⁹ Further supporting this idea would be the result from Meißner et al. in which they observed only 4% of Nano-ZnO were dissolved into Zn^{2+} after 3 days in the FBS-supplemented DMEM.³¹ One possible explanation to such conflicting points of view is that the particulate form of Nano-ZnO, after internalised by cells, may eventually release Zn^{2+} due to the highly acidic microenvironment of the lysosomes ($\sim$ pH 4). It is thus the sudden surge in the intracellular Zn^{2+} , rather than the ZnO particulate per se, is the main cause of cytotoxicity.³²⁻³³ Still, due to the complexity of the interaction of Nano-ZnO with the proteins found in the culture medium and cells, it is extremely challenging to establish the structure-activity relationship (SAR) that underpins the toxicity of Nano-ZnO. Nevertheless, the important role of Nano-ZnO induced intracellular ROS is undeniably a significant upstream molecular event that will eventually shape the biological outcomes.

2.1.3 Cellular adaption to oxidative stress: Roles of Nrf-2-Keap1 signalling

The ability for cells and organisms to adapt to their hostile environment is a fundamental tenet in evolution. As early as eighteenth century, Charles Darwin officially published his discovery on the evolution of organisms in a book called - *On the Origin of Species by Means of Natural Selection*. The theory of Natural Selection states that the phenotypes of organisms are the product of accumulated inheritable advantages (genes) that are passed on from their ancestors so that the offspring can better adapt to the ever-changing environments. This concept was eloquently expressed by López-Maury, in which he said “Cells respond to abrupt environmental changes by launching gene expression programmes that help to adjust the cellular physiology and metabolism to the new conditions and that protect against cell damage or death”.³⁴ Yet, since the diversity of genes profile in different organisms exist, case-by-case studies on adaptation that happens in various kinds of animals, plants, microbes, etc. are very much needed to truly reflect the biological facts that contributes to each adaptive response. Though people have long known that organisms can adapt to a new environment, our understanding with regards to the precise mechanism driving biological adaptation remains far from complete.

Decades of research endeavour has revealed oxidative stress as possible trigger and mediator of cellular adaption. Mild level of oxidative stress could result from exposure to oxidants (chemicals), nanoparticles, heat, radiations, etc. Once cellular adaption sets in, organisms would become more resistant to the same or other type(s) of oxidative stressor at the concentration that is initially toxic to them. The first discovery of oxidative stress-induced adaptation is in the study of bacteria and corresponding findings have gradually expanded the concept to lower eukaryotes. In 1995, Wiese and her colleagues showed that H₂O₂-pretreated HA-1 cells (a derivative of Chinese hamster ovary cell line), strikingly enhance their tolerance (40 folds compared to control) to toxic level of H₂O₂.²⁷ This seminal finding is a significant milestone in redox biology as it has laid the groundwork for subsequent demonstrations of oxidative stress-induced adaptation in mammalian cells. The same study also discovered that over 30 genes were activated during the H₂O₂ pretreatment, such as regulators of several antioxidant enzymes, DNA repair enzymes, and other proteins with unknown functions (with the technology in those days). Nevertheless, these findings implicate the important role of antioxidants production as well as DNA repair mechanisms play in cellular adaption.

One critical player regulating the adaptation process is the Nrf-2 transcription factor. Nrf-2, as previously discussed, is a key regulator of cellular antioxidant defence against oxidative stress. Under normal condition, Nrf-2 is tightly bound to Keap-1 and rapidly recycled by ubiquitination ($t_{1/2} \sim 15\text{min}$).³⁵ When cellular ROS is abnormally elevated, Nrf-2 will be activated followed by detachment from Keap-1 and stabilisation into nucleus. Once it reaches cell nucleus, it would interact with ARE, which is known to regulating multiple antioxidants-related genes, including but not limited to NQO1, HO-1, GST, UGT, and SRXN1.³⁶⁻³⁷ Activation of these antioxidants-related genes would help to maintain redox homeostasis by neutralising the excessive ROS. Although activation of Nrf-2 and its signal transduction are discovered in the context where toxic level of ROS inducers is applied, the conserved relation between oxidative stress and Nrf-2 has made the theory applicable to the case of adaptation.

To date, only a handful of papers have reported the capability of ENMs in inducing an adaptive response in cells. Interestingly, in a recent study, it was shown that exposing A549 (human epithelial cells) to non-toxic levels of silver nanoparticles could increase the cells resilience towards toxic level of silver nanoparticles as well as acrolein, a highly reactive unsaturated aldehyde formed endogenously through lipid peroxidation and a ubiquitous

Figure 2.6. Schematic illustration summarises the oxidative stress adaptation signalling pathways based on the current findings. Under oxidative stress, excessive portion of ROS in the cytosol would suppress ubiquitination of Nrf-2 and liberate it from Keap-1, resulting in rapid accumulation in cells' nucleus. Nrf-2 would subsequently act on ARE followed by upregulation of both antioxidants and proteasome. While antioxidants were implicated as a crucial factor that results in adaptation in other studies (see details in the discussion in this session), Pickering et al. suggested that proteasome, also regulated by Nrf-2, plays a conserved role in acquisition of oxidative stress oxidation. Taken together, Nrf-2 regulated antioxidants as well as proteasomes may synergistically induce such an adaptation effect.

environmental pollutant, presumably via the activation of Nrf-2 and compensation of antioxidants such as glutathione and heme oxygenase.³⁸ Similarly, one-time exposure to PEG-coated gold nanoparticles with a nontoxic concentration also led to a persistent upregulation of NQO1 (up to 20 weeks), a target antioxidant gene regulated by Nrf-2, as well as HSPA5, a gene that regulates heat shock proteins (Heat shock proteins steers cells to repair misfolded proteins during synthesis in endoplasmic reticulum).³⁹ Although neither of the two studies quantified the oxidative stress in each tested cell line after pretreatment of the nanoparticles, the upregulation of antioxidant in the cytosol as well as the genes that regulate them in the nucleus, are the proper manifestations of overexpression of oxidative stress. Therefore, it is sufficing to conclude that ROS is the first and foremost factor that initiate the adaptation process by incorporation of signal transduction of Nrf-2 and its downstream antioxidant production. Besides, the upregulation of heat shock protein observed during the gold nanoparticles-induced adaptation suggest that alternative mechanisms independently of Nrf-2 might also come into play.

Further supporting the involvement of Nrf-2 to the oxidative stress adaptation is the study by Pickering and his colleagues, in which they observed silence of Nrf-2 can inhibit the adaptation triggered by pretreatment of low dose of H₂O₂ in murine embryonic fibroblasts (MEF), *Caenorhabditis elegans*, and *Drosophila melanogaster*.⁴⁰⁻⁴¹ Surprisingly, instead of reiterating the importance of Nrf-2-targeted antioxidants in conferring the enhanced tolerance to H₂O₂ or other oxidants (e.g. menadione, paraquat, peroxynitrite, etc.), they proposed that proteolytic activity (20S proteasome), which is regulated by Nrf-2, is necessary for such an oxidative stress adaptation. Proteasomes are known to be a part of a major mechanism by which cells regulate the retention of misfolded, aged, and oxidized proteins. They are also involved in protein trafficking, immune responses, and DNA repair.³⁹ The entire complex of proteasome is called 26S proteasome, which comprises 20S core particle and 19S regulatory particle. 19S regulatory particle is in charge of the gateway that only allows ready-to-degrade proteins to enter and approach 20S proteasome, which in contrast executes the protein degradation.⁴² Prior to the degradation, target proteins need to be modified by ubiquitin (E1-3) for 19S particle to recognise. Current technology has successfully separated the ubiquitin chains that are recruited for either protein degradation

or DNA repair. While Lys48-linked polyubiquitin chain regulates the protein ubiquitination, it is Lys63-linked polyubiquitin that modulates the DNA repair process.⁴³ In Pickering's study, Nrf-2 is believed to interact with ARE which activates proteasome $\beta 5$ genes, resulting in proteasome upregulation. Due to the detoxifying function of proteasome, cells pretreated by low dose of H_2O_2 , when challenged by harmful doses of the same chemical, could become more tolerant by efficiently clearing up the damaged proteins in the cytoplasm. However, of note should be the temporal retention of such an adaptive robustness. As demonstrated by Wiese and the others, if not instantly challenged by toxic level of H_2O_2 , pretreated HA-1 would lose their adaptive capacity after 20h of normal culture. This finding implicates that oxidative stress adaptation is most likely induced by transient overexpression of redox-regulated genes which supplements the required antioxidants and/or other detoxifying molecules, leading to enhanced robustness of cells. **Figure 2.6** is a schematic illustration that summarises the current understanding of the mechanism correlated to the oxidative stress-induced adaptation.

Aside from Nrf-2, there are another two transcription factors that also belong to the family of nuclear respiratory factors and that are known to be involved in exogenous stress response, i.e. Nrf-1 and Nrf-3. Nrf-1 shares to a large extent in common with Nrf-2 with respect to its functionality particularly when cells are under stress.⁴⁴ Like Nrf-2, Nrf-1 would be translocated to cell nucleus and bind to ARE domain for production of antioxidants. Yet, majority of the currently existing publications is focused solely on Nrf-2 potentially because Nrf-2 performs its cytoprotective function much more efficiently than Nrf-1 and they function in a considerably similar way.⁴⁵ In contrast with Nrf-1 and -2, Nrf-3 has not been substantially studied and only till recent years has it been found to act conversely in response to exotic stress signals. Siegenthaler et al used a Nrf-3 deficient keratinocyte model to demonstrate that loss of Nrf-3, instead of potentiating cell death as it is in the case of Nrf-2 deficiency, suppressed ultraviolet-induced apoptosis in both vitro and vivo.⁴⁶ Together with the findings that were reported by Sankaranarayanan et al., Nrf-3 may serve as an attenuator that negatively regulate ARE-mediated gene expression to prevent accumulation of unwanted mutations in cells. However, more studies are required in the future to fully unveil the biological functions of Nrf-3 in relation to stress responses⁴⁷.

2.1.4 Paracrine signalling– an oxidative stress-driven event

Paracrine signalling ubiquitously exists in all metazoans and is of crucial importance for regulation of cellular activities such as growth, differentiation, and other developmental signalling.⁴⁸⁻⁴⁹ Decades of research endeavours have successfully identified the signalling factors that are secreted by cells during paracrine communication, including chemokines, interferons, interleukins, lymphokines, and tumour necrosis factors (growth factor is sometimes interchangeably used with cytokines, but while growth factor refers to those promoting cell proliferation, cytokine is a more neutral term encompassing all sorts of signals). These signalling molecules have been shown to strongly correlate to many diseases, such as cell growth, wound healing, cancer development, and many other diseases.⁵⁰⁻⁵² For example, interleukin-36 is secreted by lung macrophages in response to Gram-positive and –negative bacterial challenge.⁵³ Besides, evidence also suggests that paracrine signalling promotes tumorigenesis and multi-drug resistance (MDR) by allowing a cross-talk between tumours and non-neoplastic cells.⁵⁴⁻⁵⁵

Paracrine signalling can be triggered in a cell in response to excessive amount of oxidative stress that results from chemicals, toxins, or radiations. As stated earlier, under Tier 2 oxidative stress, cells could undergo inflammation by rapidly producing multiple proinflammatory cytokines. These cytokines would then be secreted to the extracellular environment and subsequently internalised to the neighbouring cells via the collective action of receptor tyrosine kinase (RTK) and cytokine-specific receptor.⁵⁶⁻⁵⁷ When entering the cells, these cytokines would interact with mitochondria as well as NADPH oxidase, by which the free radicals are produced, resulting in induction of oxidative stress. Further supporting the linkage between cellular uptake of inflammatory cytokines and oxidative stress induction is the increased pool of oxidised protein and ubiquitin observed in the proteasome-deficient mice.⁵⁸ Due to the correlation between ROS and inflammation, it was initially believed that oxidative stress is the main cause of the inflammation triggered by exogenous proinflammatory cytokines.

