Investigating the Immune and Cytotoxic Responses of Mast and Lung Epithelial Cells to Engineered Nanoparticles

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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DECLARATION

I hereby declare that except where due acknowledgement has been made, the work is that of my own; and the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Abdulkareem Elbaz

27th July, 2012
In the name of Allah the most gracious the most merciful

To my father and mother

To my father and mother in law

To my life-mate who shared with me the good and bad times during my study, my wife

To my son who entered my life in 26-04-2011, Abdullah

To my brothers and sisters,

I dedicate this work
PUBLICATIONS

Papers in Conference Proceedings


Papers in Scientific Journals


ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisors:

- **A/Prof Andreas Lopata** for his continued assistance and great guidance throughout the entire period of my study.

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ABSTRACT

The applications for engineered nanoparticles have increased dramatically in recent years. They have been introduced into the industrial, electrical, agricultural, pharmaceutical and medical fields due to their unique properties. To date, zinc oxide (ZnO) and titanium dioxide (TiO$_2$) nanoparticles (NPs) have been widely used in a number of commercial products such as cosmetics and sunscreens, due to their broad UV absorption characteristics, which protect the skin from sunburn and skin cancer. In the biomedical domain, biodegradable chitosan NPs have been assembled to carry medical compounds and genes as a vaccine delivery vector. However, currently there is limited understanding of how these nanomaterials interact with the cellular immune system, and what their potential impacts on human health might be.

The aim of this study was to investigate the possible health risks associated with the application of inorganic ZnO and TiO$_2$ NPs, as well as their in vitro cytotoxicity, inflammatory and allergic immune responses using mast cells and lung epithelial cells. This study also investigated the role of the various structural properties (particle size, wall thickness and porosity) of organic chitosan nanocapsules in drug delivery.

The in vitro assessment of the cytotoxicity and immunomodulation was carried out using two formulations of ZnO NPs including; surfactant-treated (well dispersed) and untreated (aggregated), ranging from 30 to 200 nm in size. Various inorganic metal oxide NPs were examined; ZnO and TiO$_2$ NPs and, various cell types were
studied including immune and epithelial cells, adherent and non-adherent cells, and cancer and non-cancerous cells.

The ZnO and TiO$_2$ NP-induced cytotoxicity to mast cells (P815, RBL-2H3, BMMCs) and lung epithelial cells (LA4, A549) was dependent on cell type and particle size, dispersion, composition, concentration, time and growth media. Small ZnO NPs (30 nm) caused more cytotoxicity than bulk ZnO (200 nm) particles. ZnO NPs displayed cytotoxic effects on all cancer cell types (P815, RBL-2H3 and A549), but did not induce cytotoxicity in primary BMMC cells. Surfactant-treated small ZnO NPs demonstrated strong cytotoxicity in all cell types (cancerous and primary cells). Surfactant-treated ZnO NPs showed greater toxicity to RBL-2H3 cells than their untreated NP counterparts, but had equitoxic effects to P815 and A549 cells. LA4 cells generally were more susceptible to ZnO NPs than other cell types. P815 mast cells grown in RPMI-1640 media were more sensitive to ZnO NPs than those grown in DMEM media. The solubility of ZnO NPs in cell culture was very low and soluble extra-cellular zinc alone was not sufficient to induce cytotoxicity. Whereas, the intracellular concentration of Zn ions was very high after exposure of the cells to ZnO NPs. Therefore, the cytotoxicity of ZnO NPs may be due to direct contact of the NPs with cells, leading to dissolution intracellularly, and then causing cell damage via induction of oxidative stress. Investigation of cell death pathways indicated that ZnO NPs caused cell death through apoptosis and necrosis. Overall, TiO$_2$ NPs exhibited relatively low cytotoxicity in all tested cell types.

The immune effects of ZnO and TiO$_2$ NPs were evaluated via cytokine profiles and mast cell degranulation markers. ZnO NPs caused more inhibition of IL-4 cytokine release than bulk ZnO in PMA and ionomycin-stimulated mouse P815 mast cells.
The rat RBL-2H3 and BMMC mast cells were sensitized with TIB-142 IgE mAb in the presence or absence of ZnO, TiO$_2$ NPs and zinc chloride (ZnCl$_2$) and then challenged with TNP-BSA hapten to stimulate mast degranulation. The release of degranulation products, β-hexosaminidase and histamine, was markedly inhibited by ZnO NPs, but only very weakly inhibited by bulk ZnO particulates. Interestingly, surfactant-treated ZnO NPs (30 nm) had the highest inhibitory effects, while TiO$_2$ NPs did not demonstrate any inhibition of immune modulators, but instead caused a slight but significant ($p < 0.05$) increase in β-hexosaminidase release from mast cells. However, ZnO NPs increased the release of the proinflammatory cytokine IL-8 from A549 lung epithelial cells.

Pristine organic chitosan nanocapsules were found to be highly biocompatible, showing negligible cytotoxicity. The drug delivery efficiency using chitosan capsules of varying particle size, wall thickness and porosity loaded with curcumin was examined with P815 mouse mastocytoma cells. The size of the nanocapsules played the most important role in dictating their chemotherapeutic efficiency. Furthermore, these chitosan nanocapsules displayed inhibitory effects on IL-4 release by non-stimulated and ionomycin-stimulated P815 mast cells.

This study has provided valuable information on how size and dispersion of NPs can play major roles in cytotoxicity and immune responses. These discoveries will also provide useful information which will contribute to the development of new nanomaterials with lower cytotoxicity, immunogenicity and greater therapeutic potential.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>&gt;</td>
<td>Greater than</td>
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<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>±</td>
<td>Plus or minus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>g force, equivalent to relative centrifugal force</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>kV</td>
<td>Kilovolts</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>m</td>
<td>Meter</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Milimolar</td>
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<tr>
<td>µl</td>
<td>Microliter</td>
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<tr>
<td>µm</td>
<td>Micromolar</td>
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<tr>
<td>µm</td>
<td>Micrometer</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celcius</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>pH</td>
<td>An indicator of the acidity or alkalinity of a solution</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>A549</td>
<td>Human lung adenocarcinoma epithelial cell line</td>
</tr>
<tr>
<td>BMMCs</td>
<td>Mouse bone marrow-derived mast cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscopy</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2′,7′-dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>EC50</td>
<td>Effective concentration for 50% response</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPC-AAS</td>
<td>Inductively-coupled plasma atomic absorption spectroscopy</td>
</tr>
<tr>
<td>LA4</td>
<td>Mouse lung adenoma cell line</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major-histocompatibility complex</td>
</tr>
<tr>
<td>mIgE</td>
<td>Monoclonal antibody immunoglobulin E</td>
</tr>
<tr>
<td>MIN-U-SIL® 5</td>
<td>Crystalline silica particles</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>NaCl₂</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NM</td>
<td>Nanomaterial/s</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle/s</td>
</tr>
<tr>
<td>P815</td>
<td>Mouse mastocytoma cell line</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>RBL-2H3</td>
<td>Rat basophilic leukemia</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SLIGRL−NH₂</td>
<td>Ser-Leu-Ile-Gly-Arg-Leu-amide</td>
</tr>
<tr>
<td>sZnO NPs</td>
<td>Surfactant - treated zinc oxide nanoparticles</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Th T</td>
<td>Helper cells</td>
</tr>
<tr>
<td>Th1</td>
<td>Type 1 helper cells</td>
</tr>
<tr>
<td>Th2</td>
<td>Type 2 helper cells</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNP-BSA</td>
<td>Trinitrophenyl hapten conjugated to Bovine Serum Albumin protein</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>Zinc chloride</td>
</tr>
<tr>
<td>ZnO</td>
<td>Zinc oxide</td>
</tr>
<tr>
<td>ZnO NPs</td>
<td>Pristine zinc oxide nanoparticles</td>
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Chapter 1: Introduction and Literature Review

1.1 Introduction

Modern science enables the manufacture of new materials made of particles of very small size; these particles are less than thousands of a millimetre in diameter and the science of building these tiny particles is called Nanotechnology. These materials not only have industrial uses but also have found their way into commonly used materials, such as sunscreens. The small size and unique properties of nanoparticles (NPs) enables novel applications and improvements in many areas, such as drug delivery, medical contrast agents and chemical catalysis.

Nanomaterials (NMs) in consumer products have been increased dramatically around the world. Zinc oxide (ZnO) and titania (titanium dioxide, TiO$_2$) inorganic NPs are used in sunscreens due to their broad UV absorption characteristics, which protect the skin from sunburn and skin cancer. Biodegradable chitosan organic NPs have been assembled to carry medical compounds and genes as a vaccine delivery. However, there is currently limited understanding of how these NMs interact with immune cellular systems and what the potential impact on human health may be.

This introductory chapter and literature review will provide an overview on the types of NMs and current uses of NPs for human health. It will mainly focus on inorganic
NPs that are commonly applied in sunscreens, such as ZnO and TiO₂ NPs, as well as organic chitosan NPs, as a model for drug delivery.

1.2 The nanotechnology industry

Nanotechnology includes all techniques, materials and devices that deal with the manufacture of particles on a nanometre size scale (Maynard, 2006). It represents one of the biggest engineering technologies of the 21st century, and has been considered to be a new industrial revolution (Nohynek et al., 2008). The global market of engineered NMs is estimated to be more than $1 trillion within the next decade (Maynard et al., 2006). The major products of nanotechnology are NPs, which are considered to be a subset of NMs and are defined as a single particle with a diameter below 100 nm (Nohynek et al., 2008). Recently the International Organization for Standardization (ISO) has established a universal set of definitions related to nanotechnology that include nano-structured materials and nano-objects (i.e. nanoparticle, nanoplate, nanofibre, nanotube, nanorod, nanowire and quantum dot), as illustrated in Figure 1.1. An NP is defined as being a nano-object with all three external dimensions in the nanoscale (i.e. the size range from 1 to 100 nm). Whereas, a particle is known as a small piece of material that is larger than the nanoscale (ISO, 2010).

Nanomaterials can be divided into three categories: Firstly, ultrafine particles that are naturally occurring, such as NPs arising from volcanic ash, ocean spray and mineral components. Secondly, anthropogenic ultrafine particles that have been made by human daily activity, for instance, diesel exhaust particulates (DEP) and
tobacco smoke. Thirdly, engineered NPs which have been designed for specific applications (Chang, 2010).

There are many methods to make engineered NPs – the two common approaches for synthesising engineered NPs are through breaking bulk materials down to NMs by milling, grinding, etching and pyrolysis; or through integrating atomic substances together until they are large enough to form NPs.

Engineered NPs can be classified according to their composition into: organic NPs (polymers, polymeric micelles, dendrimers), organic-inorganic hybrids (nanocomposites), carbon-rich NPs (carbon nanotubes (CNTs), fullerene (C₆₀)), liposome NPs, biological NPs (proteins, lipids, carbohydrates) and inorganic NPs including silver (Ag), gold (Au), (Platinum) Pt, iron (Fe), TiO₂, ZnO, calcium carbonate (CaCO₃), quantum dots (QDs) and silica (Teow et al., 2011).

These new engineered NPs have different physicochemical properties from their bulk material counterparts (Hsiao & Huang, 2011), thus it is unknown whether these new unique properties of NPs may be toxic to cells in biological systems and cause health diverse effects. Moreover, it is important to consider that NPs are usually present in aggregated and agglomerated forms when suspended in liquids. Aggregation occurs when the NPs combine together through strong bonds, while agglomeration occurs when NPs or aggregates join together with loose bonds. Aggregates and agglomerates contain clusters of NPs larger than single NPs; these clusters sometimes reach up to 1 micron in size (Schulling et al., 2010). A criticism of some published studies on the safety of NPs is that risk assessments have been performed on aggregate forms. As these different NP forms are likely to
have different biological properties, it is important to investigate the cytotoxic and immunological responses to both dispersed and aggregated forms of NPs.

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**Figure 1.1**: Overview of standard definitions and terms in ISO related to nanotechnology (adapted from ISO, 2010 and Frujit-Polloth, 2012).
The use of NMs has increased dramatically in recent years; they have been used in sporting goods, tyres, catalysts, electronic components, window sprays, paints, varnishes, coatings, foods, sunscreens, cosmetics and antimicrobial agents. Other applications, including vaccine trials, drug delivery and medical imaging, are still under development. The increased use of NMs may pose potential new risks to human health and the environment (Nohynek et al., 2007).

1.3 Nanoparticles in sunscreens and cosmetics

Australia has the highest rate of skin cancer in the world, and application of sunscreens is recommended to protect the skin from ultraviolet light (UV light), which damages the skin and causes skin cancer (Haywood et al., 2003). More than twelve hundred sunscreens are authorised to be used in Australia by the Australian Therapeutic Goods Administration (TGA). Approximately half of these sunscreens contain ZnO NPs or TiO$_2$ NPs, or both. These metal oxide NPs are used in sunscreens because of their ability to filter a broad spectrum of UV light (particularly UVA) from sunlight, giving better protection, particularly at the longer wavelengths, than other organic sunscreen materials (TGA, 2006).

Traditional sunscreens contained large (bulk) ZnO and TiO$_2$ particles, which did not spread well, leaving a white film on the skin, and so they were not considered to be cosmetically attractive. Recently, various sunscreen formulations containing ZnO and TiO$_2$ NPs have been made commercially available; these materials are clear or transparent when applied on the skin, and are more effective at filtering UV light from sunlight (Nohynek et al., 2007). This new formulation of sunscreens has solved the problem of the undesirable appearance of traditional sunscreens.
However, the possible toxic and immune (including allergic) consequences of exposure to these NPs are poorly characterised.

Furthermore, recent sunscreen preparations contain surfactant materials that prevent ZnO and TiO$_2$ NPs from aggregating, to ensure that the NPs are dispersed to spread well on the skin and give a more desirable transparent appearance. Therefore, it is recommended to repeat the health risk assessment of such metal oxide UV filters when used in nanosunscreen preparations (Faunce, 2010).

The production of engineered NPs in sunscreen products has spread around the world and has been estimated to be up to 100 kilotonnes during 2003 alone (Borm et al., 2006). Today the rapid expansion of NP production may bring about new risks to human health and the environment. However, there is conflicting data regarding the risks associated with application of these NPs in sunscreens, and whether skin penetration occurs (Nohynek et al., 2008). There is also concern about the possible risk from ingestion or inhalation of NPs during the application of sunscreens, especially in the case of children. There is a need to address whether there are risks from the application of NMs in sunscreens, so that new NMs can be engineered with minimal toxicity and the greatest therapeutic potential.

1.4 Chitosan nanoparticles

During the past few decades, there has been growing interest towards the design of suitable polymer-based drug delivery carriers for controlled delivery of therapeutic molecules to disease sites in the body. The choice of the carrier material plays an important role in the development of these drug delivery vehicles,
especially as the carrier comes into direct contact with the body and its immune system, which plays a major role not only in determining the therapeutic potential of the carrier-drug conjugate system, but also in its clearance from the body post-drug action.

Chitosan [(1,4)-2-amino-2-deoxy-β-D-glucan] is one such preferred carrier system with a broad range of proven biomedical applications due to its low immunogenicity, toxicity, and good mechanical properties (Zeng & Ruckenstein, 1996). Chitosan possesses most of the above properties, due to it being a polysaccharide and not a polypeptide, which are typically immunogenic. Furthermore, the chitosan polymer can be broken down in the body by lysosomes to harmless N-acetyl glucosamine (Gan & Wang, 2007), making it a highly desirable biodegradable material in designing carrier vehicles for drug delivery.

Different approaches have been undertaken in the past to prepare chitosan-based nanoparticulate systems, including chitosan nanospheres and beads, by way of emulsion cross-linking (Al-Helw et al., 1998; Kumbar et al., 2002), spray-drying (Conti et al., 1998; He et al., 1999), coacervation/precipitation (Nishimura et al., 1986), ionic gelation, (Aydin & Akbuğa, 1996; Shu & Zhu, 2000) the emulsion-droplet coalescence method (Tokumitsu et al., 1999), the reverse micellar method (Mitra et al., 2001), and the sieving method (Agnihotri & Aminabhavi, 2004). Although these particles represent very promising drug delivery platforms, most of these methods produce solid chitosan particles, thereby restricting their capacity as a drug loading and carrier system. Moreover, it is extremely difficult to control the monodispersity of the chitosan particles synthesized via these routes, thus limiting their ability to control the drug payload during administration. In contrast, the use of
a template onto which a polymer layer is deposited, followed by subsequent removal of the template matrix, can result in a polymer capsule with a hollow interior. This interior can then be utilized for further drug loading, in addition to the surface, enabling substantially increased drug capacity per carrier (Wang et al., 2007). Therefore, well-controlled synthesis of chitosan nanocapsules via a templating method provides an obvious advantage over previously reported solid chitosan nanospheres for drug-delivery applications.

### 1.5 Exposure to nanoparticles

There are several possible ways by which the body can be exposed to NPs; these include environmental pollution, workplace incidents, during transportation of NMs, and by consumption of products containing NMs. These NMs can interact with the body through common entry routes, including inhalation, ingestion and skin absorption.

#### 1.5.1 Inhalation

It is well established that particulate air pollution is associated with adverse health effects, including respiratory disorders that result in coughing, sneezing and breathing difficulties. Ultrafine particles can cause greater toxicity and respiratory damage than larger particles in test animals (Zhu et al., 2008). In addition, size and shape of NPs are very crucial for particle distribution in the airways, for instance smaller particles were found to enter deeper inside the respiratory tract than larger particles (Oberdorster et al., 2005). Access to the alveoli further facilitates the distribution of NPs, which may be capable of crossing the epithelial layer, to enter the blood circulation where they can deposit in various organs in the body.
(Semmler-Behnke et al., 2008; Furuyama et al., 2008). Thus, very small NPs have a high chance of crossing through and damaging lung epithelial cells, and interacting with mast cells, which could induce respiratory disorders such as asthma and other allergic responses.

1.5.2 Skin penetration
The skin is a very important barrier that protects the body from attack by both biological and non-biological substances. However, NPs may enter through damaged or weak skin. Some studies have reported that ZnO and TiO$_2$ NPs do not pass through the stratum corneum, and that the possibility of skin penetration is very limited or negligible over short periods of time (Gulson et al., 2010; Filipe et al., 2009). Although no specific adverse health risks associated with the use of metal oxide NPs in sunscreens have been demonstrated, it has been suggested that some factors, such as damaged skin and the thinner skin of infants and elderly people, might lead to a greater likelihood of transdermal penetration, especially with chronic exposure over several years (Gulson et al., 2010; Monteiro-Riviere et al., 2009). It has also been reported that TiO$_2$ NPs are capable of penetrating the epidermis and dermis after removing the horny layer (Lademann et al., 1999).

1.5.3 Ingestion
Nanoparticles have been introduced into food and medical applications, such as molecular imaging, site-specific targeted drug delivery, anticancer therapy and gene therapy (Gwinn & Vallyathan, 2006). Nanoparticles can be orally taken up in food additives or drug delivery vectors, and absorbed into the body through the cells of the digestive system, including the specialized epithelial microfold (M) cells.
A recent study showed that 15 nm silver NPs had greater penetration through the intestine than larger 198 nm particles in a rat model (Sonavane et al., 2008). Sunscreens containing NPs such ZnO and TiO$_2$ may be swallowed by children during application on the face or lips (Nohynek et al., 2007).

1.6 Cellular uptake of nanoparticles

It is very important to understand the interaction between engineered NPs and cells. Studying cellular uptake mechanisms are useful for nano-medicines, which are required to ensure that the NPs enter the cells to provide better diagnosis or treatment. For example, chitosan NPs have been successfully used as a model for drug-delivery applications (Oyarzun-Ampuero et al., 2009). Another essential concern is to know the undesirable cytotoxic effects of engineered NPs and their biological fate inside the cells.

There are many methods that have been established to investigate NP internalization inside cells, including flow cytometry and confocal microscopy (Faklaris et al., 2008; Thurn et al., 2010). However, labelling of NPs is necessary in these techniques to visualise the NPs inside the cells. However, the addition of labelling materials to the NPs may cause a major problems through changing their physicochemical properties, including size, shape and surface chemistry, subsequently altering cellular uptake pathways and subcellular localization of the NPs (Al-Rawi et al., 2011; Gupta and Gupta, 2005; Nativo et al., 2008; Win and Feng, 2005). Chitosan nanocapsules used in this study are autofluorescent in nature and did not require additional fluorescent labelling.
Transmission electron microscopy (TEM) is also a good technique to visualise NP internalization (Oberdörster et al., 2005), but it is difficult with this technique to determine the quantity of specific elements due to intact NPs, especially if they contain essential metal elements, such as for ZnO NPs inside the cells. Synchrotron x-ray fluorescence microscopy (SXFM) has recently been used to quantitatively estimate elements inside the cells (Carmona et al., 2008). Thus, it can be applied to investigate metal oxide NP internalization in cells.

Engineered NPs may enter cells by various mechanisms such as macropinocytosis, lipid raft-dependent endocytosis (caveolin-dependent and clathrin-dependent endocytosis), clathrin- and caveolin-independent endocytosis, and phagocytosis, as illustrated in Figure 1.2 (Geiser, 2010; Hu et al., 2009; Dawson et al., 2009; Conner & Schmid, 2003); whereas ions (e.g. Zn$^{2+}$ ions release from ZnO NPs) enter the cells through ion channels in the cell membrane (Canton & Battaglia, 2012).

Phagocytosis occurs mainly in some immune cells, such as the major antigen-presenting cell (APC) types, being the dendritic cells (DCs) and macrophages, as well as neutrophils and mast cells. It is characterised by the engulfing of large foreign antigens (e.g. large particles, bacteria, yeast or large cellular debris) that can reach up to multiple micrometers in size, by the fusion of the cell's membrane around the particles to form phagosomes. Phagosomes then bind to lysosomes and form phagolysosomes, in which the particles are digested (Champion et al., 2008).
Figure 1.2: Nanoparticles may enter the cells through different mechanisms including macropinocytosis, lipid raft-dependent endocytosis (caveolin-dependent, clathrin-dependent), clathrin- and caveolin-independent endocytosis, and phagocytosis (adapted from Conner & Schmid, 2003; Dawson et al., 2009).

Macropinocytosis and pinocytosis occur in all mammalian cells, by which small particles approximately several hundreds of nanometres in size can enter the cells. The pinocytic pathways are directed by the type of cargo molecule and its receptor (Conner & Schmid, 2003).

Both caveolae-mediated endocytosis (Wang et al., 2009; Contreras et al., 2010) and clathrin-mediated endocytosis are essential mechanisms for NP uptake (Zhang et al., 2009). These endocytosis pathways form small vesicles less than 100 nm that fuse with endosomes (Rapoport, 2008). Clathrin- and caveolin-independent endocytosis may play a major role in the uptake of negatively charge NPs (Dausend et al., 2008). For instance, polyethylene glycol-coated gold NPs (with a negatively-charged surface) have been shown to pass through cell membranes via clathrin- and caveolin-independent endocytosis, but did not enter the cells via clathrin- or caveolin-dependent endocytosis (Brandenberger et al., 2010). Very small NPs less than 40 nm can penetrate into cells and enter the nucleus (Dawson et al., 2009), and also translocate between cells (Wang et al., 2009). Therefore,
engineered NPs that are capable of crossing into the nucleus may potentially damage DNA and result in cell death or cause DNA mutation in surviving cells.

Stolle et al. (2009) suggested that TiO$_2$ NPs (39 nm) were engulfed by the cells through endocytosis and that the NPs were localized within lysosomes, the cytoplasm and a few of the mitochondria, which may alter mitochondrial function. Another study by Geiser et al. (2005) reported that TiO$_2$ NPs (41 nm) passed into the nucleus and organelles, which may enhance their toxicity. They also observed that these TiO$_2$ NPs were not taken up by the endocytic pathway because particles within the cells were not bound to membranes. In contrast, some other studies found that TiO$_2$ NPs were up taken by various endocytic pathways such as macropinocytosis, clathrin-mediated endocytosis, or caveolae-mediated endocytosis (Faklaris et al., 2009; Saxena et al., 2008; Singh et al., 2007; Thurn et al., 2010, Belade et al., 2012). Consequently, the exact mechanism of TiO$_2$ NP cell uptake is not completely known (dos Santos et al., 2011).

Cellular uptake mechanisms are also dependent on many factors, including physicochemical properties of NPs, such as size, shape, solubility, crystal structure and electrostatic charge, chemical composition, surface area, surfactant coating, particle size distribution (i.e. agglomeration state, dispersion factor and zeta potential in the system), protein adsorption, time of incubation and differences between cell lines (Belade et al., 2012; Dawson et al., 2009).

A recent study by Belade et al. (2012) examined particle size effects using fluorescent carboxyl-modified polystyrene particles (ranging from 40 nm to 2 µm) on several different cell lines, including human HeLa and A549 epithelial cells,
human 1321N1 astrocytes, human HCMEC D3 endothelial cells, and murine RAW 264.7 macrophages. They reported that NP uptake was highly dependent on their size; the greatest cell uptake occurred with smaller particle sizes in all of these cell lines. However, there are conflicting studies concerning the relationship between the cellular uptake pathways and size of NPs. For example, 500 nm NPs enter the cells via caveolin-dependent endocytosis (Rejman et al., 2004), whereas other studies demonstrated that caveolin-dependent endocytosis is a major uptake pathway for NPs less than 100 nm (Wang et al., 2009; Nishikawa et al., 2009).

Surface modification or surfactant-coating of NPs may alter the major cell uptake pathway from that used for their pristine NP counterparts. For instance, amino-functionalized polystyrene NPs mainly entered the cells via the clathrin-dependent pathway, but pristine polystyrene NPs were up taken through clathrin-independent endocytosis (Jiang et al., 2010). Therefore, surfactant-coated materials may be useful to re-engineer new safer NMs that are not able to enter specific organelles within the cell to cause cell death, such as the mitochondria and nucleus.

Surface charge of NPs also plays another important key role in determining their major cellular uptake pathway. Positively-charged NPs enter cells at a greater rate through clathrin-mediated endocytosis than negatively-charged NPs (Mailander & Landfester, 2009). The enhanced ability of positive-charged NPs to enter cells could be due to their ionic interaction to the negatively charged cell membrane (Dobrovolskaia & McNeil, 2007).
1.7 Cytotoxicity effects of nanoparticles in mammalian cells

Numerous studies have confirmed that NPs can induce cytotoxic effects in a variety of mammalian cells, as illustrated in the following reviews (Landsiedel et al. 2010; Nohynek et al. 2007; Nohynek and Dufour 2008; Schilling et al. 2010; Nohynek et al. 2012). It has also been observed that even NPs originating from nontoxic bulk materials can cause toxic effects in cells and organs (Donaldson et al. 2005).

Toxic dose responses have been shown in several different cell lines, including: human BEAS-2B bronchial epithelial cells (Huang et al., 2010), human A549 lung epithelial cells (Lin et al., 2009; Karlsson et al., 2008; Horie et al., 2009), human HaCaT keratinocyte cells (Horie et al., 2009), human A431 epidermal cells (Sharma et al., 2009), rat L2 lung epithelial cells and rat alveolar macrophages (Sayes et al., 2007, 2009), mouse RAW 264.7 macrophages (Xia et al., 2008), human MSTO-211H mesothelioma cells and rodent 3T3 fibroblast cells (Brunner et al., 2006), primary mouse embryo fibroblasts (PMEF) (Yang et al., 2009), human embryonic lung fibroblasts (HELF) (Yuan et al., 2010), mouse neural stem cells (NSC, C17.2) (Deng et al., 2009), human U87 astrocytoma cells (Lai et al., 2008), human T lymphocytes (Reddy et al., 2007; Hanley et al., 2008), and human aortic endothelial cells (HAEC) (Gojova et al., 2007). However, the cytotoxic mechanisms induced by NPs are still not understood (Nohynek et al. 2007), and generalizations are inappropriate as one needs to also consider the specific physicochemical characteristics of the NPs and the innate toxic potential of the constituent material.
There are some possible factors that have been correlated to the toxicity of NPs such as generation of reactive oxygen species (ROS), release of metal ions, and mechanical injury. Accumulation of NPs inside cells leads to excessive generation of ROS that damage the cell membrane and organelles, and causes cell death. This can happen with numerous NP types, such as TiO$_2$, carbon black, silica, Ag, magnetite, CeO$_2$ and WCo-Co (Xia et al. 2006; Hussain et al. 2009; Eom & Choi, 2009; Kim et al. 2009; Park et al. 2008; Park et al. 2009; Hsin et al. 2008; Ding et al. 2009).

Furthermore, metal oxide NPs such as NiO, ZnO, Fe$_2$O$_3$, Mn$_3$O$_4$, Co$_3$O$_4$ and Al$_2$O$_3$, are able to release metal ions in cell culture media or inside the cells. The dissolved metal ions from metal oxide NPs appear to play a critical role in cell toxicity (Brunner et al., 2006). For example, a recent study by Buerki-Thurnherr et al. (2012) showed that ZnO NP toxicity is mainly due to the release of Zn$^{2+}$ ions. Therefore, measurements of intracellular and extracellular metal ion release are necessary when investigating the cytotoxicity of soluble metal oxide NPs.

Another important issue is penetration and translocation of NPs through vital components of the cells, such as the nucleus, mitochondria and other organelles, which may cause mechanical damage and kill the cells. An in vitro study has shown that CNTs can enter the nucleus of human cells, resulting in cell toxicity in a dose-dependent manner (Porter et al., 2007). Titanium ultrafine particles (<0.1 µm) could also be found in the organelles and nucleus of the cells, which may be the reason for their cytotoxic effects (Geiser et al., 2005).
1.8 Nanoparticles and their impact on the immune system

1.8.1 The immune system

The immune response consists of two interconnected arms: innate immunity and adaptive immunity. Innate immunity is the first-line of defence against an antigen, in which microorganism recognition is mediated through pathogen-associated molecular patterns (PAMPs) on the surface of microorganisms that are recognized by pathogen recognition receptors (PRRs) on phagocytic cells, such as neutrophils, eosinophils, mast cells, macrophages and natural killer cells. This response is associated with a number of mechanisms such as opsonization, complement activation, acute inflammation, host-derived antimicrobial compounds, or phagocytosis (Look et al., 2010).

The adaptive immune response acts as a second-line of defence against pathogens. Unlike innate immunity, it is characterised by powerful antigen-specific memory responses (Kuby, 1992). The APCs, such as macrophage and DCs, process and present the antigen through class II major histocompatibility complex (MHC) molecules to CD4 T helper cells. The T helper cells may activate B cells to produce antibodies (humoral immunity), or in cell-mediated responses, CD4 T helper cells respond to APCs and produce cytokines that activate CD8 cytotoxic T lymphocytes (CTLs), which mediate the killing of infected cells (Romagnani, 1999). Mast cells have features of both innate and adaptive immunity and can also express MHC class II (Sayes et al., 2008), thus mast cells play a role in the processing and presenting of antigen to T cells similar to that of APCs.

The APCs can activate naïve CD4 T cells which are then differentiated into Th1, Th2, Th17 and T regulatory (Treg) cells (Figure 1.3). T cells of all types produce
many cytokines. Th1 cells produce interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) cytokines, while Th2 cells produce IL-4, IL-5, and IL-13 cytokines. Th17 cells are characterized by the production of the proinflammatory cytokine IL-17, while Treg cells are responsible for control of immune tolerance to self-antigens through production of the immunosuppressant cytokine, IL-10 (Look et al., 2010). Measurement of cytokine profiles can be used to monitor changes in immune responses. Table 1.1 shows some important cytokines of the innate and adaptive immune responses.

1.8.1.1 Mast cells
Mast cells are derived from haematopoietic stem cells and circulate in the blood in an immature form; when these cells reach the tissue they differentiate into mature mast cells. Mast cells are very widespread throughout the body, especially at the surface of the skin, as well as throughout the airways and gastrointestinal tract where microorganisms invade the tissue. Mast cells generally are among the first immune cells that are exposed to pathogens, along with DCs and macrophages (Galli et al., 2008).

Mast cells are phenotypically very close to the basophils in blood, and both share many similar features, including the presence of basophilic granules in the cytoplasm, the surface expression of high-affinity IgE receptor FcεRI, and the release of chemical mediators during stimulation, such as histamine (Mukai et al., 2009).
Figure 1.3: T helper cell subsets – naïve CD4 T cells can be activated by APCs; subsequently, these activated CD4 T cells can differentiate into a Th1, Th2, Th17 or T regulatory (Treg) phenotype. Th1 cells can produce IFN-γ and TNF-α cytokines, which are important in defence against intracellular pathogens, while Th2 cells produce IL-4, IL-5, and IL-13 cytokines, which are associated with allergy, extracellular and helminthic infections. Th17 cells produce the proinflammatory cytokine IL-17, whereas Treg cells are responsible in maintaining tolerance for self-antigen. Adapted from Look et al., 2010.
Table 1.1: Examples of cytokines of innate immunity and adaptive immunity (reproduced from Kuby, 1992).

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<th>Secreted by</th>
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<td>Cytokine</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Monocytes, macrophages, endothelial cells, epithelial cells</td>
<td>Vasculature (inflammation); hypothalamus (fever); liver (induction of acute phase proteins)</td>
</tr>
<tr>
<td>Tumor Necrosis Factor α (TNF-α)</td>
<td>Macrophages</td>
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</tr>
<tr>
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<td>Macrophages, dendritic cells</td>
<td>NK cells; influences adaptive immunity (promotes TH1 subset)</td>
</tr>
<tr>
<td>Interleukin 6 (IL-6)</td>
<td>Macrophages, endothelial cells</td>
<td>Liver (induces acute phase proteins); influences adaptive immunity (proliferation and antibody secretion of B cell lineage)</td>
</tr>
<tr>
<td>Interferon α (IFN-α) (This is a family of molecules)</td>
<td>Macrophages</td>
<td>Induces an antiviral state in most nucleated cells; increases MHC class I expression; activates NK cells</td>
</tr>
<tr>
<td>Interferon β (IFN-β)</td>
<td>Fibroblasts</td>
<td>Induces an antiviral state in most nucleated cells; increases MHC class I expression; activates NK cells</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Some cytokines mainly involved in adaptive immunity</th>
<th>Secreted by</th>
<th>Targets and effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin 2 (IL-2)</td>
<td>T cells</td>
<td>T-cell proliferation; can promote activation-induced cell death (AICD); NK cell activation and proliferation; B-cell proliferation</td>
</tr>
<tr>
<td>Interleukin 4 (IL-4)</td>
<td>TH2 cells; mast cells</td>
<td>Promotes TH2 differentiation; isotype switch to IgE</td>
</tr>
<tr>
<td>Interleukin 5 (IL-5)</td>
<td>TH2 cells</td>
<td>Eosinophil activation and generation</td>
</tr>
<tr>
<td>Interleukin 25 (IL-25)</td>
<td>Unknown</td>
<td>Induces secretion of TH2 cytokine profile</td>
</tr>
<tr>
<td>Transforming growth Factor β (TGF-β)</td>
<td>T cells, macrophages, other cell types</td>
<td>Inhibits T-cell proliferation and effector functions; inhibits B-cell proliferation; promotes isotype switch to IgE; inhibits macrophages</td>
</tr>
<tr>
<td>Interferon γ (IFN-γ)</td>
<td>TH1 cells; CD8+ cells; NK cells</td>
<td>Activates macrophages; increases expression of MHC class I and class II molecules; increases antigen presentation</td>
</tr>
</tbody>
</table>
1.8.1.2 Role of mast cells in innate and adaptive immune responses

Mast cells play important roles in innate and adaptive immunity, including the killing of pathogens, especially parasites, and controlling the immune system through regulating immune stimulation (Galli et al., 2010). Mast cells control immune stimulation by three ways. Firstly, by stimulating the migration, maturation, differentiation and function of immune cells, through secretion of factors such as TNF, chemokines, histamine, leukotriene B4 (LTB₄) and proteases. Secondly, by presenting antigen to T cells through MHC class I or II molecules, or activating antigen presentation by capturing IgE-bound-antigen through FcεRI and then dying through apoptosis. Thirdly, mast cells stimulate B cells to produce IgE through IL-4, IL-13 and CD40L (Galli et al., 2008). Moreover, mast cells stimulate expression of thymic stromal lymphopoietin (TSLP) on epithelial cells by the production of cell mediators such as TNF, IL-4 and IL-13, and also increase the recruitment of immune cells by production of TNF and other mediators that up-regulate adhesion molecule expression on vascular endothelial cells. Mast cells are also able to stimulate TH2 responses through action of prostaglandin D₂ on DC maturation. Finally, mast cells produce TNF, IL-4 and IL-13 that stimulate airway smooth muscle production of other inflammatory chemokines and cytokines (Galli et al., 2010; Tsai et al., 2011).

Conversely, mast cells can also induce immune suppression through production of IL-10, which suppresses cytokine production by T cells and monocytes, reduces the production of proinflammatory cytokines and chemokines by keratinocytes, suppresses sensitization for contact hypersensitivity, and enhances the ability of DCs to decrease T cell proliferation and cytokine production (Galli et al., 2008).
The interaction between mast cells and the adaptive immune response is initiated following the stimulation of Th2 cells, via mast cells’ APC function, these cells then secrete IL-4 that in turn activates B lymphocytes to produce IgE and IgG, and these antibodies bind to FcεRI and FcγRI receptors on mast cells, respectively (Figure 1.4). Activation of mast cells occurs when specific antigens bind to antibodies attached to these receptors, leading to mast cell degranulation and the release of cytokines or chemokines and other mediators, such as granulocyte macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein (MCP), macrophage inflammatory protein (MIP), “regulated upon activation, normal T cell expressed, and secreted” (RANTES), histamine and β-hexosaminidase (Tkaczyk et al., 2006). Mast cells are especially recognised for their role in IgE-associated allergic disorders, including hypersensitivity (Galli et al., 2010).

Activation of mast cells can also occur directly through many other signals, including physical and chemical injuries, exposure directly to pathogens, or some signals that are generated during innate or adaptive immune responses (Sharma et al., 2002; Tsai et al., 2011). These signals can activate the intracellular signalling cascade, which leads to the secretion of many mediators that control immune responses. Tumour mast cell lines, such as rat RBL-2H3 basophilic leukemia cells and mouse bone marrow-derived mast cells (BMMCs, primary cells that are cultured in vitro from mice bone marrow), have been widely used as models to study the IgE-mediated responses of mast cells in vitro. Primary mast cells have an advantage over tumour mast cells, by which BMMCs can be engrafted to mast cell-deficient c-kit-mutant mice (mast cell knock-in mice) to study the specific mast cell mediators and their diverse immunological responses in vivo (Kalesnikoff & Galli, 2011). In contrast, the P815 mouse mastocytoma cell line can be activated by
chemical substances, such as the calcium ionophore ionomycin, but cross-linking of high-affinity IgE (FcεRI) receptors by multivalent antigens does not stimulate degranulation in an IgE-sensitized P815 mouse cell line (Ohno et al., 1990).

**Figure 1.4:** The role of adaptive immune responses in regulating mast cell activation. Activation of mast cells occurs when specific antigens bind to antibodies attached to the receptors, leading to mast cell degranulation and release of cytokines or chemokines, such as GM-CSF, MCP, MIP and RANTES (reproduced from Tkaczyk et al., 2006).
1.8.2 Effect of nanoparticles on the immune system

Many studies have found that engineered NPs at high concentrations can cause a cytotoxic effect in immune cells, but recently researchers have also started to realize that NPs may also impact on immune system regulation (Hussain et al., 2012). Due to these potential immune interactions and the poor understanding of the novel chemistry of NMs, all engineered NPs should be investigated for their potential interactions with the immune system before introducing them for commercial use. There are two potential primary impacts resulting from interaction between NPs and immune systems: immunostimulation and immunosuppression.

Immunostimulatory NPs are defined as NPs that enable the triggering of stimulatory immune responses that may be either desirable or undesirable. Desirable immunostimulatory effects of NPs may include vaccine and antitumor effects, while undesirable immunostimulation by NPs may include hypersensitivity reactions, inflammation and anaphylaxis. Immunosuppressive NPs (NPs that down-regulate immune responses) may also be either desirable or undesirable. Desirable immunosuppression may be useful in the treatment of inflammatory disorders, autoimmune disease, prevention of allergic responses or to promote transplant acceptance, while undesirable immunosuppressive effects may cause reduced immune responses to infection and cancerous cells, myelosuppression and thymic suppression (Zolnik et al., 2010).

Some immunostimulatory reactions generated by NPs have been investigated. A number of studies have reported that NPs are capable of inducing cytokine production, both in vitro and in vivo (Fifis et al., 2004; Mottram et al., 2007; Scholer et al., 2002; Vallhov et al., 2006). NPs are also able to improve the antigenicity of a
weak antigen, and therefore act as an adjuvant that can be applied in vaccine
development (Manolova et al., 2008; Fifis et al., 2004). On the other hand, NPs can
trigger undesirable immune responses that are responsible for allergic reactions.
Some studies have argued that NPs cause allergic immune responses in animals
and humans (Nygaard et al., 2009; Toyama et al., 2008). For instance, a study in
mice has shown that CNTs promote allergic responses to the known allergen
ovalbumin (OVA) (Nygaard et al., 2009).

There are several studies showing that NPs are capable of beneficially suppressing
immune responses. For example, poly(D,L-lactic/glycolic acid) (PLGA) NPs loaded
with betamethasone sodium phosphate have been applied to treat inflamed joints
(arthritis) in a mouse model (Higaki et al., 2005). Some NPs have toxic effects on
immune cells, which can result in the lowering of immune responses, e.g. inhaled
multi-walled carbon nanotubes (MWCNT) caused systemic immune suppression in
a mouse model (Mitchell et al., 2009). To date, there has been a dramatic increase
in the quantity of manufactured NPs, however, few studies have been undertaken
to investigate the specific immune inhibitory effects of NPs. More studies are
urgently needed to evaluate such risks from the use of these NPs.

1.8.2.1 Immunotoxicity of nanoparticles

Nanoparticles initially interact with epithelial cells that are the first line of defence in
the body, which are likely to be located in the skin, respiratory and digestive
systems. Once NPs damage and invade the epithelial cells, inflammation is
triggered and NPs are engulfed by tissue-resident immune cells. During this
process a variety of inflammatory, cytotoxic and immune responses occur. Several
studies have established using in vivo and in vitro models that NPs can induce
inflammatory responses, as well as cytotoxicity via necrosis and apoptosis of cells (Sayes et al. 2006; Sayes & Warheit, 2008). There are many possible ways in which NPs cause immunotoxic effects, for instance, the toxicity of NPs depend on various factors such as the physicochemical properties of NPs, interaction with medium components, the specific cell types encountered, and the way by which NPs enter cells. Once inside cells, NPs can cause a variety of effects, such as generation of ROS, apoptosis, necrosis and activation of cell signalling (Marano et al., 2011).

The physicochemical properties of NPs play a critical role for inducing immunological responses and cytotoxicity. A number of studies have confirmed that the toxicity of engineered NPs (like cellular uptake) is due to physicochemical properties such as size, shape, solubility, crystal structure and electrostatic charge, chemical composition, and surface area, as well as particle size distribution (agglomeration state, dispersion factor and zeta potential) and the innate toxic potential of the constituent material (Padmavathy & Vijayaraghavan, 2008; Nair et al., 2008; Nel et al., 2006; Yang et al., 2008; Jiang et al., 2009).

1.8.2.2 Biological investigation of zinc ion and ZnO nanoparticles
Zinc is an essential trace element and the second most abundant transition metal ion found in mammals. However, zinc deficiency is widespread in human populations, causing harmful impacts on growth, nerve cell development, and immune system function (Tapiero & Tew, 2003; Plum et al., 2010). Rather than being a common toxic metal, zinc deficiency is a far greater risk to human health than its excess. However, many studies have reported that exposure to high levels of ZnO oxide particle inhalation in workers has lead to metal fume fever that is
characterized by airway irritation and lung inflammation (Antonini et al., 2003; Warheit et al., 2009). Therefore, safety concerns have been raised about the potential toxic effects of direct and long term skin contact to the ZnO NPs increasingly used as a broad-spectrum, UV absorber in sunscreens and personal care products (Stern & McNeil, 2010; Epstein, 2011).

Several studies on the toxicological impact of ZnO NPs in vitro have shown a toxic dose response in a number of different cell types. It has been suggested that high levels of dissolved Zn$^{2+}$ are a contributor to overall ZnO NP toxicity, with the induction of oxidative stress as the major factor in cytotoxicity (Deng et al., 2009; De Berardis et al., 2010). Brunner et al. (2006) also demonstrated that the severe cytotoxic effects of ZnO NPs on human MSTO mesothelioma or rat 3T3 fibroblast cells may be related to the release of Zn$^{2+}$ ions in the media or inside the cells. ZnO NP dissolution could occur in culture medium and reach approximately 80% saturation of dissolved Zn$^{2+}$ ions within 3 hours. It was expected that until Zn saturation was reached, the cells are mainly exposed to aqueous Zn$^{2+}$ ions, but when particle concentrations are used that exceed the maximum Zn solubility, which is around 225 µM in complete Dulbecco's Modified Eagle's Medium (DMEM), cells will be exposed to non-dissolved NPs (Xia et al., 2008). At this stage, the multiple pathways for cellular toxicity of ZnO NPs in vitro have yet to be fully elucidated, and their relevance to long-term dermal or inhalational exposures is not known. For these reasons, it is important that the biological interactions of ZnO NPs be more comprehensively assessed.