In addition to ROS, proinflammatory cytokines can also activate Nrf-2 transcription factor, which in turn, was found to inhibit inflammation evidenced by many studies. For example, inflammation caused by the abnormally high expression of proinflammatory cytokines in sickle cell disease (SCD) mice can be dramatically subsided by the constant activation of Nrf-2.⁵⁹ Also, Nrf-2 can suppress the lipopolysaccharides (LPS)-induced inflammation to murine macrophages.⁶⁰ Although it is clear that Nrf-2 is activated in the cells in response to various oxidative/xenobiotic stress, the exact mechanism as to how Nrf-2 alleviates cellular inflammation is poorly understood. As oxidative stress induction is commonly observed when cells are under inflammation, it is believed that Nrf-2 in this case is driven by the oxidative stress. Contrary to this belief, one very recent finding showed that Nrf-2 could directly instruct the expression of the genes that regulate proinflammatory cytokines in macrophage; and such regulatory action was undertaken irrespective of ROS level.⁶¹ This indicates an alternative signalling pathway in which Nrf-2 could be induced and exert its function to ameliorate the inflammation stimulated by the exotic inflammatory cytokines. On the other hand, inflammation could also be triggered via a ROS-independent activation of proinflammatory genes. Internalised by the cells, proinflammatory cytokines could transduce the pro-inflammatory signals via JAK/STAT pathway and eventually to the cell nucleus, resulting in activation of proinflammatory genes.⁶² Therefore, although the driving force for the occurrence of paracrine is oxidative stress, the stress induction in the cytokines-receiving cells is just one consequence in response to such cytokines. The inflammation observed in the receiving cells is likely due to the coordinated effect of oxidative stress-dependent and –independent proinflammatory actions.

Nanoparticles as a potent ROS stimulus could also generate inflammation to a wide range of cells. Such inflammation has been implicated because of oxidative stress induction and the subsequent activation of proinflammatory genes. Nanoparticles-induced oxidative stress could activate mitogen activated protein kinase (MAPK) through induction of NF- κ B, leading to proinflammatory genes overexpression and cytokines release (IL 1 α , IL6, TNF- α) in the extracellular environment.⁶³⁻⁶⁴ While it is known that cytokines could be produced and released from the nanoparticles-treated cells, little is known about the biological responses of the cells that internalise these cytokines. To the best of knowledge,

there is only one article mentioning the paracrine signalling between blood brain barrier endothelium and astrocytes upon the exposure of polymeric nanoparticles.⁶⁵ Yet, due to the conserved role of ROS that plays in the paracrine signalling, it could be postulated that nanoparticles might trigger a chain of cellular responses in the cytokines-receiving cells similar to that results from classical inflammatory inducing factors such as chemicals or radiation.

2.2 Questions to answer based on literature

In light of the current understanding of Nano-ZnO with regard to its physicochemical properties and biological effects, several questions are to be answered:

- What are the biological outcomes of Nano-ZnO that occur to human body at low and non-toxic concentrations?
- Does low dose of Nano-ZnO induce oxidative stress adaption in biological systems (e.g. cells)?
- Due to the limited number of Nano-ZnO particulate used at the low dose context, a heterogenous uptake of the nanoparticles is expected to happen. Based on this, could Nano-ZnO induced adaption be achieved via paracrine signalling transduction?
- Due to the cytoprotective function of Nrf-2 and its relationship with oxidative stress, will Nrf-2 be activated by Nano-ZnO induced oxidative stress and thereafter mediate the adaptive response signalling process?
- As proteasomes and antioxidants are known to be regulated by Nrf-2, will they be upregulated after the activation of Nrf-2 by Nano-ZnO and will such upregulation account for the adaptive response in cells?

2.3 Master of Engineering in context of literature

The Master of Engineering seeks to (i) explore the potential cellular adaption in human keratinocytes that could be triggered by Nano-ZnO at low and non-toxic concentrations, (ii) gain more insightful understanding of the biological events in relation to the adaption

process, and (iii) conduct proof-of-concept trial to examine whether Nano-ZnO could further aid the human keratinocytes to cross-adapt to mitigate the UV rays induced cytotoxicity.

To achieve these goals, transmission electron microscope (TEM), dynamic light scattering (DLS), and zeta potential analysis were used for characterization of the physical properties of Nano-ZnO. Next, a fine designed conditioning and challenge regime (2Cs) was applied to tackle the low signal-to-noise ratio that specifically occurs in low dose context whereby the cellular adaptive response could be revealed. Furthermore, intracellular oxidative stress, Nrf-2, antioxidant, proteasome, and DNA damage were quantified using well-established assays that are across cellular, proteomic, and transcript levels with the assistance of microplate reader, light/ fluorescence microscope, and RT-PCR.

The significance of this study lies in (i) examining the potency of nanomaterials (Nano-ZnO as an example) in the low dose range and (ii) provide further clarification with regards to the potential toxicity (if any) of Nano-ZnO in skincare products. Conventionally, the toxic potential of Nano-ZnO had been studied mostly in the high dose range and considerable short (acute) exposure duration. This thesis therefore seeks to investigate the long-term and sub-lethal dosage of Nano-ZnO, which is better approximate to the actual human exposure scenario.

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Chapter 3

Experimental Methodology

This chapter will elaborate the main techniques and chemicals that were used in the study. Starting with the introduction of the rationale for choosing the chemicals and techniques used in this thesis, the rest of this chapter will first delineate the principle of the selected techniques and chemicals for either material characterization or examination of the biological responses of human keratinocytes in various conditions, which encompasses cellular, proteomic, and transcript level. Specifically, reagents were chosen to conduct a quantitative or semi-quantitative measurement for the intracellular oxidative stress, Nrf-2 protein expression, 20S proteasome retention, antioxidant capacity, Nrf-2 target and inflammatory genes expression and DNA damage. Following which is the errors analysis methodology applied in the thesis for processing the raw data. Lastly, a short summary of how they were utilized in a cooperative fashion to achieve the aims for this study will be given.

3.1 Rationale for selection

As for characterization of the physical properties of Nano-ZnO, including its size, shape, and surface charge, transmission electron microscope (TEM), dynamic light scattering (DLS), and zeta potential (ξ) analysis were employed in this study. TEM is used to visualize the primary size and shape of the nanoparticles. DLS evaluates the particles' size in the physiological environment and zeta potential analysis reveals the surface charge and the stability of the particles in a suspension. These techniques are well established and documented for characterization of a wide range of nanomaterials in many publications.¹⁻²

As for assessment of the cell viability of human keratinocytes (HaCaTs) in various conditions, Alamar Blue was used in this study to measure the metabolic activity of the cells. As viability is known to be strongly correlated to cellular metabolism, Alamar Blue thus confers an accurate indication of the cells' viability. Besides, this dye has been applied in many other studies regarding nanotoxicity.³⁻⁴

For measuring the oxidative stress in HaCaTs, many specific and non-specific indicators can be used, including CM-H₂DCFDA and CellROX™ (General measurement of oxidative stress), lipid peroxidation kit (Membrane-related oxidative stress), MitoSOX (Mitochondrial stress), Dihydroethidium (Superoxide), DAF-FM diacetate (Nitric Oxide), and H₂O₂ sensor. As it was intended to understand the overall expression of ROS in the cytoplasm, CellROX™ Orange was chosen to detect the overall amount of oxidative stress. Besides, there are three different colours of CellROX™ reagent provided by the company (Thermofisher). The orange-colour reagent was selected due to its availability to be applied in live-cell context, which means the time-consuming fixation and washing process frequently seen in other reagents is not required.

For molecular study such as Nrf-2, total antioxidant capacity (TAC), 20S proteolytic activity, and DNA damage assay, fluorescence microscopy and high-throughput microplate reader was used to measure the expression of the target molecules. While fluorescence microscopy conducts the measurement by visualizing the target molecules,

microplate reader allows for a fast measurement of target molecules by using absorbance/fluorescence screening.

For comparison of the Nrf-2 intracellular pool between groups of specimens, using Nrf-2 target antibody to specifically reveal the locality and expression level of Nrf-2 is much better than using western blot as it could not only quantify the amount of Nrf-2 in the cells but also locate its whereabouts. Given the fact that Nrf-2 is expected to translocate to the nucleus when the cells are under oxidative stress, the immunostaining method is thus well-suited to probe the spatiotemporal activation of Nrf-2.

For quantifying intracellular antioxidant pool, it is decided to use 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to measure the overall antioxidant pool in cells. ABTS is a substrate that has been widely used for study regarding kinetics of some specific enzyme during catalysis. Due to its capability to react with hydrogen peroxide and produce green-colour substance, the reverse reduction reaction is now used for detecting the antioxidants capacity in the intracellular environment. Its colorimetric reaction allows for the measurement being conducted using high-throughput microplate reader and the rapid change of the colour in merely few minutes confer a lot of convenience for conducting the assay.

For quantifying intracellular proteolytic activity, Suc - Leu - Leu - Val - Tyr - AMC (SUC-LLVY-AMC) substrate was employed. This 7-amino-4-methylcoumarin (AMC) is a fluorescent probe that specifically targets the chymotrypsin-like sites of 20S proteasome. Using fluorescence detector with the wavelength of 353nm allows for a facile quantification of the amount of proteasome in the cytoplasm.

For measuring the DNA damage in the nucleus after the cells are exposed to UV, γ -H2AX antibody was chosen to be immune-detected using staining method. This is because when double strand of DNA breaks apart, H2AX histone protein, which wraps around DNA, will be phosphorylated and become γ -H2AX, binding to which using its antibody can serve as a meaningful indicator of DNA damage.

For quantifying the expression of Nrf-2 target as well as inflammatory genes, real time polymerase chain reaction (RT-PCR) was used in the study. This method was most widely used in many fields of cell biology when genes profiling is needed.⁵ Compared to conventional PCR which gives only endpoint results, real time PCR is more effective in monitoring the quality of the amplification process. Also, when compared to Northern blot, it confers a higher sensitivity therefore only a trace amount of cDNA sample is required for the assay.

For validation of the paracrine signalling between Nano-ZnO loaded keratinocytes and the bystander cells, inserts were used to create an artificial bilayer culture microenvironment that allows for the study on the indirect effects that the nanoparticles exert on the cells situated on the bottom layer. When cells on the inserts' membrane grow to 100% confluence, the pores beneath the cell's monolayer (sized 3 μm) will be completely blocked, therefore the para-cellular transport of nanoparticles can be deemed negligible.

For studying the cross-adaption of HaCaTs to ultraviolet (UV), 8W UVA was used. Compared to other types of UV (i.e. UVB, UVC), UVA was used preferably as it accounts for over 90% of the UV that could penetrate through Ozone layers and reach human skins.⁶ 8W was chosen over 36W during the study as 36W exerts a lot of heat thus the experimental temperature is beyond the cells' tolerance, leading to a hyperthermia-induced cell death.

3.2 Characterization of Physical Properties of Nano-ZnO

3.2.1 Transmission Electron Microscope

Transmission Electron Microscope (TEM) is an advanced microscopy technique that is widely used in multi-disciplinary fields such as material science, chemistry, biology, and medical research. Its primary function is to visualize an ultrathin specimen with a high resolution that could reach atomic level. To date, there are many derivatives of TEM developed to suit different purposes of imaging, including Scanning TEM (STEM), Cryo-TEM for imaging biological samples, Environmental TEM, and Aberration corrected TEM.