Similarities in cytotoxicity between ZnCl$_2$ and ZnO NPs, have led to the suggestion that Zn dissolved from these NPs is the main contributor to their cytotoxicity (Song
et al., 2010), other research has indicated that direct ZnO NP-to-cell contact is required for cytotoxicity (Moos et al., 2010). Whilst a definitive answer on the relative contributions to cytotoxicity between ZnO NPs and “soluble” Zn remains unresolved to date, no studies have been carried out which explain why “soluble” Zn gives varied results, or even if it is present as Zn$^{2+}$ in the cell culture systems typically used.

The cell culture media widely employed usually consists of a complex mixture of amino acids and inorganic salts, designed to mimic the electrolytic, osmotic and pH properties of blood plasma, together with the addition of a source of serum (e.g. foetal bovine serum, FBS) to provide proteins, predominately albumin, which will coat NPs introduced into the system (Vippola et al., 2009). In many studies a simple water-soluble, ionic form of Zn (e.g. ZnCl$_2$ or ZnSO$_4$) has been employed to serve as a control, to distinguish the activity of “soluble” Zn from that of ZnO NPs. However, Zn$^{2+}$ ions are well known to afford a range of poorly soluble carbonate (Chen et al., 1998; Ghose, 1964) and phosphate phases (Ziemniak & Opalka, 1994; Frost, 2004).

Examination of the solubility and physicochemical speciation of Zn$^{2+}$ in cell culture media may impact on Zn bioavailability in cell studies. Common cell culture media types (RPMI-1640, DMEM, Hank’s Balanced Salt Solution - ‘HBSS’, etc.) have relatively high concentrations of both phosphate and carbonate anions. As the Zn salts of phosphate and carbonate anions are known to be insoluble in aqueous solutions, it is reasonable to expect that the addition of Zn$^{2+}$ ions to cell culture media may also result in the formation of insoluble Zn species. Some very recent work has suggested that the solubility of ZnO NPs in various media solutions is
quite variable, and that reactions and precipitations indeed do appear to form in some of these solutions (Reed et al., 2012). Thus for in vitro cell studies, the speciation of Zn will complicate the comparison of the relative cytotoxicity of ZnO NPs compared to that of a soluble Zn control.

Numerous of studies have been conducted to investigate the effect of physicochemical properties of ZnO NPs on cytotoxicity and inflammatory responses in different mammalian cells. A recent research study has been published by our research group (Feltis et al., 2012) that investigated the effect of size and dispersion of ZnO NPs (ZnO NPs, 30 & 200 nm, and surfactant-coated ZnO NPs, 30 & 200 nm) on immune cell function and cytotoxicity of human THP-1 monocytes and macrophages. They reported that cytotoxicity was higher in macrophages than monocytes, suggesting that active phagocytosis by macrophages may be important in NP cytotoxicity. Consequently, increased ZnO NP uptake led to increased Zn\(^{2+}\) level inside the cells. In addition, the smallest NPs were more cytotoxic than bulk particles. Surfactant-dispersed ZnO NPs were also more toxic than their pristine counterparts. However, ZnCl\(_2\) showed equivalent cytotoxicity to the smallest ZnO NPs in both macrophages and monocytes. Cytokine profiles showed that IL-8 was induced by all ZnO NPs, with the smaller NPs inducing a higher amount of IL-8 release, regardless of cytotoxicity or dose. However, ZnO NPs did not stimulate type 1 (i.e. IL-2, IL-12, TNF-β and IFN-γ) or type 2 (i.e. IL-4, IL-5, IL-6 and IL-10) cytokine responses. Our research group is currently investigating the possible mechanism of Zn\(^{2+}\)-induced cytotoxicity and immune responses upon the addition of ZnCl\(_2\).
Another study by Hanley et al. (2009) reported that ZnO NP-induced cytotoxicity and immunoregulatory cytokine release were dependent on cell type, size of NPs and ROS generation. They also reported that ZnO NPs are capable of increasing the production of IFN-γ, TNF-α, and IL-12 in primary human peripheral blood cells. In addition, a study by Hsiao et al. (2011) provided detailed information about the interaction of ZnO NPs with human A549 lung epithelium cells. They reported that physicochemical properties of NPs including size, shape, surface area, phase, and composition have a marked effect on cell toxicity and production of IL-8 from A549 cells.

In contrast, other studies have reported that ZnO NPs did not cause size-dependent cytotoxic effects on mouse neural stem cells. This suggested that firstly, ZnO NPs might aggregate and form large particles with similar cytotoxicity, and secondly, that the cytotoxicity of ZnO NPs could be due to Zn$^{2+}$ ions released into the medium (Deng et al., 2009).

A number of studies have reported that particle surface area is a major determinant of cytotoxicity. Smaller particles have a larger surface area to volume ratio than larger particles, resulting in increased NP exposure and uptake in cells and more cytotoxic effects (Karakoti et al., 2006). However, others have observed that the higher surface area might not relate to higher cytotoxicity for some NP types such as TiO$_2$ NPs (Sayes et al., 2006; Gojova et al., 2007).

Many studies have tried to illustrate the effect of ZnO NPs on the mechanism of cell death but the research data are contradictory. On one hand, some studies have showed that the apoptosis pathway is induced in cells that are exposed to
ZnO NP (De Berardis et al., 2010; Lai et al., 2008), while on the other hand, other studies have reported that ZnO NPs induced cell death by necrosis (Hackenberg et al., 2010). This discrepancy may be related to differences in exposure and sampling periods and the dose-range investigated – as many toxic substances can induce apoptosis at lower concentrations, but higher concentrations can overwhelm the energy-dependent apoptotic processes to cause cell death by necrosis.

### 1.8.2.3 Biological investigation of titania nanoparticles

Zinc oxide and TiO$_2$ NPs both consist of inorganic metal oxides, but cause different biological interactivities. For instance, TiO$_2$ NPs are insoluble, whilst ZnO NPs are capable of releasing Zn$^{2+}$ ions that play a crucial role in ZnO NP-induced cytotoxicity (Brunner et al., 2006). Therefore metal ion release from NPs may contribute in the cytotoxicity and immune response effects of some soluble NPs, but other insoluble NPs have different mechanisms for inducing toxicity and other biological effects.

The significantly different cytotoxic effects seen between ZnO and TiO$_2$ NPs have been confirmed in numerous studies (Lin et al., 2009; Yang et al., 2009; Hsiao et al., 2011). Moreover, both of these inorganic metal oxide NPs generate ROS (Hussain et al., 2009).

A number of studies have been conducted to compare the cytotoxicity and inflammatory properties of TiO$_2$ NPs to their bulk counterpart. Many studies have found that ultrafine TiO$_2$ particles (20 nm) induce higher inflammatory responses in the rat lungs compared to fine TiO$_2$ particles (250 nm) (Oberdörster et al., 1994).
In addition, ultrafine TiO$_2$ particles (140 nm) caused greater cytotoxicity than fine TiO$_2$ particles (380 and 250 nm) in human A549 and rat L2 lung epithelial cells (Sayes & Warheit, 2008). Very small nanoscale (10 and 20 nm) TiO$_2$ particles can elicit oxidative damage, while larger particles (200 and >200 nm) do not induce oxidative damage (Gurr et al., 2005). Other studies have argued that there are no biological differences between ultrafine and fine particles (Sayes et al., 2006; Kim et al., 2009).

Sayes et al. (2006) illustrated that the dose of TiO$_2$ NPs required to observe cytotoxicity and change biological function in human dermal fibroblasts (HDF) cells was around 1.5 mg/mL. Caution must be used in interpreting this result, as this is a very high concentration that may have impacted directly on colourimetric readings (due to light scattering by the NPs present in the multi-well plate), as well as the potential for direct binding of NPs to assay components.

There are different forms of TiO$_2$, such as anatase, rutile, brookite, ilmenite, leucoxene, perovskite and sphene. Rutile and anatase TiO$_2$ are the most common forms used. A number of studies have shown that the crystal composition has an impact on cytotoxic and inflammatory responses. The cytotoxicity of TiO$_2$ NPs to A549 cells was greater for a mixture of anatase/rutile TiO$_2$, than for amorphous NPs (Hsiao et al., 2011). Anatase TiO$_2$ NPs have also been shown to be 100 times more cytotoxic than an equivalent sample of rutile TiO$_2$ NPs (Sayes et al., 2006). The mixture of two crystal forms together has also elicited a much higher level of cytotoxicity than those of pure rutile or anatase forms (Gurr et al., 2005). Therefore, it is very important to know the purity and composition of TiO$_2$ NPs before investigating their cytotoxic and inflammatory effects.
One of the major concerns with TiO$_2$, as with ZnO NPs, is that there is relatively little specific data regarding interaction of these NPs with the immune system. Schanen *et al.* (2009) found that TiO$_2$ NPs induced immune responses through increased production of proinflammatory cytokines, such as IL-1$\alpha$, IL-1$\beta$, IL-6, IL-8, IFN-$\gamma$ and TNF-$\alpha$, from monocyte-derived DCs, which induced proliferation in CD4$^+$ T cells. However, the TiO$_2$ bulk counterpart did not induce any responses and remained inert. Moreover, TiO$_2$ NPs also stimulated A549 cells to produce IL-8 (Sayes *et al.*, 2006). In contrast, Hsiao *et al.* (2011) did not observe an increase in IL-8 production from A549 after exposure to TiO$_2$ NPs.

A study has observed the immunogenicity of TiO$_2$ and ZnO NPs. Palomaki *et al.* (2010) reported that in the murine RAW 264.7 macrophage cell line, TiO$_2$ NPs induced expression of TNF-$\alpha$ and macrophage inflammatory protein (MIP-1$\alpha$), whereas ZnO NPs stimulated the expression of IL-1$\beta$, and the neutrophil chemoattractant chemokine (C-X-C motif) ligand 9 (CXCL-9). Both ZnO and TiO$_2$ NPs induced the production of IL-6. Only ZnO elicited the expression of IL-1$\beta$, IL-6, TNF-$\alpha$, CXCL-5, CXCL-9 and CXCL-10 in bone marrow-derived dendritic cells (bmDCs). TiO$_2$ and ZnO NPs were both able to stimulate the expression of a maturation marker (CD11c) and various activation markers, such as MHC II, CD1d, CD86 and CD40 in RAW 264.7 cells. However, no significant differences were observed in these markers for bmDCs exposed under the same conditions (Palomaki *et al.*, 2010).

A study by Warheit *et al.* (2007) provided detailed information about the toxicity and inflammatory affects of rutile and anatase ultrafine and rutile fine TiO$_2$ particles in rats. They reported that rutile ultrafine and fine particles produced pulmonary
inflammation, histopathological changes, and fibrogenic and cytotoxic effects at a lower concentration than anatase ultrafine TiO$_2$ particles. Size distribution was measured by dynamic light scattering (DLS) which showed particle sizes of 382 nm for fine rutile, 136 nm for ultrafine rutile and 129.4 nm for ultrafine anatase particles. However, high resolution scanning electron micrographs (HR-SEM) images demonstrated that the size of the ultrafine rutile was larger than the anatase, which might explain the different results from rutile ultrafine particles.

As illustrated above, TiO$_2$ in its bulk form seems to be quite inert or less bioactive. The nanoparticulate form is associated with cytotoxic and inflammatory effects, and any novel changes to the characteristics of NPs, such as shape, coating and size, will possibly create new health risks and should be tested extensively.

In addition to the different factors discussed above, the binding of proteins to NPs (protein corona) is an important consideration when investigating the effect of NPs on toxicity and immune responses, in both in vitro and in vivo studies. A number of studies have reported that protein binding to NPs can cause changes to the physicochemical properties of NPs (Moghimi et al., 2001; Chithrani et al., 2006; Nagayama et al., 2007; Lynch et al., 2009; Deng et al., 2011; Monopoli et al., 2011). These changes may have an impact on cytotoxicity, immune responses and other biological functions. It has been shown that binding of albumin to single-walled CNTs increased their uptake by RAW 264.7 cells (Dutta et al., 2007).

1.8.2.4 The effect of nanoparticles on mast cells

Mast cells, which are present at all the tissue sites where NPs are likely to first encounter, play a significant role in both activation and inhibition of innate
immunity, adaptive immunity and allergic responses, as described previously in the section 1.7.1.1. Therefore, mast cells provide a very good model for studying the interaction between NPs and cells of immunity, and how this interaction might alter the regulation of allergic immune responses. However, to date, very few studies have looked at the impact of NPs on mast cells.

A recent study by Chen et al. (2012) examined rat RBL-2H3 mast cells exposed to a mixture of anatase and rutile forms of TiO$_2$ NPs (<100 nm to 100, at 750 µg/mL) and measured histamine release. They observed that TiO$_2$ NPs can directly stimulate histamine release from mast cells via a Ca$^{2+}$ dependent pathway. They did not, however, investigate the effect of TiO$_2$ NPs on IgE-activated RBL-2H3 rat mast cells, which is considered to be a more relevant model.

A study by Yamaki et al. (2009) investigated the β-hexosaminidase inhibitory effects of ultrafine (21 nm) and fine (<5 µm) ZnO particulates on IgE-induced mast cell activation. They reported that ZnO inhibited the release of β-hexosaminidase from rat RBL-2H3 mast cells activated with anti-OVA IgE and its antigen, through inhibition of FcεRI-mediated PI3K and protein tyrosine kinase activation, without affecting calcium mobilization. Also, they demonstrated that ultrafine ZnO and ZnSO$_4$ inhibited FcεRI-mediated degranulation more efficiently than fine ZnO; this could be explained by the higher surface area and therefore greater release of Zn$^{2+}$ from ZnO, thus causing the more inhibition. Similarly, Marone et al. (1981) argued that ZnCl$_2$ inhibits histamine release from IgE-activated human basophils. Unfortunately, Yamaki et al. (2009) did not describe the cytotoxic effects of ultrafine and fine ZnO particulates, and only studied the β-hexosaminidase inhibitory of ZnO
NPs in their aggregated state, but not the dispersed state which may give stronger inhibitory effects. To date no study has been conducted to evaluate ZnO NPs on normal primary mast cells and their potential immunomodulatory effects.

In contrast to the finding of Yamaki and coworkers, Hide and Beaven (1991) found that Zn$^{2+}$ inhibited IgE-activated mast cell degranulation through inhibition of calcium influx. In addition, Musset et al. (2008) reported that the Ca$^{2+}$ influx stimulated by anti-IgE in human basophils may also be inhibited by Zn$^{2+}$. Thus, increasing the intracellular level of Zn$^{2+}$ might play a potential role in inhibiting Ca$^{2+}$ influx.

Another study examined the effect of carbon NPs on allergic responses. Ryan et al., (2007) reported that fullerene NM derivatives (polyhydroxy-C$_{60}$, N-ethyl-polyamino C$_{60}$) were able to inhibit allergic responses in human primary mast cells and peripheral blood basophils in vitro and anaphylaxis in vivo. They suggested that the IgE-dependent allergic inhibitory responses might be due to inhibition of oxidative stress and syk tyrosine phosphorylation.

Nanoparticles can also inhibit inflammatory responses; Ag NPs inhibited the IL-5 and TNF-α inflammatory cytokines produced by phytohaemagglutinin (PHA) stimulated peripheral blood mononuclear cells (Shin et al., 2007). However, Wingard et al. (2011) recently reported that 8 nm cerium oxide (CeO$_{2}$) NPs activated mouse BMMCs to produce proinflammatory mediators, such as TNF-α and IL-6, suggesting that mast cells may have an essential role in recruitment of inflammatory cells in response to NP exposures.
Organic NPs can act as negative regulatory agents for IgE-mediated allergic responses. For example, chitosan and dendrosome NPs loaded with peanut allergen inhibited IgE allergic responses in mice (Balenga et al., 2006; Roy et al., 1999). Moreover, Tahara et al. (2012) recently conducted a comprehensive study on the effect of chitosan-PLGA NPs on the allergic response of RBL-2H3 mast cells in vitro and also used the same NPs in an in vivo mouse model. They observed that chitosan-PLGA NPs were capable of inhibiting histamine allergic responses mediated by IgE-activated mast cells, both in vitro and in vivo. In contrast, another study did not observe any inhibitory effects or histamine release for chitosan-hyaluronic acid NPs loaded with heparin and stimulated with compound 48/80 in RBL-2H3 cells (Ampuero et al., 2009). A criticism of this work is that the chitosan NPs were made by Ampuero and co-workers using an ionotropic gelation method, which is not an ideal method for drug delivery.

Anthropogenic ultrafine particles can induce allergic responses, e.g. DEPs, which are a major source of atmospheric pollution. Devouassoux et al. (2002) demonstrated that DEPs can greatly stimulate IL-4 production from non IgE-activated human basophils (i.e. IL-4 production via the IgE-independent basophil cytokine pathway) – consequently DEPs can shift the adaptive immune response toward an undesirable Th2 allergic inflammatory response. Similar effects of DEPs on IgE-activated murine mast cells have been reported by Sanchez et al. (2000). Therefore, DEPs can stimulate IL-4 production from basophils via both IgE-independent and IgE-dependent pathways.
1.8.2.5 The effects of nanoparticles on lung epithelial cells

The human A549 cell line has been used in well established in vitro models to study lung epithelium responses (Don Porto Carero et al., 2001; Aufderheide et al., 2003; Schwerdtle & Hartwig, 2006). The lung is a primary area for direct exposure to NPs, or accumulation of NPs in the respiratory tract, through inhalational exposure. Inhalation is one of the most likely risks of exposure to dry powders or aerosols containing NPs, and also allows direct contact with the NPs to living cells and potential access to the body’s circulation.

Numerous studies have provided detailed information about the cytotoxic effects of ZnO and TiO$_2$ NPs in A549 cells. They report that the physicochemical properties of NPs, including size, shape, surface area, phase, composition and agglomeration, have a crucial impact on cytotoxicity (Hsiao et al., 2011; Sayes & Warheit, 2008). However, these studies investigated the cytotoxic and proinflammatory effects of these metal oxide NPs in an aggregated state and not in a dispersed form, which may induce different cytotoxicity and immune responses.

ZnO NPs display more cytotoxicity than TiO$_2$ NPs in A549 cells (Hsiao et al., 2011). TiO$_2$ NP cytotoxicity often gives contradictory results in different studies, but the general view is that TiO$_2$ NPs do not induce severe cytotoxicity (Johnston et al., 2010). This could be due to the purity of the titanium component (rutile, anatase, amorphous or crystalline mixtures), physicochemical properties (such as the lack of solubility) and concentration of TiO$_2$ NPs.

Recently, Lankoff et al. (2012) reported studies of the cytotoxic effects of TiO$_2$ and Ag NPs in three different cell lines, i.e. the human HepG2 liver cell line, A549 and
THP-1 monocytic cell lines. They observed that the NP cytotoxicity was not directly dependent on particle size only, but that there was a “complex relationship” between particle and cell properties.

Production of the inflammatory cytokine IL-8 from A549 cells has been stimulated by both ZnO NPs (Hsiao et al., 2011) and TiO$_2$ NPs (Sayes et al., 2006). In contrast, Hsiao et al. (2011) did not observe an increase in IL-8 production from A549 cells after exposure to TiO$_2$ NPs. These contradictory results suggest that there is insufficient data regarding the effect of ZnO and TiO$_2$ NPs on cytokine production from A549 cells.

1.9 Project objectives

Nanoparticles are increasingly being used in drug delivery and skin care products. ZnO and TiO$_2$ NPs are used in sunscreens due to their broad UV absorption characteristics and transparency to visible light. Most sunscreen preparations also contain surfactant materials that prevent NP aggregation and ensure NP dispersion. Therefore, it is very important to expand the health risk assessment for these preparations (Faunce, 2010). Mast cells are very important in many immune responses, especially in the skin and respiratory tract, due to their role in inflammatory and allergic reactions. However, to date, very few studies have looked at the direct impact of NPs on mast cells.

There is currently a limited understanding of how these NMs interact with cellular systems and what the potential impact on human health may be. To address the risks from the increasing application of NMs, the cytotoxicity and inflammatory
responses to NMs will be assessed with *in vitro* studies. This study aims to provide useful information to contribute to the development of new NMs with lower toxicity and greater therapeutic potential, and to help identify biomarkers that may predict the potential health hazards of NMs.

In this present study, I will examine how the size and dispersion of ZnO NPs, along with two different crystalline forms of TiO$_2$ NPs, can affect both the cytotoxicity and inflammatory profiles of mast cells and lung epithelial cells. I will investigate several NP sizes that are typical of those present in current sunscreen formulations, in both well and poorly dispersed forms. In the case of ZnO, I will also compare these NPs with both bulk and dissolved ion controls.

The study will also investigate the role of different structural properties (particle size, wall thickness and porosity) of chitosan nanocapsules used in drug delivery. Capsules will be infiltrated with curcumin, a hydrophobic anticancer molecule naturally found in the yellow curry spice turmeric. The effects of curcumin-loaded chitosan nanocapsules on cellular uptake, cell viability and allergic responses in mouse P815 mast cells will be studied.

Specifically, the aims of this study are:

- To assess the mechanism of cytotoxicity and the cytokine profiles induced by zinc oxide and titanium dioxide nanoparticles in the mouse P815 mast cell line;
• To assess the effects of zinc oxide and titanium dioxide nanoparticles on mast cell degranulation in rat RBL-2H3 and bone-marrow derived mouse mast cells;

• To assess the inflammatory and cytotoxic responses of lung epithelial cell lines (human A549 and mouse LA4) exposed to zinc oxide and titanium nanoparticles; and

• To assess the potential of chitosan nanocapsules as a nontoxic and non-allergenic drug delivery vector in vitro using mouse P815 mast cells.
Chapter 2: Investigation of the mechanism of cytotoxicity and cytokine profile response to zinc oxide and titanium dioxide nanoparticles in the P815 mouse mast cell line

2.1 Introduction

Nanotechnology represents one of the most promising technologies of the 21st century. The applications of nano-materials have increased dramatically in recent years; they have been used in many commercial products such as sunscreens, cosmetics and antimicrobial agents (Choksi et al., 2010).

To date, a variety of sunscreen and cosmetic formulations containing ZnO and TiO$_2$ NPs have been made commercially available; these recent sunscreens are effective at filtering a broad spectrum of UV light from sunlight (Nohynek et al., 2007). However, the possible toxic, immune and allergic consequences of exposure to these NPs are poorly characterised.

Mast cells are essential in the regulation of immune responses, especially those at the surface of the skin and the respiratory system, due to their role in inflammatory and allergic reactions (Galli et al., 2008). Activation of mast cells through the cross-linking of high-affinity IgE (FcεRI) receptors by multivalent antigens, or indirectly by chemical substances such as the calcium ionophore ionomycin, causes the activation of an intracellular signalling cascade, which leads to the release of
cytokines, chemokines and other inflammatory mediators that can result in an allergic response (Tkaczyk et al., 2006). Mast cells and basophils can act at many levels, some of which have undesirable outcomes; they produce Th2 type cytokines, such as IL-4, which can shift immune responses toward allergy (Galli et al., 2010). Investigation of the cytokine profiles produced in mast cells when they are stimulated in specific ways, may be a useful tool for predicting an allergic immune response.

Several studies have been conducted on the toxicological impact of ZnO and TiO$_2$ NPs in vitro. They have reported that the cytotoxicity of these NPs was dependent on their physicochemical properties, such as particle size, shape, solubility, crystal structure, electrostatic charge, chemical composition, and surface area, as well as particle size distribution (agglomeration state and zeta potential) (Nel et al., 2006; Nair et al., 2008; Padmavathy & Vijayaraghavan, 2008; Yang et al., 2008; Jiang et al., 2009).

Many studies have investigated the effect of particle size on cytotoxicity, but the research data are contradictory. Some researchers have found that smaller ZnO NPs caused greater cytotoxicity than larger particulates (Hanley et al., 2009; Feltis et al., 2011). In contrast, Deng and coworkers reported that ZnO NPs did not cause size dependent cytotoxicity (Deng et al., 2009). Likewise, other studies have found no prominent biological difference between TiO$_2$ ultrafine and fine particles (Sayes et al., 2006; Kim et al., 2009). Consequently, the size dependency of cytotoxic responses is still unclear.
Both ZnO NPs and TiO\textsubscript{2} NPs are metal oxide nanoparticulates, but ZnO NPs are known to have greater cytotoxicity than TiO\textsubscript{2} NPs (Lin \textit{et al.}, 2009; Yang \textit{et al.}, 2009; Hsiao \textit{et al.}, 2011). The greater cytotoxicity of ZnO NPs could be related to solubility of ZnO and release of Zn\textsuperscript{2+} ions into cell culture media, whereas TiO\textsubscript{2} NPs are insoluble (Burner \textit{et al.}, 2006; Deng \textit{et al.}, 2009; De Berardis \textit{et al.}, 2010). Interestingly, both of these NPs are able to generate ROS, which can induce cell death (Hussain \textit{et al.}, 2009).

Nanoparticles in a biological matrix are always present in aggregates and agglomerates that are larger than single NPs (Schulling \textit{et al.}, 2010). There is some debate about the risk assessments of NPs, which are often done on aggregate forms (Nohynek \textit{et al.}, 2007; Hsiao \textit{et al.}, 2011; Taccola \textit{et al.}, 2011). It is therefore relevant to investigate the cytotoxic and immunological responses to both dispersed and aggregated forms of NPs. In addition, ZnO and TiO\textsubscript{2} NPs in many sunscreen formulations are coated with surfactant materials, which keep NPs very well dispersed without aggregation in order to improve the UV filtering and transparent appearance on the skin. Therefore, NP risk assessments should take into account both aggregated and dispersed forms of these preparations (Faunce, 2010).

ZnCl\textsubscript{2} or ZnSO\textsubscript{4} has been used as a control for \textit{in vitro} test systems for soluble Zn ions coming from ZnO NPs. Both ZnCl\textsubscript{2} and ZnO NPs have similar cytotoxicity, which suggests that Zn\textsuperscript{2+} ions release from these NPs are responsible for cell death (Song \textit{et al.}, 2010). To date, there has been no clear definitive answer on the relative contributions to ZnO NP-induced cytotoxicity of the original ZnO NP form and subsequent “soluble” Zn\textsuperscript{2+}. 

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Mast cells provide a good model for studying the allergic immune reactions induced by NP/immune cell interaction. However, few studies have looked at the potential effects of NPs on the cytokine release profiles of mast cells in vitro (Devouassoux et al., 2002; Sanchez et al., 2000). Furthermore, the specific effects of ZnO and TiO$_2$ NPs on cytokine production from mast cells in vitro have not been investigated.

The aims of the studies described in this chapter were to evaluate the effects of the size and dispersion state of ZnO NPs (using ZnCl$_2$ as a control for Zn$^{2+}$ ions), compared to two different crystalline forms of TiO$_2$ NPs, on both the mode of action of cell toxicity and cytokine production, in non-activated and activated P815 mouse mast cells.
2.2 Materials and Methods

2.2.1 Chemicals and Reagents
Assay kits utilized CellTiter 96® AQueous Solution (MTS) (Promega, Madison, WI, USA), Annexin V / propidium iodide kit (Beckman-Coulter, Marseille, France), FlowCytomix Mouse Th1/Th2 10plex cytokine kit (Bender Medsystems, Butlingame, CA, USA) and IL-4 BD OptEIA ELISA Sets (BD OptEIA kit -BD Systems, Franklin Lakes, NJ, USA). Biochemicals included lipopolysaccharide (LPS), zinquin-ethyl ester and ionomycin calcium salt from *Streptomyces conglobatus* (Sigma-Aldrich, St Louis, USA), Flou-4 AM and 2,7-dichlorofluorescein diacetate (DCFDA) (Invitrogen, Carlsbad, CA, USA), phorbol myristate acetate (PMA) (Sapphire Bioscience, Redfern, NSW, Australia), and phosphate buffered saline.

Cell culture media included RPMI-1640 with HEPES modification (Sigma-Aldrich, St Louis, USA), DMEM (Invitrogen, Carlsbad, CA, USA), glucose and 2-mercaptoethanol (BDH, Merck, Kilsythe, VIC, Australia), pyruvate, L-glutamine, gentamycin and fetal bovine serum (FBS), (Sigma- Aldrich, St. Louis, MO, USA).

2.2.2 Nanoparticles
The nanoparticles and particulates (bulk form), with and without surfactant dispersant (polyacrylate copolymer, Orotan 731 DP), used in this study included: pristine zinc oxide at 30 nm, 80 nm and 200 nm (ZnO 30, ZnO 80, ZnO 200); surfactant-treated ZnO at 30 nm, 80 nm and 200 nm (sZnO 30, sZnO 80, sZnO 200) (Micronisers Pty. Ltd. Melbourne, Australia); 34 nm rutile (TiO$_2$ R) and 25 nm

2.2.3 Nanoparticle characterization

2.2.3.1 Nanoparticle size analysis

Nanoparticle size analysis was performed on a DC1800 disc centrifuge (CPS instruments, Stuart, FL, USA), with an RPMI-1640 media and sucrose gradient. Size distribution and agglomeration of nanoparticle suspensions were measured in RPMI-1640 media and RPMI-1640 media plus FBS.

2.2.3.2 Electron Microscopy.

Room temperature transmission electron microscopy (TEM) images were obtained on a Philips CM20 microscope at 200kv using a LaB6 filament. The samples were prepared by suspending the solid material in ethanol and placing a drop on a copper grid containing holey carbon film and then air dried.

Cryo-TEM samples were examined using a Gatan 626 cryoholder (Gatan, Pleasanton, CA, USA) and Tecnai 12 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands) at an operating voltage of 120 kV. At all times, low dose procedures were followed, using an electron dose of 8-10 electrons/A² for all imaging. Samples were prepared on 300-mesh copper grids coated with lacy formvar-carbon film (ProSciTech, Townsville, QLD, Australia) that were glow discharged in nitrogen for 15 s prior to use. A laboratory built humidity-controlled vitrification system was used to prepare the samples for cryo-TEM. Humidity was kept close to 80% for all experiments and ambient temperature was 22°C. Aliquots (4 µL) of each sample were pipetted onto a grid. After 30 s adsorption time, the grid
was blotted manually using Whatman 541 filter paper, for between 2 and 10 s. Blotting time was optimized for each sample. The grid was then plunged into liquid ethane cooled by liquid nitrogen. Frozen grids were stored in liquid nitrogen until required. Images were recorded using a Megaview III CCD camera and analysis camera control software (Olympus, Munster, Germany) at magnifications between 70,000x and 110,000x.

2.2.4 Preparation of nanoparticles
Each of the nanoparticles (ZnO30, sZnO30, ZnO80, sZnO80, ZnO200, sZnO200, TiO\textsubscript{2} R, TiO\textsubscript{2} P25) were weighed into pre-weighed sterile vials. Sterile water was then added to make a NP stock solution with a concentration of 20 mg/mL. The NP suspension was ultra-sonicated for 15 min and left to stand for 24 h, once the stock solution dropped below 1 mL volume, its use was discontinued. The stock solution was vortexed for 30 s and inverted 10-20 times, and then resuspended by pipetting up and down 3-5 times, before the desired volume of stock solution was added to the media to make the working solution. Dilutions were made by inverting the working solution 3-5 times, or until no particles were stuck to the bottom of the tube, and pipetting up and down 5 times, before the desired volume of the work solution was added to the media. A 100 µL volume of the dilutions (500, 200, 100, 60, 20 and 2 µg/mL) was added to the 100 µL in control and cell-containing wells for a total volume of 200 µL/well.

2.2.5 Cell culture
All cell culture techniques were performed under sterile conditions in a class II
biohazard hood. Mouse P815 mast cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 media containing 10% v/v FBS with gentamycin, glucose, pyruvate, 2 mercaptoethanol and L-glutamine supplementation, and maintained in a humidified incubator at 37°C and 5% CO₂. The cells had been frozen in FBS and 10% DMSO and stored in liquid nitrogen, prior to thawing at 37°C and culturing in cell growth media. Cells were maintained in 75 cm² culture flasks containing media at 37°C and 5% CO₂ in a Heraeus Instruments BB16 incubator. When cultures reached 8 x 10⁵ cells/mL, a 1/10 dilution of cells to media was added to a new 75 cm² culture flask.

2.2.5.1 Cells for NP exposure experiments
Cells were centrifuged at 200 g for 5 min using a Heraeus benchtop centrifuge and re-suspended in fresh media to obtain a working cell suspension of 10⁶ cells/mL. The working suspension was then added to the media in 96-well plates for a final concentration of 10⁵ cells/well.

2. 2.6 Cytotoxicity assays
2.2.6.1 Cytotoxicity of ZnO and TiO₂ NPs in RPMI-1640 complete media
The P815 mast cells were centrifuged (200 g, 5 min) and re-suspended in fresh RPMI-1640 media plus 10% FBS before seeding in 96-well plates at 10⁵ cells/well. These cells were incubated in quadruplicate wells of 96-well plates with a concentration range of NPs at 0, 10, 20, 30, 60, 100, 150 and 200 µg/mL in a humidified incubator (at 37°C and CO₂). Cytotoxicity was measured at 24 h, and for kinetic study at 4, 8, 12 and 24 h, by adding a soluble tetrazolium salt (MTS, Promega, USA) to the wells 4 h prior to the measurement of the optical absorbance
(490 nm) using a microplate reader (Perkin Elmer EnSpire®, Waltham, MA, USA). MTS was used to evaluate the mitochondrial function of cells and its reduction to a chromophore is proportional to the number of viable cells present in the wells. ZnO and TiO$_2$ NPs, in the presence of MTS reagent alone, were measured to control for any optical density effects from the NPs; these values were subtracted from the experimental readings. Each experiment was repeated in triplicate and the average viability results with standard error of mean, calculated. The supernatant at the 20 h time point was collected before adding MTS, and stored at -80°C for cytokine analysis.

### 2.2.6.2 Cytotoxicity of ZnO and TiO$_2$ NPs in DMEM complete media

Mouse P815 cells were cultured and incubated with NPs in DMEM media plus 10% FBS. Cytotoxicity was measured as previously described in section 2.6.1. (all other experiments described in this thesis were performed in RPMI-1640 media plus 10% FBS).

### 2.2.7 Cytotoxicity of zinc dialysate from ZnO NPs

#### 2.2.7.1 ZnO NP solubility

ZnO NPs were added to 5 mL of RPMI-1640 media in cellulose dialysis tubing (10,000 MW cut-off; Sigma-Aldrich, St Louis, USA) and dialysed for 24 h at 37°C into 30 mL of fresh RPMI-1640 media without FBS. Dialysates were collected and analysed for zinc ion content by inductively-coupled plasma atomic absorption spectroscopy (IPC-AAS) using a Perkin Elmer Model 1100 Atomic Absorption Spectrometer (Perkin-Elmer, Waltham, MA, USA). Calibration standards were
made from a zinc stock solution (1 mg/mL Zn, Spectrosol, VWR, Radnor, PA, USA).

2.2.7.2 Cytotoxicity of Zn dialysate

P815 cells were incubated with the dialysate of Zn (section 2.7.1) for 24 h in a humidified incubator (at 37°C and CO₂). Cytotoxicity was measured as previous described in the section 2.6.1.

2.2.8 Cytotoxicity of the Zinc precipitates.

2.2.8.1 ZnCl₂ precipitates in cell culture media.

A solution of anhydrous ZnCl₂ (2 g, 14.7 mmol) in water (3 mL) was thoroughly mixed with RPMI-1640 media (500 mL) or with media + 10 v/v% FBS giving a nominal [Zn] = 29.4 mM. Fluffy white precipitates formed rapidly. The mixtures were stirred briefly and the precipitates allowed to settle overnight. Most of the supernatant was carefully decanted and the remaining liquid containing precipitate was centrifuged, washed 3 times with water and dried in a vacuum oven at 40°C for 48 h.

2.2.8.2 Cytotoxicity of the zinc precipitates

P815 cells were incubated in quadruplicate wells and each of the following were added to separate wells: 1) ZnCl₂ at 1-200 µg Zn/mL (from a [Zn] = 0.31 M stock solution); 2) washed and vacuum-dried Zn precipitate from RPMI-1640 plus 10% FBS; 3) freshly prepared Zn precipitate from RPMI-1640 plus 10% FBS; or 4) ZnO 39 nm nanoparticles suspended in RPMI-1640; for a final concentration of
particulate/precipitate of 1-200 µg Zn/mL. Cytotoxicity was measured as previous described in the section 2.2.8.1.

2.2.9 Measurement of intracellular level of zinc ions
The intracellular level of zinc ions was estimated using the zinc-selective fluorophore, zinquin ethyl ester. P815 mast cells were incubated in triplicate wells of 96-well plates with a concentration range of ZnO NPs at 30 and 100 µg/mL, in a humidified incubator (at 37°C and CO₂). Intracellular levels of Zn²⁺ ions were measured at the specific time points of 1, 4, 8 and 24 h by adding zinquin ethyl ester (25 µM) and the cells incubated for a further 30 min prior to analysis. Finally, the cells were washed twice in PBS and the fluorescence intensity measured at excitation/emission wavelengths of 364/485 nm, respectively, using flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA). The qualitative analysis of intracellular zinc ions was performed by fluorescence microscope (Olympus Optical Co. Ltd., Japan).

2.2.10 Measurement of intracellular reactive oxygen species (ROS)
The level of intracellular ROS generation was detected by the method of Wilson et al. (2002) using 2,7-dichlorofluorescein diacetate (DCFDA) dye. P815 mast cells were centrifuged (200 g, 5 min) and washed with PBS and resuspended in PBS. A 100 µM of DCFDA was then added to the cells and incubated for 30 min in a humidified incubator (at 37°C and CO₂). The cells were washed twice with PBS and resuspended in RPMI-1640 (phenol red free version; Sigma-Aldrich, St Louis, USA) plus 10% FBS and seeded in 96-well black opaque plates (Corning Life Sciences, USA) at 5 x 10⁵ cells/well. A range of NP dilutions in RPMI-1640 (phenol
red free) medium were added to the cells in separate quadruplicate wells and incubated for 24 h. Fluorescence intensity was measured using a microplate reader and luminol chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) at excitation and emission wavelengths of 485 and 528 nm, respectively. ZnO and TiO$_2$ NP background in RPMI media was measured as to control for any optical density effects from the NPs; these values were subtracted from the experimental readings. Hydrogen peroxide was used as a positive control at concentration (400 µM). The qualitative analysis of intracellular ROS generation was performed using confocal laser scanning microscopy (CLSM; Nikon, A1 Confocal Microscope, Tokyo, Japan).

**2.2.11 Mechanism of cell death**
Apoptotic and necrotic cell death mechanisms were detected using the Annexin V/propidium iodide kit (Invitrogen, Carlsbad, CA, USA). In brief, the cells were washed with PBS (centrifuged for 5 min at 500 g, 4°C) and the cell pellets resuspended in ice-cold 1x Binding Buffer to between $5 \times 10^5$ and $5 \times 10^6$ cells/mL. The positive control for necrosis was performed by growing the cells without FBS, while the apoptosis positive control was done by incubating the cells with 3% formaldehyde-containing PBS for 30 minutes on ice and then washed. Finally, Annexin V-FITC solution and propidium iodide were added to the cells. Following a 15 min incubation period, the cells were analysed in triplicate wells using flow cytometry (FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA).
2.2.12 Determination of ZnO and TiO$_2$ NP cell uptake by synchrotron x-ray fluorescence microscopy

P815 mast cells were seeded at 5 x 10$^5$ cells/well in 24 well plates containing a silicon grid in each well. The cells were incubated for 24 h with 30 µg/mL of ZnO NPs doped with 5% (w/w) cobalt (Co), TiO$_2$ NPs or ZnCl$_2$.

P815 cells attached to the grid were taken from the culture and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate containing 2 mM CaCl$_2$. These cells were then washed in 2 mM CaCl$_2$ and 0.1 M sodium cacodylate in distilled water for 10 min. Postfixation was performed by immersion for 20 min in 1:1 osmium tetroxide (1% w/v) in 0.1 M sodium cacodylate with 2 mM CaCl$_2$. The cells were washed in 0.1 M sodium cacodylate in distilled water for 10 min to remove residual osmium tetroxide from the surface of the cells. The cells were then dried and stored until synchrotron x-ray fluorescence microscopy (XFM) analysis. Intracellular ZnO and TiO$_2$ NPs imaging were mapped at 300 nm resolution using x-ray fluorescent microscopy at the Australian Synchrotron, Victoria.

2.2.13 Cytokine Profiling

2.2.13.1 FlowCytomix Mouse Th1/Th2 10plex

The frozen supernatant samples from culture medium of P815 cells exposed to NPs (section 2.6.1) were analysed by flow cytometry for levels of ten different cytokines using a FlowCytomix Mouse Th1/Th2 10plex kit (GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, TNF-α; Bender MedSystems, Vienna, Austria). Samples were analysed in triplicate for two experiments.
2.2.13.2 IL-4 ELISA measurement

P815 cells were seeded onto 96-well plates at $10^5$ cells/well, and various concentrations of NPs (0, 10, 30 and 60 µg/mL) were added to the cells which were then stimulated with ionomycin (1 µM) plus PMA (20 nM) by co-incubation for 24 h at 37°C. The supernatant from the 20 h time point was collected before adding MTS, and stored at -80 °C for IL-4 cytokine analysis. Enzyme-linked immunosorbant assay (BD OptEIA kit, BD Systems, Franklin Lakes, NJ, USA) was used to measure IL-4 in thawed cell supernatants. The absorbance at 450 nm was obtained using a microplate reader (Perkin Elmer EnSpire®, Waltham, MA, USA), as per the manufacturer’s instructions. The spontaneous release of IL-4 (without the addition of the stimulants; ionomycin plus PMA) was also measured after incubating the P815 cells with range of NPs (0, 10, 30 and 60 µg/mL) for 24 h using the same procedure.

NPs alone were titrated against IL-4 standard diluted in RPMI media, to investigate the possibility of IL-4 cytokine absorption or binding onto the NPs after the 24 h incubation. IL-4 standard in RPMI without NPs was used as the control.

2.2.14 Statistical analysis

All data in this study is expressed as the mean ± SEM of two to four experiments. Samples were analysed using three to four replicates within each experiment. Statistical analysis was performed using two-way ANOVA and Bonferroni post-hoc test by Graph Pad Prism for Windows version 5 (San Diego, CA, USA). Statistical significant differences were considered as having $p$ values < 0.05.
2.3 Results

2.3.1 Particle characterisation

The ZnO NPs and TiO$_2$ NPs used in this study were characterized by using TEM, cryo-TEM and disc centrifugation. Figure 2.1 shows TEM micrographs of ZnO 30 and sZnO 30, TiO$_2$ R and TiO$_2$ P25. ZnO 30 and sZnO 30 displayed hexagonal particle morphology with an average particle size of approximately 30 nm. TiO$_2$ R and TiO$_2$ P25 were rectangular in shape and had average particle sizes of about 40 nm and 20 nm, respectively.

Figure 2.2 shows cryo-TEM micrographs of ZnO 30, ZnO 200, sZnO 30 in media (RPMI-1640) without serum after 24 h incubation. The ZnO 30 NPs formed agglomerates in media, whilst sZnO 30 NPs did not form strong agglomerates but stayed quite dispersed in media, as demonstrated in Figure 2.2 (A) and (B), respectively.

It was difficult to evaluate the mean size and aggregation state of NPs based on TEM and cryo-TEM images alone. Therefore, a more accurate evaluation of the particle size distributions was attained using the disc centrifuge and dynamic light scattering (DLS) (Table 2.1). The disc centrifuge was used to characterise particles in media and serum after 24 h. Surfactant-treated NPs stayed well-dispersed (50-100 nm), while pristine (untreated) NPs formed large agglomerations reaching up to 2 µm in size. These dispersion and agglomeration states of the NPs were further investigated for their cytotoxic and immunologic effects toward P815 mast cells.
Figure 2.1: TEM microscopy of (A) pristine ZnO 30, (B) surfactant-treated sZnO 30, (C) titanium R, and (D) titanium P25.
Figure 2.2: Cryo-TEM micrograph of ZnO 30 (A), sZnO 30 (B) and ZnO 200 (C) in media (RPMI-1640) after 24 h incubation.
Table 2.1: Characterisation of ZnO NPs used in this study by disc centrifuge and DLS (adapted from Feltis et al., 2011).

<table>
<thead>
<tr>
<th>Particle</th>
<th>Disc Centrifuge Agglomerate Size (mean [FWHM])</th>
<th>Agglomerate Surface Area (m²/g)</th>
<th>Primary Particle Size (nm)</th>
<th>Primary Particle Surface Area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnO 30</td>
<td>1.33 µm [0.68 – 1.98 µm]</td>
<td>0.80</td>
<td>DLS: 36 ± 6 nm (TEM: 25 ± 7)</td>
<td>29.73</td>
</tr>
<tr>
<td>ZnO 80</td>
<td>1.32 µm [0.65 – 1.32 µm]</td>
<td>0.81</td>
<td>DLS: 83 ± 8</td>
<td>12.90</td>
</tr>
<tr>
<td>ZnO 200</td>
<td>1.29 µm [0.88 – 1.69 µm]</td>
<td>0.83</td>
<td>DLS: 200 – 500</td>
<td>3.06</td>
</tr>
<tr>
<td>sZnO 30</td>
<td>75 nm [50 – 100 nm]</td>
<td>14.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sZnO 80</td>
<td>90 nm [50 – 130 nm]</td>
<td>11.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sZnO 200</td>
<td>460 nm [240 – 670 nm]</td>
<td>2.33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FWHM = Peak full width at half maximum
2.3.2 ZnO NP solubility

The dialysates collected after 24 h from ZnO NPs present at the highest concentration used for cytotoxicity testing (100 µg/mL), were analysed for zinc ion levels by ICP-AAS. Figure 2.3 shows that zinc ion release increased with time and was still ongoing after 24 hours (1440 min). The solubility of smaller ZnO NPs (ZnO 30, 3.6 µg/mL; and sZnO 30, 2.8 µg/mL) were higher than for larger ZnO NPs (ZnO 200, 1.9 µg/mL; and sZnO 200, 2.3 µg/mL). Surfactant-treated sZnO 30 NPs, which were very well dispersed in media, released more zinc ions (3.6 µg/mL) than untreated ZnO 30 NPs (2.8 µg/mL). This suggests that the surfactant coating around the ZnO 30 NPs may increase zinc ion dissolution into the media.