The main advantage that makes TEM superior to light microscope lies in the light source. Instead of using visible light as optical microscope, TEM utilizes electrons that possesses a much lower wavelength (6pm versus 600nm), thus according to the De Broglie hypothesis, could reach an ultrahigh magnification when imaging.⁷

Figure 3.1. Composition of Transmission Electron Microscope. *Redrawn based on the original image retrieved from The McGraw-Hill Companies, Inc.*

Unlike optical microscope which uses different sets of apertures to achieve multiple magnifications, TEM modulates its magnification by changing the strength of magnetic field with a series of electromagnetic lens, such as a couple of objective lens, intermediate lens and projector lens.⁸ As such, it requires a much more complicated design, as seen from **Figure 3.1**. First, generation of electron is very similar to the light generated by a light bulb, in which a tungsten is heated up and emit electron via thermionic process. Once emitted, the anode installed right beneath the tungsten filament would attract the electrons to escape from the trap caused by the cathode installed above the anode and also allow them to move in a top-down direction. Thereafter, the electrons will be converged by the condenser lens and “shine” on the specimens in the sample holder located slightly above

the objective lens. In fact, the interaction between the electrons and samples will then generate a variety of beams, such as backscattered electrons (BSE), secondary electrons (SE), visible light, elastically/in-elastically scattered electrons, and direct pass-through beams (**Figure 3.2**). Amongst them, only direct beams can be used in TEM. The direct beams will later pass through intermediate lens where the signal will be magnified and sent to projector lens for the final processing. The processed signal will then be projected onto a fluorescent screen for visualization.

Figure 3.2. Different electrons produced during the interaction between the original electrons generated by TEM and specimens. *Redrawn based on the original image retrieved from nanoHUB. Org.*

The analysis of TEM images is divided by two sections: pre-evaluation and post-analysis. Pre-evaluation basically refers to a preliminary assessment of the quality of samples. For instance, when using TEM to examine the primary size of a nanoparticle, pre-evaluation on its dispersity, homogeneity, morphology, and size shall be conducted prior to image acquisition. A well disperse and homogenous sample in the sample holder is of critical importance to ensure the accuracy of the information attained from TEM and it determines whether post-analysis can be proceeded. Post-analysis is carried out when the quality of the specimen is satisfied, and its primary purpose is to quantitatively evaluate the size

distribution, morphology, and the degree of aggregation of the nanoparticle based on the acquired images. To measure the primary size of the nanoparticles, software such as ImageJ might be needed.

Figure 3.3. Composition of Dynamic Light Scattering. Redrawn based on the original image retrieved from

http://www.horiba.com/fileadmin/uploads/Scientific/Documents/PSA/Webinar_Slides/TE012_V2.pdf

3.2.2 Dynamic Light Scattering

Dynamic light scattering (DLS) is most commonly and effective in measuring the hydrodynamic size (D_H) of nanomaterials (also known as submicron-size materials). The reason of calling it hydrodynamic size is that DLS in fact measures the Brownian motion of a material in dispersions. The composition of DLS machine is shown in **Figure 3.3**. Bundles of light will be released from a laser, subsequently pass through an attenuator, and interact with the samples placed in a frosted cuvette. There are two sets of interactions installed in the DLS system: 173° (backscatter) and 90° . 90° is mostly used in determination of a particles' size and molecular weight. Once interacted, the light will scatter and the extent to which it scatters (intensity) will be captured by DLS. As nanomaterials in a dispersant will undergo Brownian motion, a time-lapse probe of the scattering intensity is further collected and compared by a correlator installed in the system to calculate the hydrodynamic size of the material. The formula for the calculation of hydrodynamic size is as follow:

$$d(H) = \frac{kT}{3\pi\eta D}$$

Where¹⁰:

$d(H)$ = hydrodynamic diameter

D = translational diffusion coefficient

k = Boltzmann's constant

T = absolute temperature

η = viscosity

The correlator will collect a set of intensity data of a single position and thereafter plot the data as a function of time. As nanomaterials constantly fluctuate, the intensity of the same position as time passes will decrease gradually (**Figure 3.4**). Further, the decrease rate of the intensity at a single position is higher for a smaller material. Based on these principles, DLS could correlate the hydrodynamic size to the decay rate of the intensity signal. Finally, by collecting multiple sets of intensity data at many different positions (assuming there is

Figure 3.4. Correlation between decay time and size of nanomaterials. Redrawn based on the original image retrieved from http://www.horiba.com/fileadmin/uploads/Scientific/Documents/PSA/Webinar_Slides/TE012_V2.pdf

only one single particulate in each position), DLS could accurately evaluate the hydrodynamic size of nanomaterials by analysing the mean value and the distribution of the nanomaterials. However, the hydrodynamic size measured by DLS is not an inherently true reflection on the actual size of a material, and it becomes even more evident when a material is not in spherical shape. As seen in **Figure 3.5**, DLS could only give an estimation of the diameter based on the sphere model. Besides, DLS is sensitive enough to detect the size of the conjugate on a material's surface, therefore the hydrodynamic size calculated in DLS may differ from the results acquired from TEM or SEM.

Figure 3.5. Hydrodynamic size of nanomaterials with various morphologies, degree of aggregation and surface functionalization. Redrawn based on the original image retrieved from http://www.horiba.com/fileadmin/uploads/Scientific/Documents/PSA/Webinar_Slides/TE012_V2.pdf

3.2.3 Zeta potential

Zeta potential is the charge on a particle at the shear plane, which is essentially one or two counterion layer(s) away from the particle's surface. The electric field constituted around a particle is described in **Figure 3.6**. A negatively charged particle is surrounded by multiple layers of counterions and once an extra electric field is applied, the particle will undergo shear force thus alter its morphology, forming a slipping plane. As mentioned, what zeta potential analysis measures is the charge of the green annulus (the postulated

shear plane).

Figure 3.6. The electric field of a particle when it is into a dispersant. Redrawn based on the original image retrieved from <http://www.horiba.com/scientific/products/particle-characterization/technology/zeta-potential/>

To measure the zeta potential, the machine (integrated with DLS and zeta potential) needs to harvest the particle motion during the application of the electric field, which requires a different configuration for the measuring system compared to that for measuring D_H . As seen from **Figure 3.7**, while laser light is kept for illumination of the particles and thus inducing light scattering when it is in contact with the particles, a reference beam needs to be generated in order to exclude the light scattering effect due to particles' self-motion (Brownian motion), which is the fundamental information for the acquisition of D_H . Thereafter, the magnitude of the frequency shift can be calculated whereby the particle's velocity can be determined. At last, a proper model will be applied according to the

particle's inherent properties, such as viscosity, refractive index, and liquid dielectric constant to convert the velocity data to the zeta potential of the tested particle. The most commonly used model for determining zeta potential is the Smoluchowski model.⁹

Figure 3.7. The configuration of DLS for determination of a particles' zeta potential. Redrawn based on the original image *retrieved from* <http://www.horiba.com/scientific/products/particle-characterization/technology/zeta-potential/>

3.3 Evaluation of Cellular Responses

3.3.1 High-throughput microplate reader

High-throughput microplate reader is a device that is widely used to detect biological, chemical or physical events of samples in microliter plates. It is high-throughput because it is capable examining many parallel/ different groups of samples within a short period of time. A current microplate reader performs as an absorbance, fluorescence, luminescence, and light scattering detector. Depending on the number of sample to be tested, the assay can be conducted in 6-1536 well format microliter plates. In laboratory, 96 well plate is most commonly used with assay volume ranging from 100 to 200 microliters.

For absorbance assay, a light source will be applied on one side of the microplate reader for illuminating the samples and on the other side there is a signal detector to measure the transmittance. The transmittance value will be correlated to the concentration of the

molecule of interest. One typical application of absorbance assay conducted in a microplate reader is MTT assay for cell viability measurement.¹¹

Figure 3.8. The configuration of a fluorescence microscope. Redrawn based on the original image retrieved from <https://cam.facilities.northwestern.edu/588-2/epi-fluorescence-microscopy/>

For fluorescence assay, the configuration is different from that for absorbance detection and it is usually much more expensive. In brief, prior to the test, a specific combination of excitation/ emission wavelength is set for detecting the fluorescein of interest in the well plate. Microplate will illuminate the samples according to the chosen excitation wavelength which triggers the molecule in the sample to fluoresce. The signal of such fluorescence will then be captured by a monochromator with a specific filter to enhance the signal-to-noise ratio by eliminating signals whose wavelengths are below the pre-set emission wavelength.

As luminescence and light scattering were not involved in the study, review of these two applications will not be conducted herein. These two applications have been vigorously reviewed elsewhere.¹²

3.3.2 Fluorescence microscope

Fluorescence microscope functions similarly to microplate reader for detecting fluorescence intensity. A light source will emit a bundle of light with a wavelength typically shorter than the emission wavelength. The samples will be illuminated and then fluoresce whose signal will be captured by a detector for quantification or transduced to objective lens for observation. A dichroic mirror and a set of emission filter are very important for the microscope to successfully transduce signal of interest to either the detector or the objective lens (**Figure 3.8**). As such, only one signal of interest can be detected at a time. For detecting multiple signals, images need to be combined to yield a multi-colour image. Also, since most of the excitation light will pass through the sample, making fluorescence microscope a very powerful tool for imaging with an ultrahigh signal-to-noise ratio.

3.3.3 Real-time polymerase chain reaction

Polymerase chain reaction (PCR) is a powerful tool that has been broadly applied in DNA cloning, medical diagnostics, and forensic analysis of DNA. In laboratory, PCR is most commonly used for DNA cloning whereby the expression level of the gene of interest can be determined. Conventional PCR which could only reveal the endpoint of the amplification reaction has been replaced by real-time PCR, which allows for the detection during the early phases of the reaction.

For a successful amplification of a DNA sample, Taq polymerase, forward and reverse primers, and the cDNA sample transcribed from its RNA counterpart extracted from cell's lysate, are required. There are three steps for the amplification reaction: denaturation, annealing, and extension.¹³ Denaturation is usually conducted at around 95°C and it helps

the double strands of DNA to decompose into single strands for the coming rounds of amplification. Annealing is to cool down the samples so that primers can bind to the template (single strands). Extension is the process where double strands are synthesized. During extension, Taq polymerase has the highest avidity, so it could rapidly clone the DNA template before the PCR machine proceed to denaturation process. Conventionally, DNA copies after amplification will be used for further experiments such as sequencing, forensic examination, and gel electrophoresis. Of which electrophoresis is to visualize the expression level of the gene of interest. However, currently the advanced development of fluorescence imaging allows for the *in-situ* detection of the gene expression via usage of fluorescein (e.g. Syber Green).

Analysis of the gene expression of interest requires a calibration in which a housekeeping gene (e.g. GAPDH/ β -actin) that is known to be invariably expressed serves as a standard. Usually the expression of a gene in the tested group will be normalized to that in control and is expressed as “fold of expression”. The formula for calculating fold of expression is shown as follow:

$$\text{Fold of expression} = 2^{-(\Delta\text{Ct (Control)} - \Delta\text{Ct (Experimental)})}$$

Where¹⁴:

$\Delta\text{Ct (Control)} = \text{mean Cq value (gene of interest)} - \text{mean Cq value (housekeeping gene)}$

$\Delta\text{Ct (Experimental)} = \text{mean Cq value (gene of interest)} - \text{mean Cq value (housekeeping gene)}$

3.3.4 Cell viability assay

Resazurin salt (commercially branded as Alamar Blue) is a blue dye that will turn red when it is reduced through metabolic reactions in biological systems. Due to its pronounced change in colour, it has been widely used in colorimetric experiment for determination of cell metabolic activity. It is membrane-permeable dye that could readily enter the cytoplasm and react with NADH/ H^+ in close proximity to mitochondria. As NADH/ H^+ is a metabolite produced by mitochondria, the conversion rate of Alamar Blue from its blue form to red version (**Figure 3.9**) is a proper reflection on the metabolic activity of a cell.

As the metabolism of a cell has a strong relation to its viability, evidenced by decomposition of mitochondria in the late stage of apoptosis, Alamar Blue is a proper indicator of a cell's viability. To detect the colorimetric change of Alamar Blue, both absorbance and fluorescence assay can be applied. For absorbance assay, two wavelengths (570/600nm) are applied to determine the amount of reduced form and oxidized form of Alamar Blue respectively. For fluorescence assay, excitation/emission 530/590nm is chosen. Sensitivity of the result will be enhanced as staining duration increases. As Alamar Blue is minimally toxic to cells, it allows for time-lapse viability studies.