![Figure 2.3: Solubility of ZnO nanoparticles in RPMI-1640 media was measured over 24 h (1440 min) at 37 ºC with a concentration of 100 µg/mL. Dialysate collection was analysis using ICP-AAS (Mean ± SEM of two experiments in duplicate).](image)
2.3.3 Cytotoxicity of ZnO and TiO$_2$ NPs in RPMI-1640 and DMEM complete media

MTS assays were used to assess the loss of cell viability by measuring the formation of a chromophore formazan product as an indicator for cellular mitochondrial dehydrogenase activity. Surfactant-treated ZnO NPs and untreated ZnO NPs and TiO$_2$ NPs were tested for their effect on P815 mast cell viability, in either RPMI-1640 or DMEM media for 24 h. MTS data showed a dose-dependent cytotoxicity in all tested NPs (Figure 2.4). With concentrations up to 10 µg/mL, there was no cytotoxicity observed following exposure to any of the tested NPs, and therefore this concentration has been defined as a sub-toxic dose for all cell lines in this study. Cytotoxicity after exposure of mouse mast cells to ZnO NPs was dependent on particle size, surfactant coating, composition, concentration, exposure time and growth media (i.e. RPMI-1640 or DMEM).

Mouse mast cells grown in RPMI-1640 media were more sensitive to ZnO NPs than those cells grown in DMEM media (Figure 2.5). The EC50 (effective concentration for 50% response) of ZnO 30 NPs and sZnO 30 NPs in RPMI-1640 (EC50: 25 µg/mL and 28 µg/mL, respectively) were three to sixfold lower than for mouse mast cells that grown in DMEM media (EC50:168 µg/mL and 90 µg/mL) (Table 2.2).

Smaller ZnO NPs caused more cytotoxicity than larger NPs. ZnO 200 particulates exhibited minimal cytotoxicity. In contrast, TiO$_2$ NPs exhibited less cytotoxic effect than ZnO NPs in both RPMI-1640 and DMEM media (Figure 2.6 and Figure 2.7). Surfactant-treated NPs were equitoxic to untreated NPs on cells after 24h in RPMI-1640. Interestingly, in cells cultured in DMEM, surfactant-treated NPs had
significantly increased cytotoxicity at 30 µg/mL of 70 ± 2% (p<0.01) when compared to untreated ZnO 30 NPs (EC50: sZnO 30, 90 µg/mL in RPMI media; and ZnO 30, 169 µg/mL in DMEM media).

Cell viability was investigated over a time course in order to gather information on the kinetics of the ZnO NP-induced cytotoxicity. The mouse mast cells were incubated for 4, 8, 12 and 24 h with ZnO 30 and sZnO 30 NPs at concentrations of 30 µg/mL and 100 µg/mL (Figure 2.8). These data illustrated that the cytotoxic effects became apparent at 12-24 h at the intermediate concentration of 30 µg/mL, but was earlier at between 8-24 h for the high concentration of 100 µg/mL. ZnO NP cytotoxicity in mouse mast cells increased over time, with surfactant-treated sZnO 30 NPs causing slightly more cytotoxicity than ZnO 30 NPs at shorter exposure times (12 h) at a concentration of 100 µg/mL. This cytotoxic effect was not due to surfactant alone, which was confirmed to be not cytotoxic in the concentration range tested (Figure 2.6).

In a parallel series of experiments to determine the potential role of “soluble” Zn species from ZnO NPs, each of the ZnO NPs samples were dialysed at a range of concentrations from the high toxic dose to a ten-fold higher dose (i.e. 100-1000 µg/mL) and the dialysates examined for cytotoxicity. Interestingly, negligible dialysate cytotoxicity was observed from each of the ZnO NPs (Figure 2.9).
Figure 2.4: Cytotoxicity of untreated and surfactant-dispersed ZnO nanoparticles to P815 mouse mast cells in RPMI-1640 media after 24 exposure (Mean ± SEM of three experiments in quadruplicate).

Figure 2.5: Cytotoxicity of untreated and surfactant-dispersed ZnO nanoparticles to P815 mouse mast cells in DMEM media after 24 exposure (Mean ± SEM of three experiments in quadruplicate).
Table 2.2: EC50 (µg/mL) of ZnO NPs over 24h in RPMI-1640 or DMEM cell culture media.

<table>
<thead>
<tr>
<th>EC50/24h</th>
<th>ZnO 30</th>
<th>sZnO 30</th>
<th>ZnO 80</th>
<th>sZnO 80</th>
<th>ZnO 200</th>
<th>ZnCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cells grown in RPMI</td>
<td>25 ± 2</td>
<td>28 ± 1</td>
<td>50 ± 2</td>
<td>65 ± 2</td>
<td>N/A</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>(Mean ± SEM)</td>
<td></td>
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</tr>
<tr>
<td>Mast cells grown in DMEM</td>
<td>169 ± 2</td>
<td>90 ± 2</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>(Mean ± SEM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N/A = Non-cytotoxic.

Figure 2.6: Cytotoxicity of TiO$_2$ nanoparticles and the surfactant alone to P815 mouse mast cells in RPMI-1640 media after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).

Figure 2.7: Cytotoxicity of TiO$_2$ nanoparticles to P815 mouse mast cells in DMEM media after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).
Figure 2.8: Cytotoxicity of untreated and surfactant-dispersed ZnO 30 nm nanoparticles to P815 mouse mast cells at different incubation times over 24 h, with a concentration of (A) 30 µg/ml and (B) 100 µg/mL (Mean ± SEM of three experiments in quadruplicate).
2.3.4 Reaction of ZnCl₂ precipitates in cell culture media

When solutions of ZnCl₂ were added to RPMI-1640 media alone or to mixtures of media with 10% v/v FBS at a final Zn concentration of 30 mM, a flocculent white precipitate rapidly formed (a similar precipitate was also visible when the same concentration of Zn was added to DMEM, though we have not examined the DMEM precipitate in this study). Dialysis of these reaction mixtures showed that Zn was only minimally soluble in either media (2.8 ±1 µM) or pure FBS (12 ± 2 µM). Table 2.3 shows that the equilibrium concentration of Zn species in both the media and media/FBS, sufficiently small to pass through a 2 kDa membrane, was less than 0.5% of the corresponding [Zn] level in water alone. Based on the components present in RPMI-1640 media, the predominant anions present are carbonate (24 mM) and phosphate (5.6 mM), which will most likely form insoluble precipitates with added Zn²⁺.
Transmission electron micrographs of the cryo-vitrified amorphous precipitate obtained from the addition of ZnCl$_2$ to RPMI-1640 cell culture media ([Zn] = 150 µM) showed 10 - 50 nm sized clusters of featureless particles (Figure 2.10). Zinc-containing nanoparticles of shell-like morphology (~40 nm) were observed in dried samples isolated from RPMI cell culture media, both with or without added FBS, or in neat FBS, at an initial [Zn] of 30 µM (Figures 2.11 a and c) and fused nanometre-sized agglomerates at a higher [Zn] of 150 µM (Figure 2.11b).

**Table 2.3:** Solubility of ZnCl$_2$ in water, media and media + FBS using dialysis.

<table>
<thead>
<tr>
<th>Phase</th>
<th>[Zn] (µM in dialysate)</th>
<th>% Zn remaining in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>2436 ± 20</td>
<td>~95%</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>2.8 ±1</td>
<td>0.11%</td>
</tr>
<tr>
<td>RPMI-1640/FBS (10% v/v)</td>
<td>12 ± 2</td>
<td>0.47%</td>
</tr>
</tbody>
</table>
Figure 2.10: Cryo-TEM of amorphous precipitate obtained from RPMI-1640 cell culture media with ZnCl₂ added, \([\text{Zn}^{2+}] = 150 \, \mu\text{M}\).
Figure 2.11: TEM of the dried amorphous precipitates obtained from a) RPMI cell culture media with ZnCl\(_2\) added \([\text{Zn}] = 29.5\ \text{mM}\), b) RPMI cell culture media with ZnCl\(_2\) added, \([\text{Zn}] = 0.15\text{mM}\), c) amorphous precipitate obtained from FBS with ZnCl\(_2\) added.
2.3.5 Cytotoxicity of the zinc precipitates

The following is an examination of the cytotoxicity of amorphous precipitate, (zinc-containing NPs) that was obtained from the addition of ZnCl₂ to RPMI-1640 media.

P815 cells in RPMI-1640 + 10% v/v FBS, were exposed to the precipitate via increasing concentrations of (a) ZnCl₂, (b) isolated and dried Zn precipitate or (c) an isolated form of the Zn precipitate that had been washed but not dried. This was compared to the cytotoxicity of 30 nm ZnO NPs. The cytotoxicity of the Zn species from the added ZnCl₂ solution was slightly less than that seen for 30 nm ZnO NPs (EC50 of ZnCl₂ (50 µg/mL), which was two-fold lower than the EC50 of ZnO 30 NPs (25 µg/mL), whereas both the isolated/washed, and the washed and subsequently dried Zn precipitates were at least seven-fold less cytotoxic (i.e. when comparing concentrations causing a 20% decrease in cell viability, i.e. the EC20 values) (Figure 2.12).
Figure 2.12: The viability of P815 mast cells exposed to 30 nm ZnO nanoparticles, zinc added directly as zinc chloride or the isolated non-dried and dried precipitates from media dosed with the equivalent concentration of zinc chloride for 24 h (Mean ± SEM of three experiments in quadruplicate).
2.3.6 Intracellular level of Zinc ions

The increase in intracellular Zn ion levels were measured after exposure of cells to ZnO NPs by the zinquin ethyl ester fluorescent probe. Fluorescence microscopy images showed increases in the intracellular concentration of zinc in P815 mast cells treated with ZnO NP and ZnCl$_2$ for 24 h, compared to a negative control (Figure 2.13). Within any given ZnO NP-treated cell population, two sub-populations were observed when cells were examined by flow cytometry (Figure 2.14). These P1 and P2 populations (Figure 2.15 and Figure 2.16), had higher zinc levels than negative controls (cells not treated with NPs). The cells in population P1 had higher intracellular zinc levels than the P2 population. This suggests that cells in population P1 were more sensitive to activation by the high level of ZnO NPs and had undergone apoptosis or necrosis, which led to the change in their position on the scatter plot. The P1 population showed a time-dependent (Figure 2.15) and dose-dependent (Figure 2.17) elevation in zinc ion levels when cells were exposed to ZnO NPs and ZnCl$_2$. Surfactant-treated and untreated ZnO 30 NPs appeared to equally increase the intracellular zinc ion levels in the P1 population. Whereas, surfactant-treated sZnO 30 NPs had a greater intracellular zinc ions than untreated ZnO 30 in cells of the P2 population. Smaller ZnO NPs caused a greater increase in intracellular zinc ion levels than larger NPs in both cell populations (both P1 and P2).
Figure 2.13: Photomicrographs showing the intracellular distribution of zinc using the Zinquin ethyl ester dye in P815 mast cells; control cells (“Cells”), cells exposed to ZnO 30, sZnO 30, ZnO 200, sZnO 200 and ZnCl$_2$ at 30 µg/mL for 24 h.
Figure 2.14: Flow cytometry plot (Forward scatter (FSC) vs Side scatter (SSC)) of P815 mast cells after incubation with ZnO NPs at various times show two populations (in this case after 8 h); P1 and P2. Nanoparticles and cell debris in black colour were situated far from these cell populations.

Figure 2.15: Effect of ZnO NPs on intracellular zinc ion levels in P815 mast cells after exposure to 100 µg/mL NPs for 1, 4, 8 and 24 h (population P1). Data represented as mean ± SEM of two experiments in triplicate.
Figure 2.16: Effect of ZnO NPs on the intracellular zinc ion levels in P815 mast cells after exposure to 100 µg/mL NPs for 1, 4, 8 and 24 h (population P2). Data represented as mean ± SEM of two experiments in triplicate.

Figure 2.17: Effect of ZnO NPs on intracellular zinc ion levels in P815 mast cells after exposure to two different concentrations (30 and 100 µg/mL) of NPs at a specific time point (8 h, population P1). Data represented as mean ± SEM of two experiments in triplicate.
2.3.7 Intracellular reactive oxygen species (ROS)

The production of intracellular ROS was estimated by using DCFDA, which is a non-fluorescent dye that is absorbed by cells and hydrolyzed by esterases to produce DCFH. DCFH is then oxidized to the fluorescent dichlorofluorescein (DCF) product by intracellular ROS; consequently, fluorescent intensity levels are proportional to the intracellular generation of ROS.

The effects of surfactant-treated ZnO NPs, untreated ZnO NPs and TiO₂ NPs on intracellular ROS production in P815 mast cells were investigated after a 24 h incubation. Confocal laser scanning microscopy (CSLM) images showed a rise in intracellular ROS in P815 mast cells treated with ZnO NP and ZnCl₂ after 24 h, compared to the negative control (Figure 2.18). There was a dose-dependent ROS production seen with exposure of the cells to all tested ZnO NPs and ZnCl₂ (Figure 2.19). In contrast, TiO₂ NPs did not induce ROS production. Smaller ZnO NPs caused more ROS production than the larger NPs. Even though surfactant-treated NPs were equitoxic to untreated NPs at concentrations of 10 and 60 µg/mL, untreated ZnO 30 NPs caused slightly more ROS generation at a concentration of 30 µg/mL than the surfactant-treated sZnO 30.
Figure 2.18: Photomicrographs showing the generation of intracellular reactive oxygen species (ROS) using DCFDA dye in P815 mast cells, (A) control cells; (B-H) cells exposed to ZnO 30, sZnO 30, ZnO 200, sZnO 200, ZnCl$_2$, titanium R and titanium P25 respectively at 60 µg/mL for 24 h.

Figure 2.19: Effect of ZnO and titania NPs on the generation of intracellular ROS in P815 mast cells after exposure to 10, 30 and 60 µg/mL NPs for 24 h. The ROS generation of unexposed control cells was considered to be 100%. Data represents mean ± SEM of three experiments in quadruplicate.
2.3.8 Mechanism of cell death

The mechanism of cell death was estimated by flow cytometry via the Annexin-V and propidium iodide fluorescence kit, which is capable of measuring early apoptosis and necrosis. This kit is not suitable in differentiating the late apoptosis stage from necrosis (which can result in inflammatory responses). The cell-death pathways for P815 mast cells exposed to sZnO 30 NPs, ZnO 30 and ZnCl$_2$ showed an increase in both necrosis and apoptosis compared to control cells (unexposed cells alone). ZnO NPs also induced more cell death by necrosis or late apoptosis, than early apoptotic pathways (Figure 2.20).

![Figure 2.20: Mechanism of cell death in P815 mouse mast cells after 24 h exposure to 30 µg/mL of untreated ZnO 30, surfactant-treated sZnO 30 NPs, and ZnCl$_2$ NPS (Data represents mean ± SEM of two experiments in triplicate).](image-url)
2.3.9 Synchrotron x-ray fluorescence microscopy cell imaging

Synchrotron imaging was performed to measure the distribution of ZnO NPs doped with cobalt, and TiO$_2$ NPs, into P815 mast cells. ZnO NPs were doped with cobalt to differentiate ZnO in the NP crystalline lattice from the endogenous Zn present inside the cells. As cells contain only tiny amounts of Ti, such dual-labelling is not necessary to confirm the detection of intact TiO$_2$ NPs in intracellular compartments. Images are generated from the fluorescence signal produced by the excitation of the atoms in the sample with x-rays. As calcium was present throughout the entire cell, it was used as a cytoplasmic marker in the target cells. Elemental analysis and mapping of Zn and Co, or Ti, was then used to determine the presence and location of NPs within the cells. Figure 2.21 illustrates synchrotron images of ZnO NPs doped with Co, and TiO$_2$ NPs, up taken by the P815 cells. However, in this study the positive control ZnCl$_2$ did not increase the intracellular fluorescence intensity.

2.3.10 Cytokine profiling

The cytokine production profiles from P815 mast cells when exposed to NPs were screened using the FlowCytomix Mouse Th1/Th2 10plex kit. This kit contained 10 cytokines including Th1 cytokines (IL-2, and IFN-γ), Th2 cytokines (IL-4, IL-5, IL-6 and IL-10) and proinflammatory cytokines (GM-CSF, IL-1α, IL-17 and TNF-α). The cytokine profiles were measured at both a sub-cytotoxic dose (10 µg/mL) and a cytotoxic dose approximating the EC50 (30 µg/mL for ZnO 30 and sZnO 30).
Figure 2.21: Synchrotron imaging shows ZnO NPs doped with Co, and TiO$_2$ NPs, internalized within P815 mast cells, (A) untreated cells, (B) ZnCl$_2$ (positive control), (C) ZnO NPs doped with Co, (D) TiO$_2$ NPs.
Figure 2.22 illustrates that ZnO and TiO$_2$ NPs did not stimulate expression of any of these 10 cytokines, compared to the negative control (cells alone: measuring spontaneous release of cytokines). Whereas the positive control (PMA plus ionomycin) increased production of IL-4, IL-6, IL-5 and TNF-α. LPS was also used to simulate inflammatory cytokines, but it did not have a stimulatory effect in P815 mast cells. The other six cytokines (GM-CSF, IFN-γ, IL-1α, IL-2, IL-10, IL-17) were not expressed by the NPs or the stimulatory agents (data not shown). Smaller ZnO NPs at a concentration of 30 µg/mL caused greater IL-4 and IL-6 suppression (200 pg/mL and 400 pg/mL, respectively) when compared to control cells (IL-4: 900 pg/mL and IL-6: 1200 pg/mL).

To confirm that the observed effect of ZnO NPs on IL-4 suppression was not due to NPs binding to assay components or cell death, a more sensitive IL-4 ELISA assay was performed at a sub toxic dose that did not alter the assay by binding IL-4 (10 µg/mL). This was confirmed by incubation of NPs alone with IL-4 standard dilution in RPMI-1640 cell culture media and compared to control (without NPs) - all NPs at a concentration of 10 µg/mL did not cause any alterations in the IL-4 standard curves. Surfactant-treated ZnO NPs had greater binding or adsorption to IL-4 at a concentration of 60 µg/mL, compared to the pristine NPs (Figure 2.23).
Figure 2.22: Multiplex cytokine profile of unstimulated mast cells exposed to ZnO and TiO₂ nanoparticles, (A) small ZnO NPs induced IL-4 and (B) IL-6 inhibition, (C and D) TNF-α and IL-5 were only seen in the positive control. Data represents mean ± SEM of two experiments in triplicate.
**Figure 2.23:** Shows possible ZnO and TiO$_2$ NPs binding or adsorption to the IL-4 standard after incubation for 24 hr at 37°C (Data represents mean ± SEM of two experiments in triplicate).
The inhibitory effect of ZnO NPs on IL-4 release was investigated in both unstimulated P815 mast (i.e. spontaneous release of IL-4) and PMA+ionomycin-activated P815 cells after a 24 h exposure to NPs. The sZnO30, ZnO30 and ZnO200 particulates had significant inhibitory effects (p<0.05) on IL-4 release in PMA plus ionomycin-stimulated P815 mast cells, while TiO$_2$ NPs did not demonstrate any inhibition of IL-4 response (Figure 2.24). There were no significant inhibitory effects on IL-4 release from inactivated mast cells after exposure to both ZnO and TiO$_2$ NPs (Figure 2.25).

![Figure 2.24: Inhibitory effects of ZnO NPs on IL-4 release from PMA+ ionomycin-activated P815 cells after 24 h of exposure to sub-toxic doses of ZnO and TiO$_2$ NPs (cell viability was 100% of control). Data represents mean ± SEM of two experiments in triplicate.](image-url)
Figure 2.25: Inhibitory effect of ZnO NPs on IL-4 release from unstimulated P815 cells after 24 h of exposure to NPs. Data represents mean ± SEM of two experiments in triplicate.
2.4 Discussion

To the best of my knowledge, there are no published investigations of following studies:

- Cytotoxic effects of ZnO and TiO₂ NPs in P815 mast cells;
- Comparing the cytotoxicity of ZnO NPs in two different states (agglomerated and dispersed) in P815 mast cells;
- Comparing the cytotoxicity of ZnO NPs in two different types of cell culture media (DMEM and RPMI-1640);
- Precipitation of ZnCl₂ in RPMI-1640 culture media forming Zn nanoparticles;
- Using synchrotron x-ray fluorescence microscopy to determine cellular uptake of ZnO and TiO₂ NPs; and
- ZnO NP inhibition of IL-4 production from activated P815 mast cells.

In this study the possible modes of action of ZnO and TiO₂ NPs were investigated. Figure 2.26 illustrates some of the possible mechanisms by which ZnO NPs may induce cytotoxicity. This mode of cell death may be related directly to the size, composition and dispersion of ZnO NPs, which may be taken up by cells via different pathways, for instance phagocytosis and endocytosis. Indirect cytotoxicity can occur through Zn ion release from ZnO NPs into the culture media outside the cells (extracellular) or alternately, ZnO NPs may be taken up and then dissolved inside the cells (intracellular) – both of these mechanisms would result in a high Zn ion concentration that can then damage the cells. The other possible mechanism is due to Zn ion interaction with carbonate and phosphate anions in the media to form insoluble precipitates, which may directly result in cell toxicity. ZnO NPs or Zn ions may also induce ROS generation which can result in cell death. Furthermore, the
ZnO cytotoxicity profile may be dependent on the cell type (e.g. immune or epithelial cells, adherent or non-adherent cells, cancer or non-cancerous cells), which is explored further in chapters three and four.

In this study, P815 mouse mastocytoma cells were chosen because they represent a good model to investigate immunotoxic effects. For instance, these cells are non-adherent cells, which make them easy to manipulate and analyse by flow cytometry, and these cells are also known to produce Th1 and Th2 cytokines, including IL-4 and IFN-γ. However, in this study P815 mast cells did not respond like primary mast cells to stimulation by IgE, and then challenge with multivalent antigens or the chemical substance ionomycin, to enable measurement of mast cell activation and degranulation, e.g. using as β-hexosaminidase and histamine release.