Figure 3.9. Colorimetric reaction of Resazurin. Redrawn based on the original image retrieved from <https://www.ncbi.nlm.nih.gov/books/NBK144065/figure/mttassays.F7/>

3.3.5 Oxidative stress (ROS) detection

CellROX® Orange Reagent is an orange-colour probe that allows for a non-specific detection of the intracellular oxidative stress in cells. It weakly fluoresces when it is in the reduced form. Upon oxidation by reactive oxygen species, it will be activated and converted to its oxidized form which would emit much higher fluorescence. This dye can be detected using fluorescence microscope with emission maxima 565nm. This formaldehyde-non-fixable can be further multiplexed with other live cell-compatible probes such as Hoechst 33342, DAPI, AnnexinV, Propidium Iodide. As for a general ROS detection, this non-fixable dye allows for a rapid staining process that does not require fixation as well as washing. Upon completion of the staining, samples could be imaged by fluorescence microscope and the intensity evaluation can be conducted with the help of ImageJ.

3.3.6 Immunostaining

Immunostaining initially refers to immune-histochemical staining that is applied in tissue engineering but now is as well widely used in cell biology. Briefly, primary antibody with/without fluorophore is bound to the molecule of interest which is subsequently imaged by fluorescence microscope or confocal microscope to determine the molecular expression according to the intensity of the fluorescence. If the primary antibody does not have fluorophores, a second antibody conjugated with fluorophores will be further applied to specifically bind to the primary antibody. The immunostaining sample can be further multiplexed with other dyes such as DAPI for different imaging purposes. As most of the antibodies are not membrane-permeable, detergents (e.g. TritonX) are usually used prior to the application of the primary antibody to permeabilize the plasma membrane. As multiplexing is commonly used in immunostaining, washing appropriately is necessary for effectively reducing the noise caused by the dyes remaining in the solution.

3.3.7 ABTS assay

To test the total antioxidant capacity (TAC), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was used in the study. This dye has been widely used to test the concentration of antioxidants in plasma, serum, saliva, urine, and many other extracts from plants and herbs.¹⁵ ABTS can be rapidly reduced by cellular antioxidants into its reduced version (ABTS⁺ to ABTS), as such its colour will change from green to purple. The extent to which the colour of ABTS change can be used to measure the concentration of TAC in cells, so long as a standard antioxidant solution is used concurrently for calibration.

3.3.8 SUC-LLVY-AMC fluorescent probe

Proteasomes play a crucial role in regulating intracellular protein concentration as well as protecting cells against oxidative damage via rapid clearance of damaged/ misfolded proteins. Most of the proteasomes are composed of 19S regulatory and 20S core particles

and are coined 26S proteasomes. Of which 20S core particles is the recycle centre for degradation of wasted proteins. Recent research has suggested that 20S core particles could work individually in the cytosol and these individuals are now called 20S proteasomes. Due to the fundamental role of 20S proteasomes in maintaining cells' survival, its activity was probed by using SUC-LLVY-AMC dye in this study. 20S proteasome consists of 28 subunits, which contains β -type rings, each of which is comprised of three different proteolytically active sites, including $\beta 1$ (post-glutamyl peptide hydrolase site), $\beta 2$ (trypsin-like site) and $\beta 5$ (chymotrypsin-like site).¹⁶ SUC-LLVY-AMC is a substrate that selectively binds to the chymotrypsin-like site in the 20S proteasome. Once bound, the fluorophore (AMC) would be released thus can be detected via microplate reader with excitation/emission maxima 380/460 nm. Hence, by comparing the fluorescence intensity of each sample, the amount of 20S proteasome in each sample can be determined.

3.4 Errors Analysis and Significance Evaluation

For all the experiments, 4 replicates were concurrently tested for one specimen and mean value was calculated when at least three out of four replicates had a similar value. Erroneous value was excluded from the dataset when calculating the mean value. Afterwards, standard deviation was calculated to indicate the variation of the data.

To understand the significance when comparing different groups of data, t-test and ANOVA were applied. T-test is robust to indicate the significance of one set of data out of another set when these two sets of data are known to be sampled independently. Unpaired two-sample test was applied in this study. Unpaired two-example test uses two independent groups of data and it aims to calculate the probability that a null hypothesis, that is, the means of these two groups of data are identical, is correct. In most cases, if the probability value is greater than 0.05, the statement that the two sets of data have a significant difference will not be accepted, and vice versa. To evaluate the significance of multiple groups of data, T-test was replaced by ANOVA, which could analyse many groups of data simultaneously. ANOVA operates in a way that is similar to multiple two-sample t-tests,

which makes it compatible with t-test for evaluating the significance of the data presented in the thesis.

3.5 Overview of Methodologies

In this thesis, TEM, DLS and zeta potential analysis were combined to understand the physical properties of Nano-ZnO, including its size, shape, and surface charge, which confer a fundamental information for understanding the biological effects of Nano-ZnO on HaCaTs. To examine the biological outcome of Nano-ZnO, colorimetric assay, fluorescence microscopy, and real-time PCR, which encompass cellular, proteomic, and transcript level, were cooperatively applied in the study. Molecules of interest and the methods used for examining them were fine selected based on existing publications. Random error that deviates much from other parallel replicates was strictly excluded thus the mean values presented in the thesis are the true reflection on each group of data. Each mean value was presented concurrently with an error bar indicating the degree of variation of the data in the group and significance of the data was assessed via well-established statistical models, including t-test and ANOVA. With the help of all these techniques and tools, the biological effects of Nano-ZnO on human keratinocytes can be properly examined.

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Chapter 4

Nano-ZnO Induced Adaptive Response in Human Keratinocytes

Unravelling the biological events that results from Nano-ZnO in the full dose range (both toxic and sublethal) could lead to a better understanding of the potential hazard that it might elicit to human body. Nonetheless, research pertaining to the biological effects of Nano-ZnO at sublethal concentrations is still lacking. Therefore, this study herein examined the cellular response in human keratinocytes pretreated by low dose of Nano-ZnO. It was found that cells preconditioned by a low dose of Nano-ZnO for 48 to 144 hrs could be increasingly resistant to the toxic level of the same nanoparticle. This adaptive response has been linked to the inhomogeneous induction of intracellular oxidative stress, which subsequently led to a global-wide of Nrf-2 activation. Specifically, only around 2% of the conditioned cells with “ROS flares” could be detected and these cells were sufficient to induce the global-scale upregulation of Nrf-2 via the interleukins-mediated paracrine signalling. Further mechanistic study discovered Nrf-2 induced a sustained increase of proteolytic activity, rather than of antioxidant capacity, which indispensably contributes to the adaptive response. It was also shown that the Nano-ZnO conditioned cells could become more tolerant to the UV-A induced damage. All in all, these findings could address the fundamental knowledge gap regarding the biological events in relation to Nano-ZnO with the example of human keratinocytes.

4.1 Experimental Methods

4.1.1 Materials and measurements

ZnO nanoparticles (Nano-ZnO) (<100nm), Resazurin sodium salt (Alamar Blue), and L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Asc-2P) were purchased from Sigma-Aldrich. CellROX® Orange reagent, Hoechst 33342, Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit, Chicken anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 and Chicken anti-mouse IgG secondary antibody conjugated with Alexa Fluor 594 were purchased from ThermoFisher Scientific. Rabbit anti human Nrf-2 monoclonal primary antibody and rabbit anti human E-Cadherin monoclonal primary antibody was purchased from Cell Signaling. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay kit was purchased from Beyotime. Mouse anti human γ H2AX monoclonal primary antibody was purchased from Abcam (ab26350). Dulbecco's Modified Eagle's Medium (DMEM-High glucose), Fetal Bovine Serum (heat inactivated), and Antibiotic-Antimycotic were purchased from GE Hyclone. Trypsin (0.25%, with 1mM EDTA, 4Na) and HEPES buffer solution were purchased from Gibco. Immortalized human keratinocytes (HaCaTs) were obtained from German Cancer Research Center (DKFZ). All chemicals were used without further purification. DI water was used to prepare nanoparticles stock suspension for the *in-vitro* cytotoxicity study. All experiments in this study were carried out in triplicates. Data are presented by mean $\pm$ standard deviation (SD) with *p* value indicated where necessary.

4.1.2 Characterization of Nano-ZnO

The primary physical size of Nano-ZnO was characterized based on the images obtained using transmission electron microscope (TEM, Carl Zeiss Libra 120 Plus). Briefly, 30 μ l of Nano-ZnO (100 μ g /ml) in absolute ethanol was pipetted onto the carbon-coated TEM copper grid and the samples were allowed to be air dried. The samples were then imaged using TEM (Carl Zeiss Libra 120 Plus) with a voltage of 120KV and magnification ranging from 10000 to 70000. Hydrodynamic diameter (D_H) and zeta potential (ζ) of the

nanoparticles were characterized using the Zetasizer Nano ZS (Malvern). To better understand the physiochemical properties of the Nano-ZnO in the biological milieu, D_H and ζ of the nanoparticles in the complete cell culture medium dispersant were also analysed.

4.1.3 Cell culture and cytotoxicity assay

Human keratinocytes (HaCaTs) was cultured in DMEM supplemented by 10% FBS and 1% antibiotics. The cells were routinely maintained in a cell culture incubator (Thermo) at 37°C, 5% CO₂ and 95% relative humidity. Cell morphology and confluency were visualized using Carl Zeiss Primo Vert inverted bright field microscope. Upon confluence, HaCaT cells were trypsinized and seeded into 96 well plates with a density of 18,750 cells/cm². (This seeding density applies to the following experiments unless otherwise stated) Cells were cultured for 24h to reach a targeted confluency of ~ 80%. Thereafter, the cells were treated with Nano-ZnO at various concentrations. Cell viability was determined using the alamarBlue® cell viability assay. After 4h of incubation with alamarBlue® in cell culture incubator, raw data was obtained from the high-throughput microplate reader (Molecular Devices SpectraMax M2).

4.1.4 Annexin V/PI apoptosis assay

Cells were cultured in 12 well plate with the pre-determined seeding density as described for 48h. Thereafter, the cells were exposed to 50 µg/ml Nano-ZnO for 2h before the cells were trypsinized, washed thoroughly three times in cold PBS and collected for further analysis. To examine the molecular basis of Nano-ZnO induced cell death, we employed the Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit (Thermo Fisher) as per the staining protocol provided by the manufacturer. Samples were then incubated in ice bath for 15 min prior to examination via fluorescence microscope (Carl Zeiss AxioObserver Z1). Fluorescence images of all the different experimental groups were taken using the same optics and imaging settings.

4.1.5 Nano-ZnO “conditioning and challenging” (2Cs) regime

To verify whether Nano-ZnO could induce oxidative stress adaptation in HaCaTs, we designed a Nano-ZnO “conditioning and challenging” regime. Briefly, cells were cultured in 6 well plate with as-described seeding density for 48h. At the onset of the culture, cells were conditioned with 0.5µg/ml Nano-ZnO. After 48h, cells were subcultured into a fresh 6 well plate with the same seeding density while a portion of the cells were retrieved for cytotoxicity assay. Each round of conditioning is defined as 48h of Nano-ZnO (0.5 µg/ml) exposure and is denoted in the subscript (i.e. C₀, C₁, C₂, C₃). To clarify the role of Zn²⁺ ions in this adaption process, cells were also conditioned with equivalent concentration of ZnCl₂ (6µM) with the same procedure as described above.

4.1.6 Detection of intracellular reactive oxygen species (ROS) expression

CellRox® Orange reagent was used herein to visualize and measure the intracellular ROS expression level in HaCaTs cells. Following the exposure of the cells to varying concentrations of Nano-ZnO, the cells were counterstained with CellRox® Orange reagent (5µM) and Hoechst 33342 (2µg/ml) in cell culture medium for 30 min at 37°C. Thereafter, the samples were washed thoroughly with PBS, replenished with fresh cell culture medium and imaged using an inverted fluorescence microscope (Carl Zeiss AxioObserver Z1). Fluorescence images of all the different experimental groups were taken using the same optics and imaging settings. ROS Fluorescence intensity were calculated via ImageJ without any preceded modification of the captured images.

4.1.7 Immunofluorescence staining and imaging

All samples were fixed with 4% paraformaldehyde for 15 min at room temperature and the cells were further permeabilized using TritonX (0.2%) for 10 min. Thereafter, the samples were washed 3 times with PBS, and further blocked with 2% BSA (blocking buffer) for 1h at room temperature. Rabbit anti human Nrf-2 monoclonal primary antibody/ Rabbit anti human E-Cadherin monoclonal primary antibody/ γ-H2AX mouse monoclonal primary

antibody solution was diluted in the blocking buffer (1:200) and incubated with the cell samples overnight at 4 °C. Following which, the samples were washed 3 times with PBS, counterstained with 10µg/ml Hoechst 33342 (for all samples) and 66nM rhodamine phalloidin (Nrf-2)/ Chicken anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (1:200 v/v, E-cadherin)/ Chicken anti-mouse IgG secondary antibody conjugated with Alexa Fluor 594(1:200 v/v, γ -H2AX) in the dark at room temperature for 1h. Samples were then imaged using a fluorescence microscope (Carl Zeiss AxioObserver Z1).

4.1.8 Total antioxidant Capacity (TAC) and proteolytic assay

Cells were cultured in 6 well plate with the pre-determined seeding density as described above for 48h. Thereafter, the cells were treated with the conditioning dose of Nano-ZnO (0.5 µg/ml) for further 48h. Following this step, the samples were washed 3 times with PBS before the cells were harvested with 200µl cold PBS using a cell scraper. Upon the completion of cell harvesting, the cell suspension was sonicated for 1h to attain the cells lysate. 10µl of the cell lysate was then placed into hemocytometer and observed with microscope to ensure complete lysis of the cells. Next, cells lysate was centrifuged at 12000×g for 5 min to remove the cell debris. BCA assay (ThermoFisher) was conducted to determine the protein concentration for normalization purpose. The cellular TAC was subsequently quantified using the ABTS assay kit (Beyotime) as per the manufacturer's protocol. Briefly, the cells lysate samples and bovine serum albumin (BSA) was incubated in 37°C with the working buffer for 30min before quantification. 10µl of the samples and 90µl of the working solution were added to each well in 96 well plate. 10µl of Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] with different concentrations were used for normalization purpose. Absorbance values were determined 562nm and 734nm using a microplate reader (Molecular Devices SpectraMax M2) for BCA assay and ABTS assay respectively. Final TAC result was normalized by Trolox (mM) as well as BSA (mg/ml), and presented with the unit "mmol/g". To determine the proteolytic activity of the cells, the cell lysate samples were further diluted to 50µg/ml (protein concentration) and 90µl of each sample was mixed with 10µl SUC-LLVY-AMC (100µM, Enzo Life Science) substrate in 96 well plate. Once mixed, samples were incubated in the dark at

37°C for 1h. Upon completion of the reaction, fluorescence values were determined at excitation/emission wavelength of 355/444nm using a microplate reader (Molecular Devices SpectraMax M2).

4.1.9 Real-time PCR

Cells were cultured as described. Upon confluence, cells were exposed to 0.5µg/ml Nano-ZnO for 4h prior to harvest. Total RNA of HaCaT cells were isolated using PureLink RNA Mini Kit (Life Technologies). Reverse transcription of RNA samples was done with iScript cDNA synthesis Kit from Biorad, in accordance to the manufacturer's protocol. Quantitative real time PCR was conducted using the CFX96 real time PCR detection system from Biorad and SYBR FAST qPCR Master Mix (2X) Universal from KAPA with the following thermal cycling condition: Enzyme activation at 95°C for 3 min; followed by 40 cycles of denaturation at 95°C for 3s and annealing/extension/data acquisition at 60°C for 20s. Melt-curve analysis was also done to assess the purity of the amplicon/ product. All the primers were obtained from primer bank (<https://pga.mgh.harvard.edu/primerbank/>) as listed in **Table 4.1**.

4.1.10 Statistical analysis

Origin 9 (OriginLab) was used for statistical analysis. Experimental data were subjected to either Student's t-test or one-way analysis of variance (ANOVA) where applicable. Statistical differences are indicated with probability value (p value) in the associated text or figure captions.

Table 4.1. Primers used for examination of inflammatory cytokines and Nrf-2 downstream antioxidant-related genes

Gene target	RT-PCR primer sequence	
	Sense	Antisense
<i>Housekeeping gene</i>		
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
<i>Inflammatory genes</i>		
IL-1 β	AAATACCTGTGGCCTTGGGC	TTTGGGATCTACACTCTCCAGCT
IL-1 α	TGGTAGTAGCAACCAACGGGA	ACTTTGATTGAGGGCGTCATTC
IL-6	GTAGCCGCCCCACACAGA	CATGTCTCCTTTCTCAGGGCTG
IL-8	ATAAAGACATACTCCAAACCTTTCCAC	AAGCTTTACAATAATTTCTGTGTTGGC
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
IL-12	AGGACGAGACACCCACTTATAC	ATTGACAGCAGTAACCTTGGC
IL-15	CATCCATCTCGTGCTACTTGTGTT	CATCTATCCAGTTGGCCTCTGTTT
IL-18	TCTTCATTGACCAAGGAAATCGG	TCCGGGGTGCATTATCTCTAC
IL-23	TGCAAAGGATCCACCAGGGTCTGA	TAGGTGCCATCCTTGAGCTGCTGC
IL-33	GTGACGGTGTTGATGGTAAGAT	AGCTCCACAGAGTGTTTCCTTG
TNF- α	CCCAGGGACCTCTCTCTAATCA	GCTTGAGGGTTTGCTACAACATG
TGF- α	AGGTCCGAAAACACTGTGAGT	AGCAAGCGGTTCTTCCCTTC
VEGF	GAGGAGCAGTTACGGTCTGTG	TCCTTTCCTTAGCTGACACTTGT
<i>Nrf-2 targeted antioxidant genes</i>		
HMOX-1	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCTG
NQO1	GAAGAGCACTGATCGTACTGGC	GGATACTGAAAGTTCGCAGGG
GSTM-3	TACCTCTTATGAGGAGAAACGGT	AGGAAAGTCCAGGTCTAGCTTG
UGT	CATGCTGGGAAGATACTGTTGAT	GCCCGAGACTAACAAAAGACTCT
GSS	GGGAGCCTCTTGACAGATAAA	GAATGGGGCATAGCTCACCAC
γ -GCS	TGTCTTGGAATGCACTGTATCTC	CCCAGTAAGGCTGTAAATGCTC
CAT	TGGAGCTGGTAACCCAGTAGG	TGGTACCTTTGCCTTGGAGTATT
SOD	GCTCCGGTTTTGGGGTATCTG	GCGTTGATGTGAGGTTCAG

4.2 Results

4.2.1 Physical characterization of Nano-ZnO

The physiochemical properties of Nano-ZnO were characterized using transmission electron microscope (TEM), dynamic light scattering (DLS) and zeta potential analysis (ξ). **Figure 4.1a** shows the representative TEM image of the Nano-ZnO that was employed in this study. The majority of the Nano-ZnO displayed a rod-like shape that was irregularly sized. The average primary particle size was approximately 100nm. Conversely, the measured average hydrodynamic diameter (D_H) of the Nano-ZnO in H₂O and complete cell culture medium were 195 nm and 215 nm respectively (**Figure 4.1b**). The difference of size detected from TEM and DLS could be due to their fundamentally different principles. While TEM reveals the primary size of Nano-ZnO, DLS measures the hydrodynamic size

Figure 4.1. Physical characterization of Nano-ZnO. (a) Representative TEM image of Nano-ZnO particles. (b) Compiled hydrodynamic parameters including primary particles size, hydrodynamic diameter and zeta potential of Nano-ZnO in various dispersants.

(D_H) of the nanoparticles according to the rate of doppler shift induced by Brownian motion.

¹ Zeta potential of Nano-ZnO in DI water was +24 mV and it switched to -31.3 mV when the particles were suspended in cell culture medium. This could be attributed by the particles' absorption of negatively charged proteins onto its surface.²⁻³

4.2.2 Low dose of Nano-ZnO induces adaptive response in HaCaTs

One fundamental question to answer in this study is whether low dose of Nano-ZnO that approximates the penetrated portion of the particles contained in the sunscreen could induce cellular adaption in skin cells through daily applications. Therefore, an *in vitro* “conditioning and challenge” (2Cs) experimental regime was established for emulating the repeated application of sunscreens (**Figure 4.2**). In brief, HaCaTs were first conditioned by a low (non-toxic) dose of Nano-ZnO for 48h. Thereafter, the cells (C_1) were challenged by a range of Nano-ZnO concentrations to obtain the cell viability dose-response profile. In the meantime, some conditioned cells were further conditioned for another 48h or

Figure 4.2. The “conditioning and challenge” (2Cs) regime devised to study adaptive response of HaCaTs to Nano-ZnO.

48+48h (C₂, C₃) followed by the same challenge mentioned above. The subscript presented in the different experimental group denotes the number of rounds of conditioning (C). Prior to conditioning the cells with a low dose of Nano-ZnO, a dose-response profile for the cells without conditioning were acquired and it served as a control to determine whether adaption occurred.

As shown in **Figure 4.3a**, cell viability of the unconditioned experimental group (i.e. C₀) decreased with increasing concentrations of the Nano-ZnO. Significant toxicity set in at around 30 µg/ml that corresponded to ~35% killing rate as compared to the untreated control. Cells treated with 40 µg/ml and 50 µg/ml of Nano-ZnO resulted in a further decrease in cell viability to ~50 and 40% respectively. Further Annexin-V and PI analysis revealed that the Nano-ZnO treated cells died primarily via apoptosis (**Figure 4.4a, b**).

Figure 4.3. Cytotoxicity of HaCaTs cells exposed to various concentrations of Nano-ZnO. a) Cell viability of unconditioned and conditioned HaCaT keratinocytes plotted against Nano-ZnO challenged doses. (b) Representative optical images of HaCaTs correspond to the cell viability data shown in part a. Data are presented as mean $\pm$ SD. n=3. Asterisk * denotes statistical significance. $p < 0.05$.

Strikingly, cells that were pre-conditioned with a non-cytotoxic dose (0.5 µg/ml) of Nano-ZnO exhibited an increased resilience towards the toxic levels of Nano-ZnO. This is most evident when comparing the measured cell viability and the phase contrast images (**Figure 4.3b**) of the different experimental groups that were treated with 30, 40 µg/ml of Nano-ZnO. It was observed that approximately 20-40% tolerance was enhanced for the

conditioned cells (i.e. C₁, C₂ and C₃) relative to the unconditioned control cells (i.e. C₀). It was also noted that the tolerance level increased with the duration of conditioning.

Figure 4.4. Apoptosis examination of Nano-ZnO exposed HaCaTs. (a) Representative images of annexin V (green) / PI (red) staining for HaCaTs exposed to 50 µg/ml of Nano-ZnO for 2h. (b) Percentage of early apoptotic (annexin V (+) / PI (-)), late apoptotic (annexin V (+) / PI (+)) and healthy (annexin V (-) / PI (-)) cells. 100 cells were randomly selected for the measurement. Data are presented as mean ± SD. n=3. Asterisk * denotes statistical significance. p<0.05.