2.4.1 Cytotoxicity of ZnO and TiO₂ nanoparticles

Several studies in mammalian cells have confirmed that ZnO NPs caused cytotoxicity in a variety of epithelial and immune cell types, such as human BEAS-2B bronchial epithelial cells (Huang et al., 2010), human A549 lung epithelial cells (Karlsson et al., 2008; Lin et al., 2009; Horie et al., 2009), mouse RAW 264.7 macrophages (Xia et al., 2008) and human T lymphocytes (Reddy et al., 2007; Hanley et al., 2008). A cytotoxic dose response was observed in each of these cell lines, with ZnO NPs exhibiting a more significant cytotoxicity profile than TiO₂ NPs (Lin et al., 2009; Yang et al., 2009; Hsiao et al., 2011). Surprisingly, there are no published reports of cytotoxicity studies of ZnO and TiO₂ NPs in mast cells.
ZnO NPs → Zn^{++}

- Size, Dispersion & aggregation of NPs
- Extracellular Zn^{++} dissolved from NPs
- Intracellular Zn^{++} dissolved from NPs
- Zn^{++} interact with carbonate & phosphate anions in media and form precipitates
- Generation of ROS
- Cell type

Figure 2.26: Schematic outline of zinc oxide nanoparticle-induced cytotoxicity.
The present study investigated the cytotoxicity, ROS, apoptosis and cytokine profiles in the P815 mouse mast cells of pristine and surfactant-treated ZnO at 30, 80 and 200 nm, ZnCl₂, titania rutile at 34 nm and rutile/anatase at 25 nm.

The cytotoxicity experiments showed that a dose-dependent cytotoxicity was observed for all tested NPs (Figure 2.4 and 2.5). Mouse mast cells grown in RPMI-1640 media were more sensitive to ZnO NPs than those grown in DMEM media (Table 2.2). Surfactant-treated ZnO 30 NPs were equitoxic to uncoated NPs in the P815 cells after 24 hr in RPMI-1640 media. However, surfactant-treated ZnO 30 NPs had higher cytotoxicity in P815 cells than untreated ZnO 30 NPs in DMEM media. This difference can be assigned to the increased permeability of surfactant-treated ZnO NPs into the P815 cells with slow growth log phase in DMEM, whereas the P815 cell in RPMI media with fast growth rate, may be able to uptake equally high amount of both surfactant treated and untreated ZnO 30 NPs which caused equitoxic effects. Similar conclusions have been reach in previous studies, for example, Taccola et al. (2011) found that the mechanism of ZnO NPs cytotoxicity might relate to the cell proliferation rate. Another possibility is that the different concentration of carbonate and phosphate anions present in RPMI-1640 and DMEM media have an effect on the state of the particles in media. Sodium bicarbonate and sodium phosphate concentrations in RPMI-1640 are 200 and 80 mg/L, respectively, while their concentrations in DMEM are 3700 and 125 mg/L, respectively. These differences may have differing effects on the solubility of ZnO NPs altering their cytotoxicity.

Cytotoxicity of ZnO NPs was also dependent on the size of the NPs. Smaller ZnO NPs (30 nm) caused more cytotoxicity than the larger particulates (200 nm). This
study clearly confirmed that altering the size of ZnO particles has a great impact on their cytotoxicity. Figure 2.5 showed that the cytotoxicity of ZnO NPs were increased in the following order; 30 nm > 80 nm > 200 nm. This can result from increased uptake of smaller NPs by mast cells in comparison to larger particles, increasing the exposure of cells to the smallest nanoparticles with a larger surface area relative to mass and greater dissolution rate. These results were in agreement with previous studies (Reddy et al., 2007; Hanley et al., 2008; Nair et al., 2009) that revealed ZnO NPs had greater cytotoxicity than ZnO microparticles. However, Lin et al. (2009) and Deng et al. (2009) found that small and large NPs had similar cytotoxicity, suggesting that small NPs formed aggregates that had similar sizes to larger particles and therefore caused similar cytotoxicity. To eliminate the effect of aggregation, well-dispersed surfactant-treated ZnO NPs were employed in this study (Table 2.1), and the cytotoxicity of these surfactant-treated ZnO particles have shown a clear size dependency in the following order: sZnO 30 nm > sZnO 80 nm > sZnO 200 nm, confirming that ZnO nanoparticles are more cytotoxic as they decrease in size.

Overall, TiO$_2$ NPs (1-250 µg/mL) exhibited low cytotoxicity in both RPMI-1640 and DMEM culture media (Figure 2.6 and Figure 2.7). TiO$_2$ NPs are insoluble and may require a very high dose to initiate cell biological change. Sayes et al. (2006) illustrated that the dose of TiO$_2$ NP required for observing cytotoxicity and change in the biological function of human dermal fibroblasts cells is around 1500 µg/mL.
2.4.2 The role of dissolved zinc ion in the cytotoxicity of ZnO nanoparticles

To investigate the role of extracellular zinc ions in the cytotoxicity of ZnO NPs, each of the ZnO NPs samples were dialysed at a range of concentrations from a high toxic dose to an above toxic dose (100 -1000 µg/mL) for 24 h at 37°C. Cells were then exposed to the dialysates for 24 h and examined for cytotoxicity. Negligible dialysate cytotoxicity was observed from each of the ZnO NPs (Figure 2.9). This suggests that the solubility of ZnO NPs in cell culture was very low and that the soluble Zn ions in solution were not at sufficient concentration to induce cytotoxicity. Therefore, the cytotoxicity of ZnO NPs may require direct contact of ZnO NPs with the cells. Moos et al. (2010) also reported that direct nanoparticle to cell contact was required for toxicity. However, Song et al. (2010) found that dissolved zinc ions in the supernatant severely inhibited cell viability. However, they isolated the ZnO NPs (10 nm) from supernatants by centrifuging the sample at 20,000 rpm for 30 min, a speed that may still allow some of 10 nm NPs to remain in the supernatants, which may cause cytotoxicity.

In the current study, the ZnO NPs cytotoxicity were compared to the direct cytotoxicity of a similar concentration of Zn\(^{2+}\) (ZnCl\(_2\)). This was done to investigate if the mechanism of cytotoxicity may be attributed to solubilized Zn\(^{2+}\) ions in culture media. Small ZnO NPs (30 nm) had higher cytotoxicity that was closer to that of zinc ions (EC 50: ZnO 30, 25 µg/ mL; and Zn\(^{2+}\) ions, 42 µg/ mL) in RPMI-1640. Therefore, Zn\(^{2+}\) ions are highly likely to contribute to the cytotoxic response of ZnO NPs. The cytotoxicity caused by ZnCl\(_2\) and ZnO NPs is reported as very similar, which has lead to the suggestion that Zn dissolved from these nanoparticles is the main contributor to their cytotoxicity (Song et al., 2010).
The addition of Zn$^{2+}$ ions to cell culture media may also result in the formation of insoluble Zn species. Some very recent work has suggested that the solubility of ZnO NPs varies with different media solutions, and that precipitates do indeed form in some of these solutions (Reed et al., 2012). Most cell culture media used in biological studies (e.g. RPMI-1640) contain large concentrations of carbonate and phosphate anions, which are known to form insoluble precipitates with Zn$^{2+}$. Interestingly, when ZnCl$_2$ was used as a control for a ZnO solubility experiment in RPMI-1640, only a minute fraction (2.8 µM) of the Zn passed through a dialysis membrane. This initial finding then led to studying the precipitation of Zn under biological conditions. Examination of these precipitates showed them to consist of amorphous ~30 nm nanoparticles (Figure 2.11). However, both the isolated/washed and the washed and subsequently dried Zn precipitates were at least seven-fold less cytotoxic than ZnO 30 nm (Figure 2.12). It may be that Zn$^{2+}$ interacts with carbonate and phosphate anions in culture media, which then forms an insoluble precipitate that protects the cells from Zn ion damage.

Further information can be gained on how this precipitate interacts with cells by investigating the effect of the Zn precipitate on intracellular Zn ion levels. It is also very important to know that the cytotoxicity of ZnO NPs is due to intracellular release of Zn ions. Therefore, the contribution of intracellular zinc ions to the cytotoxicity of ZnO NPs was investigated by exposing the cells to ZnO NPs or ZnCl$_2$ and measuring the signal from the zinquin ethyl ester fluorescence probe. In this study, for all ZnO NP-treated cells, higher zinc levels relative to the negative control were observed (Figure 2.15 and Figure 2.17). Moreover, the increased level of zinc ions was dependent on NP size, the concentration of ZnO NPs, and time period of incubation. These three factors were correlated to MTS data for
cytotoxicity effects of ZnO NPs. The study indicated that the increase in intracellular solubilized zinc ion concentration may be an important factor in cell death. Therefore, the finding of this study suggested that small ZnO NPs are readily taken up by the cells, and inside the lysosomes (with their low pH), the ZnO NPs are easily dissolved to Zn ions that damage cell membranes and other cell components. Some of these ZnO NPs may also escape into the cytoplasm and cause further damage to cell organelles, mitochondria and the nucleus. Similar results have been reported by Taccola et al. (2012) who found that the hydrolysis of ZnO NPs to zinc ions within the lysosomes and their release into the cytosol resulted in cell damage. Further techniques are required to visualise the ZnO NP distribution inside the cells.

2.4.3 Generation of intracellular reactive oxygen species (ROS)

It has been suggested that the mechanism of cytotoxicity at high concentrations of ZnO NPs is their dissolution into soluble Zn$^{2+}$ and the subsequent induction of direct or indirect oxidative stress via killing the cells (De Berardis et al., 2010; Sharma et al., 2011; Taccola et al., 2011). TiO$_2$ NPs also generate free radicals (Hirakawa et al., 2004), which may damage DNA by oxidation, nitration, methylation or deamination reactions (Schins and Knaapen, 2007). The intracellular ROS data in the present study demonstrated that a dose-dependent ROS production was observed in all tested ZnO NPs and ZnCl$_2$ (Figure 2.19). In contrast, TiO$_2$ NPs did not induce ROS production, suggesting that the dose or crystalline form of the TiO$_2$ NPs used in this study may not be sufficient to induce ROS. Smaller ZnO NPs caused more ROS production than larger NPs. Correlation of the ROS, intracellular Zn ions and MTS data showed that ZnO NPs caused an increase of ROS generation, combined with an increase of intracellular Zn ions and
a decrease in cell viability. This suggests that ZnO NPs entering the cell leads to increasing intracellular Zn ion levels, as well as some ZnO NPs escaping in cytoplasm. Both the increase of intracellular Zn ions and movement of ZnO NPs into the cytoplasm, stresses the cells to generate ROS, as well as damage the mitochondrial, which can result in cell death. Thus, intracellular ROS may be generated as a consequence of cell death, but not from ZnO NPs directly.

2.4.4 Mechanism of cell death

Cell death occurs through apoptosis or necrosis. Apoptotic mechanism (programmed cell death) involves cell degradation without inflammation, whereas necrosis occurs through the release of pro-inflammatory factors that attract other immune cells and causes inflammation.

The effect of ZnO NPs on cell membrane damage (necrosis) was measured via permeability of cells to propidium iodide. Annexin V-FITC binds to the membrane phosphatidylserine (PS) that is expressed on the surface of the apoptotic cells. Therefore, the Annexin V-FITC flow cytometric assay was performed to assess cell death via apoptosis.

In this study, the sZnO 30 NPs, ZnO 30 NPs and ZnCl₂ showed an increase in necrosis and apoptosis compared to the controls (cells alone). ZnO NPs induced higher levels of necrosis than apoptosis (Figure 2.20). This could be the result of either: the late apoptotic cells, which could not be differentiated from necrotic cells; or high doses of ZnO NPs overwhelming apoptotic processes and thereby inducing a greater degree of necrosis than apoptosis. In addition, the incubation of the cells with ZnO NPs for 24 h may result in the early apoptotic signal being missed if the
cells began apoptosis relatively early after exposure. Therefore, kinetic investigation at earlier incubation time points for ZnO NP exposure of cells (for 1, 2, 4, 8 and 24 h) can give a better understanding of the relationship between cell death by apoptosis and necrosis. There are conflicting results from different studies regarding the mechanism of cell death induced by ZnO NPs. On one hand, De Berardis et al. (2010) and Lai et al. (2008) found that the apoptotic pathway was predominant. On the other hand, Hackenberg et al. (2010) reported that ZnO NPs induce cell death primarily by necrosis.

2.4.5 Visualisation of ZnO and TiO$_2$ NPs by synchrotron X-ray fluorescence microscopy

Synchrotron x-ray fluorescence was used to detect the distribution of intracellular ZnO and TiO$_2$ NPs in P815 mast cells. Whilst this was a preliminary result, ZnO and TiO$_2$ NPs appeared to be taken up by the cells and distributed in the cytoplasm (Figure 2.21). However, this technique alone cannot confirm that the NPs are not merely attached to the cell surface. Therefore to confirm this result, further study will be required, employing x-ray tomography or focussed ion-beam ablation to confirm that the NPs are within the cytoplasm.

2.4.6 Effects of ZnO and TiO$_2$ NPs on the cytokine profiles of P815 mast cells

Studying the cytokine profiles provides very important information about the changes in the immune responses when a stimulus is applied to a cell system (immune stimulation and/or inhibition). Some reports suggested that NPs are capable of inducing cytokine production in vitro (Scholer et al., 2002; Fifis et al., 2004; Vallhov et al., 2006; Motttram et al., 2007). For instance, carbon nanotubes
(CNTs) can promote allergic responses (Nygaard et al., 2009). In contrast, inhaled multiwalled carbon nanotubes (MWCNT) caused systemic immune suppression in a mice model (Mitchell et al., 2009).

The multiplex cytokine profiles in the present study (Figure 2.22) showed that ZnO and TiO$_2$ NPs did not provoke immunological responses for the ten cytokines investigated. However, inhibition of IL-4 and IL-6 by ZnO NPs was detected. The IL-4 cytokine is one of the main cytokines that is responsible for Th2 responses (Devouassoux et al., 2002). Therefore, more sensitive IL-4 ELISA assays were performed at a sub-toxic and sub-binding dose (10 µg/mL) of ZnO NPs (Figure 2.24) to confirm our findings about the immuno-inhibitory effects of ZnO NPs. The results demonstrated that sZnO30, ZnO30 and ZnO200 particulates had significant inhibitory effects on IL-4 release in the PMA plus ionomycin-stimulated P815 mast cells. This could be a result of increased intracellular zinc ions, which could lead to competitive inhibition along with calcium influx. Thus, ZnO NPs may have an essential role to inhibit undesirable Th2 allergic inflammatory response, and may be applied for treatment of allergic diseases such as asthma. Zinc oxide ointments are well known for their applications for different types of dermatitis (Heimall et al., 2012).

Other types of NPs may have diverse immunological reactions. For example, Devouassoux et al. (2002) found that diesel exhaust particulates (DEPs) increased IL-4 production from mast cells. Consequently, these NPs can shift the adaptive immune response toward an undesirable Th2 allergic inflammatory response.
In conclusion, the results in this chapter indicate that NP size, composition, concentration, time of exposure and growth media are critical in determining the degree of cytotoxicity of NPs. The solubility of ZnO NPs in cell culture media is not sufficient to induce cytotoxicity. Release of intracellular zinc ions from ZnO NPs that have been taken up by the cells, maybe represent a very important factor in cell death. ZnO NPs also demonstrated immune inhibitory effects in PMA- and ionomycin-activated P815 mouse mast cells. Finally, we observed only a very little biological reactivity with the TiO$_2$ NPs, which exhibited minimal cytotoxicity and did not cause immune inhibition in mast cells.
Chapter 3: Effects of zinc oxide and titanium dioxide nanoparticles on cytotoxicity and degranulation in RBL-2H3 rat mast cells and mouse bone marrow-derived mast cells

3.1 Introduction

Metal oxide ZnO and TiO$_2$ NPs are currently used in various commercial products, such as sunscreens and cosmetics. Many studies have investigated the cytotoxicity of ZnO and TiO$_2$ NPs in cancer cell lines, the details of which have been reviewed in section 1.5. However, there have been few studies conducted in primary (non-cancerous) cells. Aktar et al. (2012) studied the cytotoxicity of ZnO NPs in three types of cancer cells (HepG2 human hepatocellular carcinoma, A549 human lung adenocarcinoma, and BEAS-2B human bronchial epithelial cells) and two primary rat cell types (astrocytes and hepatocytes). They reported that ZnO NPs induced death of cancerous cells, whilst not causing cytotoxicity in primary cells. Similarly, Taccola et al. (2011) demonstrated that ZnO NPs induced cell death in proliferating pluripotent mesenchymal stem cells, but did not exhibit cytotoxic effects to non-proliferating osteogenically-differentiated mesenchymal stem cells. In addition, it has been observed that ZnO NPs and Zn$^{2+}$ increased the cellular uptake of anticancer drugs by a leukaemia cell line (Guo et al., 2008). Thus, cell type is clearly important when considering the cytotoxicity of ZnO NPs. However, the selective sensitivity of TiO$_2$ NPs toward cancer cells has not been investigated in the literature. Moreover, there are no published studies on the effects of ZnO and
TiO$_2$ NPs related to their potential cytotoxic and immunomodulatory effects in primary mast cells.

The RBL-2H3 rat basophilic leukemia cancer cell line and primary mouse bone marrow-derived mast cells (BMMCs) have both been widely used as models of IgE-mediated responses in mast cells (Kalesnikoff & Galli, 2011; Elbaz et al., 2012). Degranulation assays have been carried out to quantify the biological activity of sensitized mast cells with a recombinant IgE monoclonal antibody (mIgE) and challenged with multivalent antigen (Yamaki et al., 2009). The β-hexosaminidase is stored in the mast cell granules and is released parallel to histamine when mast cells are activated by cross-linking IgE with antigens (Tkaczyk et al., 2006). Therefore, β-hexosaminidase release in cell culture media is a very useful marker for assessing mast cell degranulation to evaluate mast cell activity. However, the role of β-hexosaminidase in allergic diseases is not well understood (Tomasiak et al., 2008). Histamine is another mast cell degranulation mediator that is essential for inducing the major symptoms of type I allergic responses, such as vasodilation and mucous secretion, itching, and bronchoconstriction in allergic rhinitis, atopic dermatitis and asthma, respectively (Huang et al., 2009; Yamaki et al. 2009). Figure 3.1 illustrates the mechanism of mast degranulation via mIgE.

A study by Yamaki et al. (2009) found that ZnO NPs inhibited the release of β-hexosaminidase from an anti-ovalbumin IgE + antigen-activated RBL-2H3 mast cell line, but they did not study the cytotoxic effects of ultrafine ZnO and fine ZnO particulates in these cells. Another recent study conducted by Chen et al. (2012) observed that TiO$_2$ NPs can directly stimulate histamine release from RBL-2H3
mast cells via a Ca\(^{2+}\) dependent pathway, but they did not investigate the effects of TiO\(_2\) NPs on IgE-activated RBL-2H3 rat mast cells. Neither of these studies took into consideration the effect of dispersed ZnO NPs on mast cell degranulation.

**Figure 3.1**: Mast cell degranulation; multivalent antigen binds to IgE antibodies that are attached to the IgE receptors leading to mast cell degranulation and the release of various mediators, which are responsible for allergic responses. Adapted from Kuby, 1992.

The mechanism of ZnO NP inhibitory effects on mast cells is still unclear; with some studies demonstrating that these are related to Ca\(^{2+}\) ion inhibition. For example, Hide and Beaven (1991) found that zinc inhibited IgE-activated mast cell degranulation through inhibition of Ca\(^{2+}\) influx. However, there are conflicting reports about the interaction between ZnO NPs and Ca\(^{2+}\) ions, e.g. Yamaki et al. (2009) explained that ZnO NP inhibitory effects were not due to Ca\(^{2+}\) ion inhibition, but were a result of the inhibition of protein phosphorylation.
The purpose of the study described in this chapter was to investigate the effects of size and dispersion state of ZnO NPs, ZnCl$_2$ (as a control for Zn$^{2+}$ ions), and two different crystalline forms of TiO$_2$ NPs, on cytotoxicity and degranulation in both the RBL-2H3 cancer cells and the non-cancerous mouse BMMCs.
3.2 Materials and Methods

3.2.1 Chemicals and Reagents

This study used the following: 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA); TIB-142 IgE monoclonal antibody (mlgE) – a gift of Dr Graham Mackay, The University of Melbourne; release buffer (1×Hanks buffer, 0.14% NaHCO$_3$, 10 mM HEPES, 5.5 mM glucose, 0.05% BSA, 0.73 mM MgSO$_4$, 1.8 mM CaCl$_2$, pH 7.3); 2,4,6-trinitrophenyl hapten conjugated to Bovine Serum Albumin protein (TNP-BSA) (BioSearch technology, USA); Triton-X 100, p-nitrophenyl N-acetyl-β-D-glucosaminide, and glycine (Sigma Aldrich, USA); Fluo-4, AM fluorescence probe (Invitrogen) and histamine-ELISA kit (Life Science Format, Oxford Biomedical Research, USA).

Protein gel reagents included (details shown in Appendix 1): a separating gel (12.5%), stacking gel (4%), 5x SDS sample buffer, 10x SDS electrophoresis buffer (Tris – glycine), Coomassie stain (0.1% w/v), destaining solution (10% v/v ethanol and 10% v/v acetic acid), Bradford reagent, and x10 Tris Buffer Saline (TBS).

Western blot items included: the blocking solution of 5% (w/v) skim milk (Bonlac Foods Limited, Australia) in TBS; western blot primary antibodies of anti-phospho-Akt (Thr308) mouse mAb, phospho-Akt (Ser473) mouse mAb and Akt (pan) mouse mAb; western blot secondary antibody of anti-mouse IgG (whole molecule) – peroxidase antibody produced in rabbit (Sigma-Aldrich); protein standard of “Precision plus” prestained standard (Biorad), stored at -20°C; wash buffer of 25 mM Tris, pH 8.0, 0.5 M NaCl, 80 mM imidazole (Sigma), pH 8.0; extraction reagent of Phosphosafe™ extraction reagent (Novagen, USA); iBlot® Dry Blotting System (Invitrogen); western blot developing solution using chemiluminescence substrate
(Sigma-Aldrich); the developer and fixer (Kodak, USA); and the photosensitive films (Amersham Bioscience, Germany).

3.2.2 Nanoparticle characterization and preparation

The methodology used is described previously in section 2.2.3.

3.2.3 Cell culture

In the present study, two different types of mast cells were used, i.e. the cancerous RBL-2H3 cells and the non-cancerous (primary cells) BMMCs.