Specifically, cell viability remained relatively high (>90%) for the C₃ cells that were exposed to Nano-ZnO at concentration as high as 40 µg/ml. However, any acquired resistance to the Nano-ZnO induced toxicity was absent when the cells were exposed to 50 µg/ml of Nano-ZnO, suggesting that there was an intrinsic limitation to this adaptive response. Since Nano-ZnO is known to partially dissolve into Zn²⁺ in the solution, it was therefore tested whether the dissolved Zn²⁺ could account for the Nano-ZnO induced cellular adaption. Using ZnCl₂ with the same molar concentration (6 µM) of Zn as the

nanoparticles (assuming 100% Nano-ZnO turned to Zn^{2+}) failed to reproduce the result obtained in the context of Nano-ZnO (**Figure 4.5**). This suggests the cellular adaption was due to a nanoparticle-specific effect.

Figure 4.5. ZnCl_2 conditioned cells displayed marginal adaptive response. HaCaTs were exposed to varying rounds (i.e. C₀-C₃) of conditioning with Zn^{2+} (6 μM), before the cells were subsequently exposed to toxic concentrations (≥ 30 $\mu\text{g/ml}$) of Nano-ZnO. Biological replicates = 3. Data is presented as mean $\pm$ SD.

4.2.3 Nano-ZnO significantly induces oxidative stress (ROS) at toxic levels

As excessive production of intracellular ROS is an established hallmark of ENMs-induced cell death,^{4,7} it was hypothesized that at the early time points of the particles exposure, toxic levels of Nano-ZnO could lead to an elevation of ROS in HaCaT cells. As seen from **Figure 4.6a, b**, a significant increase of the intracellular ROS was observed in the cells that were exposed to cytotoxic doses (i.e. 30, 40 and 50 $\mu\text{g/ml}$). 800 μM tert-Butyl hydroperoxide (TBHP) was used concurrently in the experiment to demonstrate that the fluorescence indeed resulted from ROS. Also, it was noted that the increase of ROS level in HaCaTs through exposure to Nano-ZnO was dose-dependent. Furthermore, using 1mM ascorbic acid 2-phosphate (Asc-2P), an established antioxidant, could significantly reduce around 20-40% loss of cell viability that was induced by 30-50 $\mu\text{g/ml}$ of Nano-ZnO

(Figure 4.6c). This suggests that the Nano-ZnO induced apoptosis in HaCaT cells was primarily due to elevation of intracellular oxidative stress.

Figure 4.6. a) Representative fluorescence images of intracellular ROS expression in cells treated with different concentrations of Nano-ZnO. Tert-butyl hydroperoxide (TBHP) serves as a positive control to induce ROS production in the cells. Cells were counter stained for ROS (orange) and nucleus (blue). b) Measurement of ROS level in the cells. c) Cell viability data of the Nano-ZnO challenged cells, with or without Asc-2P pretreatment. Biological replicates = 3. Data in b and c are presented as mean $\pm$ SD. Asterisk * denotes significant difference between the tested group and the control. $p < 0.05$. Scale bar = 100μm.

4.2.4 Low dose of Nano-ZnO induces ROS flares in a subpopulation of HaCaTs

Aside from being an established hallmark of cell death, oxidative stress also regulates a wide range of signal transduction in cells.⁸⁻¹¹ Therefore, it was hypothesized that oxidative stress may be induced by a low dose of Nano-ZnO. A time-lapse examination (1-3h) of ROS expression in the cells that were treated with 0.5 $\mu\text{g/ml}$ was conducted. Interestingly, compared to the ROS staining pattern of the cells that were exposed to high concentrations of Nano-ZnO, samples treated with the conditioning dose (0.5 $\mu\text{g/ml}$) of Nano-ZnO displayed a non-uniform distribution of cells with enhanced ROS level (**Figure 4.7a**). Specifically, ~21% of the cells with higher than baseline level of ROS could be detected

Figure 4.7. Nano-ZnO (0.5 $\mu\text{g/ml}$) induced oxidative stress cellular adaption via non-uniform ROS generation. a) ROS expression profile of Nano-ZnO treated and untreated control. $n=100$ for each experimental group. Cells were counter stained for ROS (orange) and nucleus (blue). b) representative images showing the ROS flares observed from the cells treated with a low dose of nano-ZnO. Scale bar=100 μm

in comparison to the untreated control (**Figure 4.7b**). Of which, it was noted that there was a bimodal distribution of the ROS positive cells, with ~2% of these cells expressing high level of ROS (>40 RFU). (**Figure 4.7b**) Under the microscope, these clusters of cells appeared as a series of ROS hotspots that could be detected sporadically within the cell population as early as 1 h after Nano-ZnO conditioning (**Figure 4.8a**). By 2 h post Nano-

Figure 4.8 Low dose Nano-ZnO treatment induces non-uniform transient elevated ROS level in HaCaTs keratinocytes. (a) Representative fluorescence images of the ROS expression in HaCaTs after Nano-ZnO (0.5 $\mu\text{g/ml}$) exposure at various time points. Cells were counter stained for ROS (orange) and nucleus (blue). (b) Number of hotspots measured from three independent experiments. Hotspots were defined as cells with ROS intensity above 7. Scale bar = 200 μm . The inset shows a magnified view of the ROS ‘hotspot’. Scale bar = 100 μm .

ZnO treatment, a significant decrease was observed in the occurrence of these ROS hotspots and beyond this time point, the difference between the ROS staining of the conditioned cells and the unconditioned controls were negligible (**Figure 4.8b**). Coupled with the limited amount of Nano-ZnO used for the conditioning experiment and the confined solubility that Nano-ZnO possessed in the cell culture media, it is likely that the cells with “ROS flares” were the ones that had internalised the Nano-ZnO. To differentiate Nano-ZnO loaded keratinocytes and the others, the former is herein coined Nano-ZLK while the latter is denoted bystander cells.

Figure 4.9. Low dose Nano-ZnO (0.5 $\mu\text{g/ml}$) treatment upregulates expression level and accumulation of Nrf-2 in the cell nucleus. (a) HaCaT cells were counter stained for Nrf-2 (green), nucleus (blue) and F-actin (red). (b) Nrf-2 expression after 4h of Nano-ZnO treatment. The average Nrf2 expression level is denoted by the dashed line. $n=50$ for each experimental group. Scale bar = 50 μm . Asterisk * denotes statistical significance. $p<0.05$.

4.2.5 Oxidative stress triggers a global upregulation of Nrf-2 in HaCaTs via paracrine

As Nrf-2 is a master regulator of redox homeostasis that acts downstream of ROS and provides cells with cytoprotection¹², it was presumed that Nrf-2 could be activated by the inhomogeneous enhancement of ROS and account for the adaptive responses that is shown in **Figure 4.3a**. Indeed, after 4 h of exposure to the conditioning dose (0.5 $\mu\text{g/ml}$) of Nano-ZnO, there was a significant increase in the expression of Nrf-2, indicating that the Nrf-2 stress response pathway was activated (**Figure 4.9a**). Specifically, ~ 2-fold overall increase in the mean Nrf-2 expression level was detected in the Nano-ZnO conditioned cells as compared to the unconditioned control (**Figure 4.9b**). This overall-population increase in the Nrf-2 activation despite the limited Nano-ZnO, suggests that the effects of the ROS flares may be amplified via a paracrine dependent manner. To validate the aforementioned postulation, a modified transwell co-culture experimental model was next employed to delineate the signal transmission dynamics between the Nano-ZnO and the bystander cells. (**Figure 4.10a**) A confluent layer of HaCaT cells was first established in the upper chamber of the transwell insert, which was spatially separated from another layer of HaCaT keratinocytes in the bottom chamber. Thereafter, Nano-ZnO (0.5 $\mu\text{g/ml}$) was directly added to the cells in the upper chamber. Interestingly, compared to the untreated control, there was an increase in the Nrf-2 expression level of the cells (bottom layer) that were cultured in close proximity to the Nano-ZnO (0.5 $\mu\text{g/ml}$) conditioned confluent HaCaT cells (upper layer). (**Figure 4.10b, c**) A continuous E-cadherin staining along the cell boundaries suggests that the barrier integrity was intact and thus, para-cellular transport of Nano-ZnO across the upper skin epithelium was deemed to be negligible (**Figure 4.10d**). Therefore, these results suggest that low dose Nano-ZnO treatment was sufficient to activate the Nrf-2 signalling pathway via direct and indirect means.

Exactly how a subpopulation of cells with ROS Flares led to a population-wide Nrf-2 activation is still unclear. One possibility is that such phenomenon was realised by both direct stress-induced cellular response and stress-mediated paracrine signalling transduced from the Nano-ZnO to the bystander cells. Apart from direct induction by oxidative stress, Nrf-2 can be alternatively activated in the inflammatory cytokines-receiving cells through

Figure 4.10. Indirect activation of Nrf-2 stress response pathway by Nano-ZnO. a) Schematic of the co-culture set up employed in this study. The "targeted" cells (upper chamber) is designed to come in direct contact with the Nano-ZnO, whereas accessibility of the Nano-ZnO to the "bystander" cells (lower chamber) is restricted. b) Dot plot showing the mean Nrf-2 expression level of the HaCaTs under different experimental conditions. $n=50$ for each experimental group. c) Representative immunofluorescence images of the "bystander" cells counter-stained for Nrf-2 (green), nucleus (blue) and F-actin (red). Samples were retrieved 4 h after the "targeted" cells were exposed to the conditioning dose of Nano-ZnO (0.5 μ g/ml). d) Representative immunofluorescence images of the "targeted" cells counter-stained for E-cadherin (green) and nucleus (blue). Data are presented as mean $\pm$ SD. Scale bar = 50 μ m for all the images. Asterisk * denotes significant difference compared to control. $p < 0.01$.

either ROS-dependent or -independent pathway.¹³⁻¹⁴ According to the hierarchical oxidative stress model, excessively high ROS (Tier 2-3) could prompt cells to secrete

inflammatory signals to the extracellular environment which could further trigger inflammation to the neighbouring cells.¹⁵ These proinflammatory signals could thereafter rapidly activate Nrf-2 translocation via either generation of ROS or RAC-NF- κ B crosstalk. Therefore, it was hypothesized that the global-scale Nrf-2 activation was achieved by a battery of inflammatory cytokines that were secreted by the Nano-ZLK and that effected in the bystander cells for activating Nrf-2 hereinto. To validate this assumption, real time PCR was used to examine several inflammatory genes that have been reported elsewhere to be activated by oxidative stress in HaCaT cells.¹⁶⁻¹⁹ Amongst the twelve genes tested, we noted that there was a significant overexpression in IL -1 α , -6, -8, -15, -23, -33, and Tumour necrosis factor-alpha (TGF- α). (**Figure 4.11**) Of which, the upregulation of IL-6 and -8 were the most noticeable. This corresponds to previous work, as IL-6 and -8 were constantly elevated in an abundance of in-vitro/ vivo skin inflammation studies relative to human keratinocytes.²⁰ Given their role in promotion of inflammation in HaCaT cells, it is reasonable to presume that these inflammatory cytokines, secreted by Nano-ZLK cells, were then internalised by the bystander cells in which inflammation occurs, leading to Nrf-2 activation. Therefore, this finding further lends support to the presumption that Nano-

Figure 4.11. Expression of cytokines and growth factors transcripts by Nano-ZnO treated HaCaTs cells. Data are presented as mean $\pm$ SD. n=3. Asterisk * denotes statistical significance. $p < 0.05$.

ZnO triggered population-wide adaptive response through inflammatory cytokines-regulated paracrine signalling.