3.2.3.1 Culture of RBL-2H3 mast cells

The RBL-2H3 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 media containing 10% v/v FBS with gentamycin, glucose, pyruvate, 2 mercaptoethanol and L-glutamine supplementation, maintained in a humidified incubator at 37°C and 5% CO₂. Cells were maintained in 75 cm² culture flasks containing media at 37°C in 5% CO₂ incubator. When cultures reached 90% confluence, cells were detached by 0.05% trypsin-EDTA then neutralized with media. A small sample of cells was taken for counting cell number using trypan blue (Sigma-Aldrich, MO, USA) exclusion (to indicate cell viability) and a haemocytometer. Then a 1/10 dilution of cells to media was added to a new 75 cm² culture flask, and the sub-cultured cells were passaged every two days.
3.2.3.2 Preparation of BMMCs

All mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation. Bone marrow cells from the C57Bl6 mice were flushed from the femurs using RPMI-1640 and a 1 mL syringe and 20G needle (Terumo). Cells from each mouse were cultured independently in RPMI-1640 medium, supplemented with 10% v/v FBS, gentamycin, glucose, pyruvate, 2-mercaptoethanol, L-glutamine and IL-3 (5 ng/mL). The cells were cultured in a 5% CO₂ humidified atmosphere at 37°C. The cells in suspension were removed from the adherent cells in the flask, centrifuged and replenished with fresh media and transferred to a fresh flask every week. After 5 weeks of culture, cells were phenotypically characterised via FACS analysis, by measuring CD117 and FcεRI expression. Experiments were conducted on wild-type (WT) BMMCs that were age matched and cultured under identical conditions. Prior to all experiments, cell viability was assessed using trypan blue exclusion. Cultured cells with >90% viability were used in experiments.

3.2.4 Cytotoxicity assays

The RBL-2H3 cells were harvested via incubation with trypsin and EDTA, and then resuspended in medium. The RBL-2H3 and BMMCs mast cells were centrifuged (200 g, 5 min) and resuspended in fresh RPMI-1640 media before seeding in 96-well plates at 10⁵ cells/well. These cells were incubated in quadruplicate wells of 96-well plates with a concentration range of ZnO and TiO₂ NPs at 0, 10, 20, 30, 60, 100, 150 and 200 µg/mL in a humidified incubator at 37°C and 5% CO₂ atmosphere. Cytotoxicity was measured at 24 h by adding a soluble tetrazolium salt (MTS, Promega, USA) to the wells 2 h prior to the optical absorbance measurement at 490 nm on a microplate reader (Perkin Elmer EnSpire®,
Waltham, MA, USA). MTS was used to evaluate the mitochondrial function of cells, and its reduction to a chromophore is proportional to the number of viable cells in the well. The ZnO and TiO$_2$ NPs were measured alone in the presence of MTS reagent, as a control for any optical density effects from the NPs. These values were subtracted from the experimental readings. Each experiment was repeated in quadruplicate and the average and standard error of mean of viability results calculated.

3.2.5 Measurement of β-hexosaminidase

The RBL-2H3 cells were treated in two different ways; the first by incubating the RBL-2H3 cells with a range of particulates (10, 30 or 60 µg/mL) and TIB-142 mouse IgE (mlgE; 300 ng/mL) in 96 wells plates ($10^5$ cells/well) in a humidified 5% CO$_2$ incubator at 37°C for 24 h. In the second method, RBL-2H3 cells were incubated with mlgE (300 ng/mL) solely in 96 wells plates ($10^5$ cells/well) for 20 h. The cells were then washed with media to remove mlgE and then incubated with a range of particles for 4 h. In both cases, the cells were washed twice in release buffer (1×Hanks buffer, 0.14% NaHCO$_3$, 10 mM HEPES, 5.5 mM glucose, 0.05% BSA, 0.73 mM MgSO$_4$, 1.8 mM CaCl$_2$, pH 7.3), and stimulated with 100 ng/mL 2,4,6-trinitrophenyl hapten conjugated to bovine serum albumin protein (TNP-BSA) for 45 min at 37°C. The same procedure was followed, but without the addition of the stimulant (TNP-BSA), to investigate the effect of NPs on spontaneous release.

The BMMCs were pre-incubated with mlgE in the tissue culture flask for 3 days because these cells required more time to sensitize with mlgE. The cells were then incubated with a range of particulates in 96 well plates ($10^5$ cells/well) for 24 h. The
cells were washed twice in release buffer, and stimulated with 10 ng/mL TNP-BSA for 45 min at 37°C.

The β-hexosaminidase production was measured in both RBL-2H3 and BMMCs as follows: spontaneous levels of β-hexosaminidase release were measured in cells incubated with Hanks release buffer only, whereas total β-hexosaminidase release was determined through lysing cells with Triton-X 100 (0.1%) solution. After the cells were incubated with TNP-BSA in release buffer for 45 mins at 37°C, the plate was centrifuged (200 g, 5 mins). A 50 µL aliquot of the supernatant was stored at -80°C for histamine analysis and another 50 µL transferred to a new 96-well flat-bottom plate (BD Falcon) and 50 µL of p-nitrophenyl N-acetyl-β-D-glucosaminide (pNAG; 2 mM in phosphate/citrate buffer, pH 4.5) added and incubated for 90 min at 37°C. The enzymatic reaction was terminated by adding 100 µL of 0.2 M glycine solution (pH 10.7, Sigma). Absorbance was measured using a microplate reader (Perkin Elmer EnSpire®, Waltham, MA, USA) at 405 nm. The percentage of β-hexosaminidase release was calculated as:

\[
\text{Release (\%) = } \frac{\text{Test}}{\text{Total}} \times 100
\]

Cytotoxicity in the remaining cells was measured after discarding the release buffer and adding 200 µL of pre-warmed media to the cells in the wells with soluble tetrazolium salt (MTS, Promega, USA), 2 h prior to the optical absorbance measurement at 490 nm using a microplate reader. The cells were washed twice with release buffer before adding TNP-BSA to remove non-binding mIgE as well as the NPs. The supernatants were also centrifuged after incubating the cells with TNP-BSA to remove any NPs remaining before measuring the β-hexosaminidase release.
A range of NPs alone were titrated against supernatant containing β-hexosaminidase for 45 min (the time of β-hexosaminidase release experiment) at 37°C, in order to measure whether any NPs bound to (or interfered with) the β-hexosaminidase assay components.

### 3.2.6 Histamine detection

The -80°C stored supernatant was thawed and the level of histamine production was measured by using a histamine – ELISA kit, as per the manufacturer’s instructions (Life Science Format, Oxford Biomedical Research, USA). Optical absorbance was measured using a microplate reader at 450 nm. Triplicate samples were generated from two experiments for each treatment.

Nanoparticles alone were titrated against the histamine-containing supernatant to investigate the possibility of histamine absorption or binding onto the NPs after the 45 min incubation period.

### 3.2.7 Measurement of intracellular level of zinc and calcium ions

The levels of intracellular level of Zn$^{2+}$ and Ca$^{2+}$ ions were estimated by zinquin ethyl ester (Sigma-Aldrich) and Fluo-4 AM (Invitrogen) fluorescence probes, respectively. The RBL-2H3 mast cells were incubated in triplicate wells of 96-well plates with 300 ng/mL mlgE and 10 µg/mL ZnO NPs in a humidified 5% CO$_2$ incubator at 37°C for 24 h. The cells were then washed twice in release buffer and stimulated with 100 ng/mL TNP-BSA for 45 min at 37°C, except for wells used to measure the spontaneous release from cells alone.
Intracellular levels of Zn\(^{2+}\) and Ca\(^{2+}\) ions were measured by adding zinquin ethyl ester (25 µM) and Fluo-4, AM (5 µM) and incubating the cells for a further 30 min prior to analysis. Finally, the cells were washed twice in PBS and the fluorescence intensity was measured at excitation/emission wavelengths of 364/485 nm for zinquin, and 485/520 nm for Flou-4 Am, using flow cytometry (FACS Canto II, Becton, Dickinson and Co., Franklin Lakes, NJ, USA).

3.2.8 Western blot analysis for Akt and tyrosine protein phosphorylation

3.2.8.1 Cell culture

The RBL-2H3 cells were seeded into 6-well culture plates at 1.5 x 10\(^6\) cells/well for 24 h in a humidified incubator (5% CO\(_2\) and 37°C). The cells were then washed in media and 30 µg/mL of ZnO and TiO\(_2\) NPs were added to the cells and incubated for a further 30 min period.

3.2.8.2 Cell extraction

The RBL-2H3 cells were washed twice with ice-cold PBS and lysed by phosphosafe extraction reagent (Novagen), as per the manufacturer’s instructions. The solution was then collected and centrifuged (4°C; 16,000 g; 5 min) and stored at -80°C.

3.2.8.3 Determination of the protein concentration by Bradford Method

Albumin standard solutions of 3, 6, 9, 12, 15, 18 and 21 µg/100 µL in 0.15 M NaCl were prepared. Twenty microlitres of each protein solution was put in the wells and diluted to 100 µL using 0.15 M NaCl. One millilitre of Coomassie Blue reagent was
added to each tube, mixed and left for 2 min at room temperature. The samples were aliquoted (200 µL) in duplicate into 96-well microtitre plates and read on a microplate reader at 600 nm.

3.2.8.4 Western blot
Equal amounts of extracted protein from the different samples were calculated and used in the western blots. The protein lysates were reduced in SDS sample buffer (0.05 M Tris-HCL, 0.1% w/v SDS, 10% v/v glycerol, and 50 mM dithiothreitol), and separated by SDS-polyacrylamide gel electrophoresis, and then the resolved proteins were transferred onto cellulose membranes (iBlot® Dry Blotting System; Invitrogen). The membranes were blocked in 5% (w/v) skim milk in TBS/0.05% Tween 20 for 1 h and then coated with primary antibodies; anti-phospho-Akt (Thr308) mouse mAb, anti-phospho-Akt (Ser473) mouse mAb, anti-Akt (pan) mouse mAb and β-actin mouse mAb (Cell Signalling Technologies) overnight at 4 °C. Consequently, membranes were washed three times in TBS with 0.05% Tween 20 and incubated with the anti-mouse IgG peroxidase secondary antibodies (Sigma-Aldrich) at room temperature for 1 h, and then washed three times with TBS Tween 20. The membranes were incubated with chemiluminescence substrate (Sigma Aldrich). Immunoreactive protein phosphorylation was detected by the enhanced chemiluminescence technique (Lopata et al., 2005).

3.2.9 Statistical analysis
Statistics were performed using the same methods described in the section 2.2.13.
3.3 Results

3.3.1 Cytotoxicity of ZnO and TiO$_2$ NPs in RBL-2H3 and BMMC mast cells

Surfactant-treated sZnO NPs, untreated ZnO NPs and TiO$_2$ NPs were tested in cell culture for their effects on mast cell viability and proliferation. RBL-2H3 and BMMC mast cells were incubated with a range of NPs (1-250 µg/mL) in RPMI-1640 for 24 h. In RBL-2H3 cells with NP concentrations up to 30 µg/mL, no cytotoxicity was seen with any of the tested ZnO and TiO$_2$ NPs (i.e. sub-toxic dose). Small ZnO NPs (30 nm) had greater cytotoxic effects than large ZnO particulates (200 nm). Surfactant-treated ZnO 30 had more significant cytotoxicity at a concentration of 60 µg/mL compared to untreated ZnO 30 (p<0.001). Surfactant-treated ZnO 30 had greater cytotoxic effects than all of the other ZnO NPs and ZnCl$_2$ (Figure 3.2). In BMMC cells, with concentrations up to 60 µg/mL, cytotoxicity was not seen with any of the tested ZnO and TiO$_2$ NPs (i.e. sub-toxic dose). Interestingly, all untreated ZnO NPs (including ZnO 30) did not have cytotoxic effects in BMMC mast cells, whilst surfactant-treated sZnO 30 showed significant cytotoxicity at a concentration 100 µg/mL (p<0.001) to BMMC cells. Moreover, sZnO 30 caused cell proliferation at a concentration of 60 µg/mL before inducing BMMC cell death (Figure 3.3).

ZnO NPs had different cytotoxic effects between RBL-2H3 and BMMC cells. Table 3.1 shows that RBL-2H3 cells were more sensitive to sZnO 30 NPs than BMMCs. The EC50 of sZnO 30 NPs in RBL-2H3 cells was 57 µg/mL, which was two-fold more sensitive than that of BMMC mast cells (EC50:122 µg/mL). The EC50 of untreated ZnO 30 NPs in RBL-2H3 cells was 187 µg/mL, but ZnO 30 NPs did not cause cytotoxicity to BMMCs. ZnCl$_2$ was cytotoxic to both RBL-2H3 and BMMC
cells and caused higher cytotoxicity to RBL-2H3 (EC50 = 114 µg/mL in RBL-2H3 and 140 µg/mL in BMMCs). In contrast, large ZnO particulates (ZnO 200) were not cytotoxic to BMMCs and RBL-2H3 cells. Furthermore, TiO₂ NPs did not induce cytotoxic effects toward RBL-2H3 or BMMCs cells (Figure 3.4 and Figure 3.5).

**Figure 3.2:** Cytotoxicity of untreated and surfactant-treated ZnO NPs in cultured RBL-2H3 rat mast cells after 24h exposure (Mean ± SEM of three experiments in quadruplicate).

**Figure 3.3:** Cytotoxicity of untreated and surfactant-treated ZnO NPs in BMMCs after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).
Table 3.1: EC50 (µg/mL) of ZnO NPs in RBL-2H3 and BMMC cells after 24 h.

<table>
<thead>
<tr>
<th>EC50 at 24 h</th>
<th>ZnO 30</th>
<th>sZnO 30</th>
<th>ZnO 200</th>
<th>sZnO 200</th>
<th>ZnCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBL-2H3 mast cells (Mean ± SEM)</td>
<td>187 ± 4</td>
<td>57 ± 3</td>
<td>N/A</td>
<td>N/A</td>
<td>114 ± 4</td>
</tr>
<tr>
<td>BMMC mast cells (Mean ± SEM)</td>
<td>N/A</td>
<td>122 ± 1</td>
<td>N/A</td>
<td>N/A</td>
<td>142 ± 1</td>
</tr>
</tbody>
</table>

N/A = Non-cytotoxic

Figure 3.4: Effect on cell viability of TiO₂ NPs in RBL-2H3 cells after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).

Figure 3.5: Effect on cell viability of TiO₂ NPs on BMMCs cells after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).
3.3.2 Effect of ZnO and TiO\textsubscript{2} NPs on $\beta$-hexosaminidase release from RBL-2H3 and BMMCs mast cells

Rat RBL-2H3 and BMMC mast cells were sensitized with TIB-142 IgE mAb in the presence or absence of ZnO particulates, TiO\textsubscript{2} NPs or ZnCl\textsubscript{2} for 24 h, and then challenged with TNP-BSA hapten to stimulate degranulation. Figure 3.6 and 3.7 illustrate that the release of the degranulation product, $\beta$-hexosaminidase was inhibited by ZnO NPs in both RBL-2H3 and BMMC mast cells. At a concentration of 30 µg/mL, both dispersed and pristine 30 nm ZnO NPs inhibited $\beta$-hexosaminidase release more than the bulk ZnO particulates; while sZnO30 was more inhibitory than ZnO 30 NPs to RBL-2H3 cells. In contrast, TiO\textsubscript{2} NPs did not demonstrate any inhibition of degranulation, but instead caused a slight but significant increase ($p<0.01$) in $\beta$-hexosaminidase from mast cells.

The RBL-2H3 cells were sensitized with mlgE mAb for 20 h, mlgE was then washed (to remove excess mlgE that might bind to NPs) and incubated with ZnO, TiO\textsubscript{2} NPs and ZnCl\textsubscript{2} for 4 h and challenged with TNP-BSA. Figure 3.8 shows that the inhibitory effects of ZnO NPs after a 4 h incubation were similar to the inhibitory effects of ZnO NPs after a 24 h incubation in RBL-2H3 cells.

The effects of ZnO, TiO\textsubscript{2} NPs and ZnCl\textsubscript{2} on spontaneous $\beta$-hexosaminidase release from RBL-2H3 cells without the addition of the stimulant (TNP-BSA), confirmed that basal $\beta$-hexosaminidase was also inhibited by ZnO NPs, and again that TiO\textsubscript{2} NPs caused a significant increase ($p<0.01$) (Figure 3.9).

The cell viability was not affected in these experiments as the particulate concentrations used were sub-toxic (i.e. 10 and 30 µg/mL). Therefore, $\beta$-
hexosaminidase inhibition was not induced by NP-induced cell death. Furthermore, a range of NPs alone were titrated against supernatant containing β-hexosaminidase in order to account for NPs binding to β-hexosaminidase assay components. All NPs at concentrations of 10 and 30 µg/mL did not cause any alterations in the detection of β-hexosaminidase concentration levels (Figure 3.10).

Figure 3.6: Inhibitory effects of ZnO NPs on β-hexosaminidase release from IgE/antigen-activated RBL-2H3 cells after 24 h of exposure to NPs (Mean ± SEM of two experiments in triplicate).
Figure 3.7: Inhibitory effects of ZnO NPs on β-hexosaminidase release from IgE/antigen-activated BMMC cells after 24 h of exposure to NPs (Mean ± SEM of two experiments in triplicate).
Figure 3.8: Inhibitory effects of ZnO NPs on β-hexosaminidase release from IgE-activated RBL-2H3 cells after 4 h of exposure to NPs (Mean ± SEM of two experiments in triplicate).
Figure 3.9: Inhibitory effects of ZnO NPs on β-hexosaminidase release from non-activated RBL-2H3 cells after 24 h of exposure to NPs (Mean ± SEM of two experiments in triplicate).

Figure 3.10: ZnO and TiO$_2$ NPs did not bind to β-hexosaminidase or the assay components at concentrations of 10 and 30 µg/mL (Mean ± SEM of two experiments in triplicate).
3.3.3 Effect of ZnO and TiO\textsubscript{2} NPs on histamine release from RBL-2H3 mast cells

The RBL-2H3 rat mast cells were sensitized with mIgE mAb in the presence or absence of ZnO particulates, TiO\textsubscript{2} NPs and ZnCl\textsubscript{2} for 24 h and challenged with TNP-BSA to stimulate mast cell degranulation. The effect of NPs on histamine release was investigated by histamine ELISA kits. Small ZnO NPs strongly inhibited mIgE-mediated histamine release in a concentration-dependent manner (Figure 3.11). These small ZnO NPs caused more effective histamine inhibition than larger ZnO in triplicate when compared to the positive control. Surfactant-treated sZnO 30 caused more a significant histamine inhibition at the concentration of 30 µg/mL (p<0.001) than untreated ZnO 30 NPs. Interestingly, ZnCl\textsubscript{2} slightly increased histamine release at 30 µg/mL, and then strongly inhibited histamine at concentrations of 60 µg/mL. In contrast, TiO\textsubscript{2} NPs did not demonstrate any inhibition of histamine release.

The cell viability unaltered from control levels for all ZnO NPs doses (10, 30 and 60 µg/mL) except sZnO 30, which at a concentration of 60 µg/mL displayed cytotoxicity, and therefore this concentration data point was excluded from the graph. In addition, a range of NPs alone were titrated against supernatant containing histamine in order to account for any NP binding to components of the histamine assay. All NPs across the experimental concentration range did not cause any alterations in the detection of histamine concentration levels (Figure 3.12). This suggests that the inhibition of histamine release by ZnO NPs was not due to cell toxicity or NPs binding to histamine.
Figure 3.11: Inhibitory effects of ZnO NPs on histamine release from IgE/antigen-activated RBL-2H3 cells after 24 h of exposure to NPs (Mean ± SEM of two experiments in triplicate).

Figure 3.12: ZnO and TiO$_2$ NPs did not bind to histamine or assay components at concentrations of 10, 30 and 60 µg/mL (Mean ± SEM of two experiments in triplicate).
3.3.4 Intracellular level of zinc and calcium ions

The intracellular Zn$^{2+}$ and Ca$^{2+}$ ion levels were measured after ZnO NP exposure of the RBL-2H3 cells that had been challenged with TNP-BSA. The Zn$^{2+}$ and Ca$^{2+}$ ions levels were detected using zinquin ethyl ester (Zn-specific) and Fluo-4 Am (Ca-specific) fluorescence probes that were analysed by flow cytometry (Figure 3.13). All ZnO NP-exposed cells exhibited a strong increase in intracellular Zn$^{2+}$ compared to control stimulated cells (Figure 3.14 and 3.15). Surfactant-treated and untreated ZnO 30 NPs had equal effect, with both causing an increase in intracellular Zn$^{2+}$ ions. Smaller ZnO NPs caused a greater increase in intracellular Zn$^{2+}$ ions than larger ZnO particulates. Figure 3.16 illustrates the effect of ZnO NPs on intracellular Ca$^{2+}$ concentration; the histogram shows that the fluorescence intensity was decrease by ZnO NPs compared to the stimulated cells. The intracellular Ca$^{2+}$ levels were significantly inhibited by small ZnO NPs, ZnO 200 and ZnCl$_2$ (p<0.05) (Figure 3.17). The 30 nm ZnO NPs caused a greater decrease of intracellular Ca$^{2+}$ than the other ZnO particulates.

Figure 3.13: Flow cytometry plot (Forward scatter (FSC) vs Side scatter (SSC)) of RBL-2H3 mast cells (A), and after incubation with ZnO NPs at 10 µg/mL for 24 h (B) and challenged with TNP-BSA. (P1; population 1 cells had two different phenotypic sizes which did not alter by the low exposure concentration of 10 µg/mL of ZnO NPs).
Figure 3.14: The effects of ZnO 30 (B), sZnO30 (C), ZnO 200 (D), sZnO200 (E) and ZnCl$_2$ (F) on the intracellular Zn$^{2+}$ concentration in RBL-2H3 cells, after challenge with TNP-BSA. Fluorescence intensity was increased by ZnO NPs compared to the stimulated cells (A).

Figure 3.15: Effects of ZnO NPs on intracellular Zn$^{2+}$ ions in RBL-2H3 mast cells after exposure to 10 µg/mL NPs for 24 h and challenged with TNP-BSA. Data is represented as the mean ± SEM of two experiments in triplicate.
Figure 3.16: The effect of ZnO 30 (B), sZnO30 (C), ZnO 200 (D), sZO200 (E) and ZnCl$_2$ (F) on the intracellular Ca$^{2+}$ concentration in RBL-2H3 cells, after challenge with TNP-BSA. Fluorescence intensity was decreased by ZnO NPs compared to stimulated cells (A).

Figure 3.17: Effects of ZnO NPs on intracellular Ca$^{2+}$ ions in RBL-2H3 mast cells after exposure to 10 µg/mL NPs for 24 h and challenge with TNP-BSA. Data is represented as the mean ± SEM of two experiments in triplicate.
3.3.5 The effect of ZnO and TiO$_2$ nanoparticles on the level of Akt in RBL-2H3 cells.