4.2.6 A sustained increase of proteasome rather than antioxidants accounts for Nano-ZnO induced cellular adaption.

Thus far, the mechanism by which Nrf-2 functioned to contribute to the increased cells defence capacity against noxious level of Nano-ZnO remains unclear. Nrf-2 is known to regulate a variety of antioxidants (e.g. Glutathione, NADPH) which are responsible for redox homeostasis.¹⁹ Therefore, real-time PCR was first employed to test out the expression of Nrf-2 regulated antioxidant genes. As expected, several representative downstream targets of Nrf-2 such as CAT, γ -GCS, NQO1, GSTM-3, SOD, and UGT were upregulated at the transcript level 6 h post Nano-ZnO (0.5 μ g/ml) conditioning (**Figure 4.12**). Consistently, assessment of the total antioxidant capacity (TAC) in cells identified a significant increase of TAC at the early time points (4, 6h) of Nano-ZnO treatment. These

Figure 4.12. Quantitative RT-PCR analysis showing mRNA levels of Nrf-2 dependent anti-oxidant genes in HaCaTs keratinocytes after 6 h of Nano-ZnO (0.5 μ g/ml) exposure. Expression for each of the target genes were normalized to the untreated control (dashed line). Asterisk * denotes significant difference: $p < 0.05$.

suggest that antioxidants were indeed upregulated by the activation of Nrf-2 to re-establish the dysregulated homeostasis caused by Nano-ZnO exposure. However, it was also noted that such increase of antioxidants did not sustain till the end of the conditioning (48h) (**Figure 4.13a**). This implies that the upregulation of antioxidants by Nrf-2 was not the main cause to enhance the resistance of HaCaTs against toxic levels of Nano-ZnO.

Recent reports have successfully identified the regulation role of Nrf-2 in proteolytic activity.²²⁻²³ As proteasome is the “recycle centre” in a cell to efficiently degrade damaged or misfolded proteins, which helps maintain the normal function of the proteins in cells, increase of proteasome by Nrf-2 may account for the cellular adaption induced by Nano-ZnO. SUC-LLVY-AMC, a fluorescent substrate that specifically targets the chymotrypsin-like sites of 20S proteasome, was used to measure the proteolytic activity of HaCaTs post 0.5 µg/ml Nano-ZnO conditioning. Compared to the unconditioned control cells, the proteolytic activity was increased by ~ 20% for the C₁ and C₂ group, and further up to 50% for the C₃ group, suggesting upregulated proteolytic activity was involved in the Nano-ZnO induced cellular adaption (**Figure 4.13b**).

4.2.7 Sustained upregulation of proteolytic activity is required to induce cellular adaption

Since sustained increase of proteolytic activity was detected when HaCaTs were conditioned by a low dose of Nano-ZnO, it was further asked whether such increase was required for the occurrence of cellular adaption. To answer this question, Mg132, a potent inhibitor of proteasome, was pretreated to the cells prior to the Nano-ZnO conditioning. A repeated dose-response profile experiment showed that 1µM Mg132 was sufficient to abolish the enhancement in tolerance to the toxic level of Nano-ZnO in the conditioned cells (**Figure 4.13c**). In other words, the increased overall capacity to degrade oxidized/damaged proteins via proteolysis, is an indispensable aspect of the Nano-ZnO induced adaptive response in HaCaT cells.

4.2.8 Nano-ZnO induces cross-adaption in to Ultraviolet-A in HaCaT cells

As Nano-ZnO is broadly applied in sunscreen products for skin protection against Ultraviolet (UV), it would be practically important to know if Nano-ZnO induced cellular adaption could aid the cells to better resist to the oxidative damage caused by UV. Among

Figure 4.13. a) Total antioxidant capacity of human keratinocytes exposed to low dose of Nano-ZnO particles for various durations. b) Overall proteolytic activity of C₀-C₃ human keratinocytes. c) Cell viability of C₀-C₃ human keratinocytes with/without pretreatment of 1 μ M Mg132. d) Cell viability of C₀-C₃ human keratinocytes after exposure to 8W UVA lamp for 1 h. Cells without both conditioning and UV exposure will serve as a control which represents 100% viability. Data are presented in mean $\pm$ SD. N=3. $p < 0.05$. *: Significant change compared to the untreated counterpart unless otherwise specified in the image. Dotted line in part c denotes cell viability of C₀ cells.

the different types of UV, UV-A should be a preferable candidate to study as it accounts for over 90% of the UV that could reach human skins.²⁴ To verify the “cross-adaption” postulation, C₀-C₃ cells were further exposed to 8W UVA rays for 1h followed by cell viability measurement. After 1 h of UVA exposure, a steep decline (~ 38%) in cell viability could be detected for the unconditioned group (**Figure 4.13d**). In contrast, it was found that conditioning the cells with Nano-ZnO prior to UVA exposure could effectively suppress the irradiation-induced cell death. Specifically, it was noted that there was a ~ 7% increase of survival seen in C₁ group as compared to the C₀ and the cells’ revival continually improved as prolonging the conditioning, evidenced by the 15% and 20% increased viability seen in C₂ and C₃ group, respectively. This further lends support to the cross-adaption hypothesis. As UVA is known to induce cell death by dissociating the double strand of DNA and DNA repair requires proteasome to locate the damaged sites,²⁵ the enhanced viability of the Nano-ZnO conditioned cells in this case may be due to the reduction of DNA damage. UVA exposure experiment was repeated, and the cells post UV exposure were immunostained by γ -H2AX antibody, an established marker for DNA double strand breaks (DSBs). As expected, a moderate reduction of γ -H2AX could be detected in C₁ group (~ 20%) and it became more obvious in C₂-C₃ groups, in which γ -H2AX expression reduced to ½ of the C₀ group (**Figure 4.14**). Taken together, the sustained increase of intracellular pool of proteasome by a low dose of Nano-ZnO allowed the cells to further mitigate the oxidative damage of UVA rays via the reduction of DNA damage.

4.3 Conclusion

In summary, this chapter mainly elaborates the experimental results that were found in the study and the experimental procedures that were employed. Results suggested a low dose of irregularly-sized ZnO nanorods could markedly enhance the overall resistance of human keratinocytes (HaCaTs) to both toxic levels of Nano-ZnO and ultraviolet-A damage via the propagation of pro-survival signals (Interleukins) transduced from Nano-ZnO to Bystander cells. The signal transduction could subsequently lead to the global-wide activation of Nrf-2 in the cells. Further mechanistic study suggested that proteasome, instead of antioxidants,

acted downstream of Nrf-2 and provided an intense pro-survival signal to the cells primarily via enhanced clearance of damaged proteins in the cytoplasm and accelerated

Figure 4.14. Nano-ZnO conditioning alleviate UVA induced DNA damage. a) Representative immunofluorescence images of cells subjected to different experimental conditions counter-stained for cell nucleus (blue) and phosphor- γ -H2AX (red). Scale bar = 50 μ m. b) γ -H2AX expression in the cells relative to the fluorescence intensity measured from the images shown in a. N=50 and mean value in each group is indicated by dotted line in black. $p < 0.05$.

repair rate of DSBs in the nucleus. A detailed discussion will be given in the following chapter.

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Chapter 5

Discussion and Future Work

In this study, it was found that chronic exposure to a low dose of Nano-ZnO could significantly enhance the overall fitness of human keratinocytes and these conditioned cells could further mitigate the toxicity induced by UV-A rays. Key effectors that mediate the cellular adaptive response were found including ROS, Nrf-2, interleukins, and 20S proteasome. This chapter will offer a detailed discussion that explains the findings displayed in the previous chapter on the basis of literatures. Besides, some reconnaissance work that are necessary yet not included in the main result will be presented here, such as batch-to-batch variation examination regarding the cytotoxicity of Nano-ZnO solution, “fast response” hypothesis verification, and optimization of the core parameters of the UV rays employed in the “cross-adaption” study. Lastly, a brief conclusion to summarize the findings and the significance for this study will be given together with several outstanding questions which would serve as a guidance for the potential future relevant research.

5.1 General Discussion

Zinc oxide nanoparticles (Nano-ZnO) are comprehensively applied into skincare industry due to its excellent efficacy in prevention of ultraviolet (UV) rays.¹ A myriad of previous work has successfully revealed the behind principle for the UV blockage, i.e. physical scattering and absorption.²⁻³ Nonetheless, due to its relatively high potency to induce cytotoxicity, Nano-ZnO has raised considerable concern with regard to its potential harm towards human when applied to skins.⁴⁻⁵ Extensive investigations have been conducted and as of now the “safety issue” of Nano-ZnO has been well addressed. Although the topical application of Nano-ZnO has been demonstrated sublethal,⁶ it remains unclear what are the biological outcomes that would result from the nanoparticles within the sublethal range in a long run. In order to address this fundamental question, this study sought to emulate the repeated application of Nano-ZnO containing sunscreen onto skin in vitro for up to 6 days. It was found that low dose of Nano-ZnO (0.5 µg/ml) was able to significantly improve the overall tolerance of HaCaT cells to the toxic level of the same nanoparticles (**Figure 4.3a**). The cells conditioned by low dose of Nano-ZnO could effectively reduce the ROS-mediated apoptosis triggered by toxic levels of the same nanoparticles. Since Nano-ZnO is known to partially dissolve into Zn²⁺ when it is dispersed in cell culture media, we tested whether its dissolved ions could induce the same effect seen in low dose of Nano-ZnO. It was found that using ZnCl₂ with the same molar concentration (6µM) as Nano-ZnO to condition the cells failed to reproduce the Nano-ZnO induced adaptive response (**Figure 4.5**). This suggests that the adaptive response was achieved via a particle-specific effect, which could be attributed to the different modes of uptake when cells encounter these two substances.

In accordance with the previous findings, it was found that the Nano-ZnO induced cellular adaptive responses were regulated by a mild level of oxidative stress.⁷ Nonetheless, contrary to many other studies in which the overall oxidative stress in the entire population of cells was significantly elevated,⁸⁻⁹ the ROS fluorescence probe revealed that only a subpopulation of cells with enhanced ROS levels could be detected under fluorescence microscope (**Figure 4.7a,b**). These ROS flares suggest that compared to other oxidants

such as paraquat, menadione, and hydrogen peroxide, Nano-ZnO possesses a unique nano-bio interaction when it is internalized by cells. Due to its limited solubility in the cell culture medium, it is likely that those cells with ROS flares (Nano-ZLK) are the ones that take up the majority of Nano-ZnO. As Nrf-2 is a key redox regulator that is downstream of ROS for maintaining the normal function of cells, the intracellular Nrf-2 expression level was then examined. It was found that these Nano-ZLK, surprisingly, activated Nrf-2 in a global-wide manner (**Figure 4.9**). Given the fact that Nrf-2 is primarily activated by oxidative stress, this data strikingly contrasts with the heterogenous distribution of the intracellular ROS in the cells, suggesting that the Nrf-2 activation is realized via direct and indirect means.

In order to further validate the paracrine signalling (indirect means) hypothesis, a transwell culture was used to spatially compartmentalize nanoparticles from the cells. A fully confluent monolayer of cells was grown into the upper chamber, which was validated by E-cadherin immunostaining (**Figure 4.10d**), to prevent the paracellular transport of Nano-ZnO. It was found that even without a direct contact with Nano-ZnO, Nrf-2 in the cells situated in the lower chamber of the device was as well activated (**Figure 4.10b,c**) as the cells which were directly exposed to the nanoparticles (**Figure 4.9**). This further confirms that Nano-ZLK could transduce pro-survival signals to the Bystander cells via paracrine to trigger the global-wide activation of Nrf-2 in HaCaT cells.

One possible driving force of the paracrine signalling is the activation of inflammatory cytokines-related genes in the Nano-ZLK. According to the well-established hierarchical oxidative stress model, oxidative stress that reaches Tier-2 level could result in intracellular inflammation, leading to secretion of multiple inflammatory cytokines towards the extracellular environment, such as interleukins.¹⁰ These cytokines can be later recognized and subsequently uptaken by the neighbouring cells via a receptor-mediated pathway for the pro-inflammatory signalling transduction¹¹, in response to which the neighbouring cells would self-activate Nrf-2 by RAC1-NF κ B pathway.¹²⁻¹³ Indeed, it was found that several interleukins (IL -1 α , -6, -8, -15, -23, -33) and TGF- α with a low-grade upregulation were

overexpressed and thus might be accountable for the initiation of the paracrine signalling (**Figure 4.11**).

Pre-treating the cells with Asc-2P managed to suppress Nano-ZnO induced cell death in the high dose range, suggesting that ROS is the main culprit and that the Nrf-2 stress response pathway may be implicated in the adaptive response (**Figure 4.6c**). When Nrf-2 is activated by oxidative stress, it will rapidly dissociate from the Keap-1 protein complex and translocate into nucleus, where it would bind to the ARE domain through interaction with the small Maf proteins.¹⁴⁻¹⁵ Thereafter, antioxidants-related genes will be activated to increase the intracellular pool of antioxidants for the redox re-establishment. Indeed, real-time PCR successfully identified that some downstream targets of Nrf-2, such as CAT, γ -GCS, NQO1, GSTM-3, SOD, and UGT were overexpressed (**Figure 4.12**). Consistent with this result is the ABTS assay data where we observed a significant increase in the total antioxidant capacity (TAC) in the cells at the early timepoints (4, 6h) post Nano-ZnO (0.5 μ g/ml) exposure. (**Figure 4.13a**). This suggest that Nrf2-antioxidants signalling was activated and may serve as an explanation for the gradual disappearance of the ROS hotspots (**Figure 4.8**). However, we did not observe a sustained upregulation of TAC at the end of the conditioning period (48h) (**Figure 4.13a**). It can thus be inferred that the global-wide activation of Nrf-2 induced by low dose of Nano-ZnO may engage a different signalling axis to exert a cyto-protective effect to the HaCaT cells.

Proteasomes play a critical role in maintaining cellular function due to its capability in degrading damaged proteins. Recent studies have identified that proteasomes are partially regulated by Nrf-2.¹⁶ Binding of the Nrf-2 with the ARE domain can regulate expression of the proteasome β 5 subunit gene for proteasome biosynthesis.⁷⁻⁹ In this study, it was shown that in contrast with the unsustainable improvement of TAC, a significant increase of 20S proteasome could be detected in the cells post Nano-ZnO conditioning, suggesting that proteasome may perform as a critical mediator downstream of Nrf-2 to induce the cellular adaptive response (**Figure 4.13b**). Furthermore, inhibition of proteasome by Mg132 could abolish the adaptive response that is induced by Nano-ZnO (**Figure 4.13c**), suggesting that the contribution of the proteasome to the Nano-ZnO adaptive response is

indispensable. Yet, such moderate extent of improvement also complies with the absence of adaptive response in the condition of 50 $\mu\text{g/ml}$ (**Figure 4.3a**), highlighting the inherent limitation of such cellular adaptability.

Proteasome-regulated ubiquitination is essential for cells to repair damaged DNA. Cells are able to self-repair DNA damage primarily via homologous recombination. And this process requires proteasome to assist BRCA2 and Rad51 to locate the damaged sites.¹⁷ It is known that UV could serve as a potent inducer of DNA double strand breaks (DSBs).¹⁸ Therefore, we postulated that Nano-ZnO conditioned HaCaT cells could become more resistant to the UV-induced damage. Results showed that exposure to 8W UVA rays for 1h, viability of the conditioned cells was progressively elevated (7-20% from C₁ to C₃) as the round of conditioning increased (**Figure 4.13d**). DNA damage assay using $\gamma\text{-H2AX}$ antibody further confirmed the contribution of 20S proteasome to the DNA repair function. Specifically, ~ 20% decline of DSBs could be detected in C₁ group and it further descended to ~ 50% in C₂ and C₃ groups. These results collectively demonstrate that Nano-ZnO conditioned cells could resist to UVA damage via reduction of the DSBs (**Figure 4.14**). Taken together, the findings in this study indicates that the adaptive response of HaCaT cells might not be limited to the damage that results from Nano-ZnO, but to a more general sense of oxidative stress-regulated toxicity. All these Nano-ZnO induced adaptive responses are achieved via enhancement of the intracellular concentration of proteasome that is mediated by the ROS-Nrf2 signalling pathway.

Titanium dioxide nanoparticles (TiO_2) are also widely used in the skincare product manufacturing. Like Nano-ZnO, it is able to generate oxidative stress once internalised by cells and could further cause apoptosis if the concentration is too high to be withstood by the cells.¹⁹ Previous report by Shukia et al. has shown that TiO_2 induced oxidative stress-mediated DNA adduct formation and apoptosis in human keratinocytes.²⁰ Hence, it is possible that TiO_2 in a sublethal range could also induce adaptive response in HaCaTs, albeit the dose range may differ from that of Nano-ZnO given their discrepant toxicity profile. Further study is required to examine whether low dose of TiO_2 is able to generate cellular adaption.

5.2 Reconnaissance work not included in main chapters

5.2.1 Verification of the batch-to-batch variance of Nano-ZnO stock dispersion regarding the cytotoxicity profile of HaCaT cells.

The batch-to-batch variation of the stock Nano-ZnO dispersion to induce the adaptive response on HaCaTs was evaluated. As seen from **Figure 5.1**, the cytotoxicity of toxic levels of Nano-ZnO (i.e. 30-50 $\mu\text{g/ml}$) to HaCaTs is virtually identical in the two batches, suggesting that there is minimal variation for different batches of the Nano-ZnO stock

Figure 5.1. Batch-to-batch variance of Nano-ZnO stock dispersion regarding their cytotoxicity and effects on cellular adaption. a) and b) stands for batch 1 and batch 2, respectively. $p < 0.05$.

dispersions to induce this phenomenon. More importantly, similar to batch 1, the second batch of the stock dispersion was also capable of inducing cellular adaption in HaCaTs. This further confirms that the improvement in the Nano-ZnO conditioned cells' (C_1) ability to resist the toxic levels of Nano-ZnO in cell viability is highly reproducible.

5.2.2 “Fast response” hypothesis regarding upregulation of TAC.

As mentioned in *Chapter 4*, it was found that whereas TAC was markedly upregulated at the early timepoints, it gradually declined and became no different from the untreated control (**Figure 4.13a**). it was then hypothesized that some unknown mechanisms that

might assist the cells to accelerate the antioxidant production process when challenged by toxic doses of Nano-ZnO. It is possible in that zinc ions, which could result from dissolution of internalized Nano-ZnO in the lysosomes, is known to be essential for many functionally-important proteins or enzymes (e.g. Superoxide dismutase) that are involved

Figure 5.2. Verification of “Fast Response” hypothesis. Cells were conditioned in 0.5 µg/ml Nano-ZnO for 48h prior to challenge by 30 µg/ml of the same nanoparticles. Cells cultured in complete medium serve as a negative control while unconditioned cells that were challenged by the same toxic doses of Nano-ZnO serve as positive control. N=3.

in cell metabolism. To verify this hypothesis, cells (conditioned and unconditioned) with 0.5 µg/ml Nano-ZnO for 48h, followed by a challenge by 30 µg/ml of the same nanoparticles for 2h. Thereafter, the TAC of each sample was quantified via ABTS assay. Unfortunately, results did not identify any observable difference in the TAC level in the conditioned cells as compared to the unconditioned cells, suggesting that the “fast response” postulation may not be valid, and that the kinetics of the TAC production may not be responsible for the adaptive response (**Figure 5.2**). This finding further lend support to the hypothesis that there is some other mechanism that is downstream of Nrf-2 which accounts for the Nano-ZnO induced adaptive response in HaCaT cells.

5.2.3 Effects of varying parameters of UV lamp on “cross-adaption” hypothesis verification

When studying the potential cross-adaption effect that is exerted by the low dose of Nano-ZnO on HaCaT cells, factors such as type of the UV applied, power efficiency of the UV lamps, and exposure time are required to carefully consider. The optimal condition is ought to find out before the start of the study. As mentioned, UVA was chosen as the candidate

Figure 5.3. Cell viability of human keratinocytes exposed to 36W UVA in various conditions. a) Representative optical images indicate the status of the cells under various conditions. b) Cell viability data corresponds to the images shown in part a. Data are presented as Mean $\pm$ SD. N=3.

to study is because it accounts for the majority of the UV that could reach human skin. The power efficiency of a UV lamp shall be taken into consideration in that generation of UV rays from the lamp is in fact an exothermic process. Preliminary result shows that using 36W UV lamp for 1h exposure could significantly reduce cell viability to 55% and further down to 25% for 2h exposure (**Figure 5.3a, b**). However, the loss of cell viability is not due to oxidative damage, as evidenced by an obvious “cell burst” detected at 2h of the UV exposure (**Figure 5.3a**, indicated by blue arrows), which is a typical characteristic of necrosis. In addition, exposure time also needs to be carefully fine-tuned as it has been shown that the Nano-ZnO induced enhanced resistance in HaCaT cells to the toxic doses of the same nanoparticles was moderate (**Figure 4.3a**). Indeed, it was noted that even using 8W UV lamp, 2h of exposure could generate so much damage to the cells (~ 40% cell death) that any benefits in the conditioned cells that were observed in the case of Nano-ZnO challenge would be negated or not observable (Data not shown). Therefore, it was decided to use 8W UV lamps to produce UV-A rays and expose them to the cells for shorted time points (e.g. 1h).

5.3 Conclusion and Outstanding Questions

In summary, this study showed that heterogeneity is a pervasive feature of nano-bio interaction in the low dose/ non-toxic level range. Functional dynamics and direct dialogue via a complex interplay between the ROS flares production and the paracrine signalling from the Nano-ZnOs to the “bystander” HaCaT cells drives the collective spatiotemporal adaptive response of HaCaT to sublethal level of Nano-ZnO. The generation of localized Nano-ZnO induced ROS flares is sufficient to activate the population-wide of the Nrf-2 stress response to impart the cyto-protective effects against toxic level of oxidative stress. This is achieved via upregulation of the proteolytic capacity of the cells to facilitate efficient removal of oxidized/ damaged proteins. (**Figure 5.4**) Furthermore, it was revealed that Nano-ZnO conditioning can increase the range of stress signal processing (cross-adaption) to mitigate UVA induced DNA damage in human keratinocytes. This further lends support to the fundamental role of Nrf-2 in the maintenance of redox homeostasis for protecting cells against a wide range of oxidative stress.

Along with the major findings made in this study, are questions that have not been addressed and warrant future studies. Firstly, whether the cellular adaption induced by low dose of Nano-ZnO is temporary or permanent is still unclear. Secondly, it is still unknown whether physiochemical properties of Nano-ZnO such as surface modification, shape, and

Figure 5.4. Schematic to illustrate the complex interplay between the Nano-ZnOs and the ‘bystander cells’ to induce adaptive response to Nano-ZnO induced sub-lethal oxidative stress. In the sub-lethal dose regime where Nano-ZnO is limiting, the internalization of Nano-ZnO is expected to be non-uniform and only restricted to a small population of the cells (i.e. Nano-ZnOs). A unique characteristic of the Nano-ZnOs is the enhanced level of intracellular ROS (ROS flares) due to the dissolution of Nano-ZnO in the lysosome. In response to the augmented oxidative status, the cells will activate the Nrf-2 stress response pathway to restore oxidative homeostasis and to secrete pro-inflammatory interleukins to influence the “bystander cells”. The net effect is the population-wide upregulation of 20s mediated proteolytic capacity of the cells to counteract toxic level of oxidative stress in a Nrf-2 dependent manner.

size would affect the outcome of the nano-bio interactions of Nano-ZnO with HaCaT cells. Along the same vein, the ability to establish the structure-activity-relationship will provide us with a clearer “better by design” approach to tap onto this unique adaptive response inducing capability of Nano-ZnO for skin repair. Implicit in this notion, is the need to attain a controlled spatiotemporal delivery of the Nano-ZnO to the viable layer of the epidermis. Lastly, since TiO₂ nanoparticles is also commonly employed in sunscreen, it remains to be seen whether TiO₂ nanoparticles could similarly induce an adaptive response in human keratinocytes.

Collectively, the critical findings featured in this study have unravelled the novel concept of Nano-ZLK as a nano-bio hybrid for the application in skin protection. Furthermore, the findings are posited to bridge a pivotal knowledge gap in the field of nano-bio interaction studies and provide a mechanistic framework for future examination of ENMs induced adaptive response in the sub-lethal range.

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