The effect of ZnO and TiO$_2$ NPs on the common phosphorylation pathway through threonine and serine residues was investigated in this study using the western blot technique. The Akt enzyme is essential in regulating cell survival and apoptosis. Full activation of Akt occurs through the phosphorylation of its threonine 308 and serine 473 residues. Figure 3.18 illustrates that a high level of Akt phosphorylation through serine 473 residues was induced by surfactant-treated and untreated ZnO 30 NPs in non-IgE/antigen-stimulated RBL-2H3 cells. More interestingly, surfactant-treated ZnO 30 NPs induced strong Akt phosphorylation at both serine 473 and threonine 308 residues. Both ZnCl$_2$ and ZnO 200 caused a slight expression of pAkt Ser 473. In contrast, the surfactant-treated large ZnO 200 particulates and TiO$_2$ NPs did not exhibit an increase in Akt phosphorylation. A 50 µM concentration of PI3 Kinase Inhibitor was used as an experimental control to inhibit the expression of pAkt phosphorylation that was induced by ZnO 30 NPs (Figure 3.19).
Figure 3.18: Effect of ZnO and TiO$_2$ NPs on Akt phosphorylation. Non-antigen/IgE-stimulated RBL2H3 cells were exposed to ZnO and TiO$_2$ NPs for 30 min. Phosphorylated-Akt through serine (pAkt Ser) or through threonine amino acid, were determined by western blot. Both untreated ZnO 30 and surfactant-treated sZnO 30 NPs increased the level of phosphorylation of pAkt Ser 473. Whereas, sZnO30 was the only particulate that increased the level of pAkt Thr 308.

Figure 3.19: The PI3 Kinase Inhibitor inhibited the phosphorylation of pAkt induced by ZnO 30 NPs, and was used here as an experimental control.
3.4 Discussion

To the best of my knowledge, the current study is the first to compare the cytotoxicity and allergic immunological inhibitory effects of ZnO NPs in their agglomerated and dispersed states. The effects of these compounds were compared with two different crystalline forms of TiO$_2$ NPs for their abilities to induce cell death and inhibit degranulation in both RBL-2H3 mast cells and primary BMMC mast cells.

The present study demonstrated that untreated ZnO 30 nm NPs were cytotoxic to the RBL-2H3 cancer cells at concentrations ranging from 100 to 250 µg/mL (Figure 3.2). However, untreated ZnO 30 nm NPs did not demonstrate cytotoxic effects in normal BMMCs at concentrations up to 250 µg/mL (Figure 3.3). Therefore, ZnO NPs may have a degree of selective cytotoxicity toward certain cancer cells over normal cells. A similar conclusion has been reached in previous studies. For instance, Aktar et al. (2012) and Hanley et al. (2008) found that ZnO NPs were cytotoxic to cancerous cells, but did not cause cytotoxicity in primary cells (astrocytes, hepatocytes and T cells). The cytotoxicity in RBL-2H3 cells is maybe due to the increased uptake of particles, because of their rapid growth compared to the slower growth of BMMC primary cells. This finding supports the previous results in section 2.4.1, in which P815 mast cells grown in DMEM culture media were more tolerant to ZnO NPs than the highly proliferating P815 mast cells grown in RPMI-1640 media. These results are in agreement with Hanley et al. (2008), who reported that ZnO NPs induced cytotoxicity in proliferating and activated T cells, while quiescent T cells displayed the most resistance to ZnO NPs.
Surfactant-treated sZnO 30 NPs were the only ZnO NPs showing significant cytotoxicity (p<0.01) toward BMMCs. The cytotoxicity level from treated sZnO NPs was increased in the adherent RBL-2H3 cancer cells in comparison to the untreated ZnO 30 NPs. This may suggest that the surfactant is increasing the permeability of the cell membrane toward ZnO 30 NPs, or that the surfactant’s ability to decrease the particulate aggregation is allowing the NPs to maintain their small size, and hence the more effective cellular uptake. However, surfactant-treated sZnO 30 NPs were equitoxic to untreated ZnO 30 NPs in the non-adherent cancerous P815 mast cells when in the same RPMI-1640 growth media (section 2.4.1). Thus, the cell type, i.e. adherent or non adherent cells, are another important factor that should be considered when studying NP cytotoxicity. Interestingly, ZnCl$_2$ demonstrated strong cytotoxicity in both cell types (cancerous RBL-2H3 and normal BMMC cells); this could be due to the very high concentration of Zn$^{2+}$ ions present in both the culture media and intracellularly, which can result in cellular damage.

The RBL-2H3 and BMMC cells were also used to assess the effect of NPs on mast cell degranulation markers ($\beta$-hexosaminidase and histamine) in vitro, in order to study their potential to interact with allergic responses. Primary BMMC mast cells have an advantage over tumour mast cells, in which BMMCs can be transferred to mast cell-deficient c-kit-mutant mice (mast cell knock-in mice) to study the specific mast cell mediators in vivo for comparison with wild-type mice (Kalesnikoff & Galli, 2011). Therefore, BMMC in vitro studies help enable correlation with future in vivo studies of NP actions on mast cells.
The current study demonstrated that β-hexosaminidase and histamine were highly inhibited by ZnO NPs from IgE-activated RBL-2H3 and BMMC mast cells after 24 h of incubation (Figure 3.6, 3.7 and 3.11). Small ZnO 30 NPs inhibited β-hexosaminidase and histamine release more than bulk ZnO particulates. ZnO NPs also inhibited spontaneous β-hexosaminidase release from non-activated RBL-2H3 cells. More interestingly, surfactant-treated sZnO 30 showed the greater inhibition of β-hexosaminidase and histamine than untreated ZnO 30 NPs in IgE-activated RBL-2H3 cells. Activation of mast cells via IgE/antigen cross-linking causes an increase in Ca^{2+} influx, which leads to an increase of free cytosolic Ca^{2+} that triggers mast cell degranulation. Therefore, it is very important to evaluate the effect of Zn^{2+} ions on Ca^{2+} ion influx after incubation of the mast cells with ZnO NPs.

The impact of ZnO NPs on intracellular Zn^{2+} and Ca^{2+} levels in IgE-activated RBL-2H3 cells were investigated. In this study, we found that there was an inverse correlation between Zn^{2+} and Ca^{2+} ions. For all tested ZnO NPs, intercellular Zn^{2+} levels were increased (Figure 3.15). In contrast, ZnO NPs diminished intercellular Ca^{2+} levels (Figure 3.17). In addition, these elevations in Zn^{2+} ions and reductions in Ca^{2+} ions were particle size-dependent, with small ZnO NPs having a more significant impact on intracellular Zn^{2+} and Ca^{2+} levels than larger particulates. This finding suggests that dissolved Zn^{2+} ions play an important role in the inhibition of mast cell immune mediators (β-hexosaminidase and histamine) via diminished Ca^{2+} influx, which inhibits mast cell degranulation. However, surfactant-treated sZnO 30 NPs induced the strongest inhibition, but had a lower Ca^{2+} inhibition (p<0.05) and equal intercellular Zn^{2+} levels to untreated ZnO 30 NPs. Therefore, there are other possible mechanisms causing surfactant-treated ZnO 30 NPs to
induce the strongest inhibition of mast cell degranulation. This could be due to surfactant-treated sZnO 30 NPs having a different impact on the cell signalling pathways. Moreover, surfactant-treated sZnO 30 NPs may escape from the cytoplasm and interact with organelle compartments such as the endoplasmic reticulum, Golgi apparatus and secretory granules, and thereby interfere with the mast cell degranulation process.

Herein, ZnCl₂ exhibited a significant inhibitory effect on both β-hexosaminidase at 30 µg/mL and histamine at a high dose of 60 µg/mL (p<0.05). This confirms that Zn²⁺ ions may have an inhibitory effect on mast cell degranulation. Similar results have reported that Zn²⁺ ions inhibited IgE-activated mast cell degranulation through inhibition of Ca²⁺ influx (Hide and Beaven, 1991: Musset et al., 2008).

The TiO₂ NPs did not demonstrate any inhibition of histamine, but instead caused a significant increase in β-hexosaminidase release (p <0.05) from IgE-activated and non-activated RBL-2H3 mast cells at 30 µg/mL. Chen et al. (2012) also observed that TiO₂ NPs can directly stimulate histamine release from non-activated RBL-2H3 mast cells at between 250 to 750 µg/mL. In this study the maximum concentration was 60 µg/mL, which might not be enough to induce histamine release. The β-hexosaminidase release was elevated but histamine was unchanged could also be because the detection of β-hexosaminidase release as a marker for activation mast cells is more sensitive than the detection of histamine release.

Another important finding in the present study was that surfactant-treated sZnO 30 NPs had the strongest impact on the Akt signalling phosphorylation that regulates cell survival and apoptosis. A high level of Akt phosphorylation through serine 473
residues was induced by untreated ZnO 30 NPs in non-stimulated RBL-2H3 cells. Whereas, surfactant-treated sZnO 30 induced strong phosphorylation through both serine 473 and threonine 308 residues (Figure 3.18). This complete activation through serine and threonine residues at 30 min may be the trigger for apoptotic programmed cell death that is induced by the highly cytotoxic effects of sZnO 30 NPs. As ZnO 30 NPs had less cytotoxicity than sZnO 30 NPs, the amount of phosphorylation was only partial and via serine 473 alone. Also, ZnCl₂ slightly increased the expression of Akt phosphorylation. However, large ZnO particulates (200 nm) and TiO₂ NPs did not express Akt phosphorylation because they were not cytotoxic. A recent study showed that PI3K/Akt pathway contributes in the ZnCl₂-induced increase in proliferation of mouse embryonic stem cells. However, this proliferative effect of Zn is reduced at higher doses of ZnCl₂ (200 μM) (Ryu et al., 2009). Further research is required to investigate the impact of ZnO NPs on cell signalling phosphorylation pathways in non-activated and IgE-activated mast cells.

In conclusion, the experiments discussed in this chapter showed that ZnO NPs have selective cytotoxicity toward cancerous cells (RBL-2H3) but not to normal mast cells (BMMCs). Surfactant-treated sZnO 30 exhibited the highest cytotoxic effects to both cell types (RBL-2H3 and BMMCs). Furthermore, histamine and β-hexosaminidase release from mast cells was also markedly inhibited by surfactant-treated sZnO 30 NPs. ZnO NPs can inhibit IgE-activated mast cell degranulation through inhibition of calcium influx. It was also observed that TiO₂ NPs had very low reactivity in this test system, in which they did not induce cytotoxicity or immune inhibition of mast cells, but only slightly increased β-hexosaminidase release.
Chapter 4: Inflammatory and cytotoxic responses of lung epithelial cell lines (A549 and LA4) to zinc oxide and titania nanoparticles

4.1 Introduction

Nanoparticulate ZnO and TiO$_2$ are very important commercial nanomaterials. They have been widely used in many processes and products including rubber manufacture, sunscreens, cosmetics, pigments, food additives and medicines, as well as in the chemical fibre and electronics industries (Ji and Ye, 2008). Production of engineered ZnO and TiO$_2$ NPs in sunscreen products alone was estimated to be up to 100 kilotonnes during 2003 (Borm et al., 2006). There are more than 1300 consumer products containing nanoparticles and the global market of engineered nanomaterials is estimated to be worth US$2.5 trillion by 2015 (Lanone and Boczkowski, 2011). This rapid expansion in the use of nanomaterials has raised concerns about the potential health risks from NP exposure.

Inhalation is one of the most likely exposure routes for NPs. Inhaled NPs includes ambient ultrafine particulates of many metals (such as Fe, Zn, V and Ni), and metal oxides (Fe$_2$O$_3$, ZnO, V$_2$O$_5$ and NiO), all of which can cause lung inflammation and respiratory irritation (Fond and Meyer 2006; Schaumann et al., 2004). Inhalation of very high doses of ZnO NPs in the workplace has been known to cause acute metal fume fever, which is characterized by fever, chills, nausea, headache, fatigue, and muscle aches (Gerberding, 2005; Warheit et al., 2009). Chronic
inhalation of TiO$_2$ NPs is also commonly associated with development of inflammation, pulmonary damage and lung tumours in rats, most likely as result of overload of the particle clearance mechanisms (Hext, 1994; Warheit and Frame, 2006). These inflammatory disorders might be due to the aggregation of NPs on the epithelial cell layer of the lung resulting in chronic inflammation, fibrosis, hyperplasia formation, and overall disruption of multiple biological systems. Non-toxic and non-immunological surface coatings or treatments of NPs may reduce their health impacts (Mano et al., 2012).

The A549 human lung epithelial cell line is a well-established model system that is commonly used to investigate the impact of NPs on the human lung epithelium (Don Porto Carero et al., 2001; Aufderheide et al., 2003; Schwerdtle and Hartwig, 2006). Previous *in vitro* studies have provided detailed information about the cytotoxic effects of ZnO and TiO$_2$ NPs toward A549 cells. These studies demonstrated that the physicochemical properties of NPs, including particle size, shape, surface area, phase, composition and agglomeration, have a crucial impact on cell toxicity and inflammatory responses (Hsiao et al., 2011; Sayes and Warheit, 2008). However, these studies examined the cytotoxicity and proinflammatory effects of NPs in an aggregated state, but not in a dispersed form. Nanoparticles tend to clump together in culture media and forming big aggregates, sometimes reaching up to >1 micron in size (Schulling et al., 2010), while dispersed NPs are most likely remain closer to their corresponding nano-sized primary particle when suspended in culture media. Thus, the cells may interact differently with these various states of NPs to cause different cytotoxicity and immune responses.
In addition, there have been few studies examining the impact of ZnO NPs on LA4 mouse lung epithelial cells. In one such study, Beyerle *et al.* (2009) demonstrated that 70 nm ZnO nanoparticles had greater cytotoxic effects in MH-S cells than in LA4 cells. Currently, there are no published studies investigating the effect of TiO$_2$ NPs on LA4 cells.

The aim of the study described in this chapter was to evaluate the effects of the particle size and dispersion state ZnO NPs (pristine NPs in an aggregated state vs. surfactant-treated in a dispersed state), ZnCl$_2$ (a control for Zn ions), along with two different crystalline forms of TiO$_2$ NPs; and to compare these with the crystalline form of silica particles (Min-U-Sil 5), a well-known respiratory toxicant. For all these particulates, the cytotoxicity and inflammatory responses in human A549 lung epithelial cells and mouse LA4 lung epithelial cells were measured.
4.2 Materials and Methods

4.2.1 Chemicals and Reagents
Materials and assay kits utilized specifically in this chapter included: Cell dissociation solutions (Sigma-Aldrich); Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL–NH2) (Sigma); human Th1/Th2/Th9/Th17/Th22 13plex Kit FlowCytomix (Bender MedSystems, Vienna, Austria); and IL-6, IL-8 and TNF-α BD OptEIA ELISA Sets (Bioscience).

4.2.2 Nanoparticle characterization and preparation
The nanoparticle characterization and preparation was as per section 2.2. In addition, Min-U-Sil 5 particles (Fine Ground Silica, U.S. Silica Co., Berkeley Springs, WV, USA) were also used as a reference particle (positive control) in this chapter. Crystalline silica particles are well known for their lung toxicity and have been used as a benchmark substance to study toxicity and inflammatory responses in the lung (Beyerle et al., 2009).

4.2.3 Cell culture
Cell culture experiments were carried out using two adherent cancer cell lines; LA4 mouse lung adenoma cell line and A549 human lung adenocarcinoma epithelial cell line. The cells were grown in RPMI-1640 medium containing 10% v/v FBS with gentamycin, glucose, pyruvate, 2-mercaptoethanol and L-glutamine. All cells were grown in 75 cm² culture flasks containing media in a humidified incubator at 37°C and 5% CO₂. At 90% confluence, cells were detached using the cell dissociation solution, and subsequently washed with media. A 1/10 dilution of cells to media
was then added to a new 75 cm² culture flask. All cells were passaged every 2-3 days.

4.2.4 Cytotoxicity assay

The A549 and LA4 cells were harvested by cell dissociation solutions instead of trypsin and EDTA, to avoid activation of cells via trypsin which increases the production of cytokines such as IL-8 and IL-6. The cells were resuspended in fresh RPMI-1640 medium, and centrifuged (200 g, 5 min) before seeding in 96-well plates at a cell density of 2 x 10⁴ cells/well. After 24 h of cell attachment and culture, the media was discarded and replaced with fresh media; the cells were then exposed to various concentrations of NPs (ZnO or TiO₂) and crystalline silica (Min-U-Sil 5) particles at 0, 10, 20, 30, 60, 100, 150 and 200 µg/mL for 24 h in a humidified incubator (at 37°C and 5% CO₂). Cytotoxicity was measured by adding soluble tetrazolium salt (MTS, Promega, USA) to the wells. After 4 h, the optical absorbance was measured at 490 nm on a spectrophotometer (Perkin Elmer EnSpire®, Waltham, MA, USA). Aliquots of supernatants were collected from wells before the addition of MTS and stored at -80°C for cytokine analysis.

4.2.5 Cytokine profiling

4.2.5.1 Quantification of human cytokines by multiplex flow cytometry

Supernatants of A549 cells exposed to the various NP concentrations for 24 h were assessed for presence of 13 different cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-9, IL-12, IL-13, IL-17, IL-22 and TNF-α) with a multiplex kit (Th1/Th2/Th9/Th17/Th22 13plex FlowCytomix Kit, Bender MedSystems, Vienna, Austria) using flow cytometry. The peptide SLIGRL–NH₂ (100 µM) was used as
positive control exposure for releasing IL-6 and IL-8. Samples from triplicate wells were analysed for each experiment.

4.2.5.2 Quantification of IL-6 and IL-8 by ELISA

The A549 cells were seeded in 96-well plates at a cell density of $2 \times 10^4$ cells/well. After 24 h of cell attachment (70% confluence), various concentrations of ZnO or TiO$_2$ NPs and Min-U-Sil 5 particles (at 10, 60 and 100 µg/mL) were added to the cells and incubated for 24 h. For kinetic studies, cells were exposed to 60 µg/mL of NPs for 4, 8, 12 and 24 h. The supernatant was then collected at different time points and IL-6 and IL-8 levels were analysed using enzyme-linked immunosorbant assay (ELISA, BD OptEIA kit; BD Systems, Franklin Lakes, NJ, USA). The absorbance at 450 nm was obtained using a microplate reader (Perkin Elmer EnSpire®, Waltham, MA, USA) as per manufacturer’s instructions. The cytotoxicity was also measured by the MTS assay.

To control for the possibility of particulates interfering with the ELISA assays, NPs without cells were titrated against IL-6 or IL-8 standard that was diluted in RPMI-1640 media and incubated for 24 h at 37°C. The IL-6 or IL-8 standard in RPMI-1640 without NPs was used as a negative control.

4.2.6 Statistical analysis

Statistics were performed using the same methods described in the section 2.2.13.
4.3 Results

4.3.1 Cytotoxicity of ZnO and TiO$_2$ NPs in A549 and LA4 cells

The A549 human lung epithelial cells and LA4 mouse lung epithelial cells were incubated with surfactant-treated ZnO (dispersed, “sZnO”) NPs, untreated ZnO NPs, TiO$_2$ NPs or Min-U-Sil 5 particles in RPMI-1640 plus 10% FBS for 24 h to evaluate cell viability. The cell viability data showed a dose-dependent cytotoxicity in A549 cells exposed to ZnO and sZnO NPs (Figure 4.1). At concentrations up to 10 µg/mL, no cytotoxicity was observed for any of the tested ZnO and TiO$_2$ NPs (“sub toxic” dose). At higher doses (>10 µg/mL), the smaller ZnO NPs (30 nm) caused more cytotoxicity than larger particulates. Small sZnO 30 NPs were equitoxic to untreated NPs on cells after the 24h exposure period. Large sZnO 200 particulates demonstrated greater cytotoxicity than the large untreated NPs after 24 h of exposure. The LA4 cells were more sensitive to all tested ZnO NPs than the A549 cells (Figure 4.2). The EC50 values of ZnO 30 and sZnO 30 NPs in LA4 cells were four-fold more sensitive than for A549 cells (Table 4.1). Interestingly, the large ZnO particulates (200 nm) were equitoxic to the small ZnO NPs (30 nm) in LA4 cells. In contrast, TiO$_2$ NPs and the crystalline silica particles were not cytotoxic to either A549 or LA4 cells after 24 h of exposure (Figure 4.3 and 4.4).
Figure 4.1: Cytotoxicity of untreated and surfactant-dispersed ZnO NPs in A549 human lung epithelial cells after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).

Figure 4.2: Cytotoxicity of untreated and surfactant-dispersed ZnO NPs in LA4 mouse lung epithelial cells after 24 hr exposure (Mean ± SEM of three experiments in quadruplicate).

Table 4.1: EC50 (µg/mL) of ZnO NPs in A549 and LA4 cells after 24 h.

<table>
<thead>
<tr>
<th></th>
<th>ZnO 30</th>
<th>sZnO 30</th>
<th>ZnO 200</th>
<th>sZnO 200</th>
<th>ZnCl2</th>
</tr>
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<tbody>
<tr>
<td>A549 cells</td>
<td>55 ± 2</td>
<td>56 ± 2</td>
<td>N/A</td>
<td>N/A</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>(Mean ± SEM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LA4 cells</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
<td>25 ± 1</td>
<td>13 ± 0</td>
</tr>
<tr>
<td>(Mean ± SEM)</td>
<td></td>
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N/A = Non-cytotoxic
**Figure 4.3:** Cytotoxicity of TiO$_2$ NPs and crystalline silica (Min-U-Sil 5) to A549 human lung epithelial cells after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).

**Figure 4.4:** Cytotoxicity of TiO$_2$ NPs and crystalline silica (Min-U-Sil 5) to LA4 mouse lung epithelial cells after 24 h exposure (Mean ± SEM of three experiments in quadruplicate).
4.3.2 Cytokine profiling

4.3.2.1 Multiplex cytokine profiling

The effects of ZnO, TiO$_2$ NPs and crystalline silica particles on cytokine expression in A549 lung epithelial cells were screened using a human Th1/Th2/Th9/Th17/Th22 13-plex flow cytometry bead-based kit. This kit simultaneously quantifies 13 cytokines, including Th1 cytokines (IL-2, IL-12 and IFN-$\gamma$), Th2 cytokines (IL-4, IL-5, IL-13 and IL-10), a Th9 cytokine (IL-9), Th17 (IL-17), Th22 (IL-22) and proinflammatory cytokines (IL-1$\alpha$, TNF-$\alpha$, IL-6).

The cytokine profile was measured at both sub-cytotoxic (10 $\mu$g/mL) and cytotoxic doses at approximately the EC50 (60 $\mu$g/mL for ZnO 30 and sZnO 30) after a 20 h incubation. Figure 4.5 demonstrates that ZnO and TiO$_2$ NPs and crystalline silica did not stimulate the expression of these 13 cytokines, except for the sZnO 200 particulate and SLIGRL, which increased the expression of the IL-6 cytokine. However, as the important proinflammatory cytokine IL-8 was not part of the multiplex kit, it was measured by ELISA.
**Figure 4.5:** Multiplex cytokine profile of A549 cells exposed to ZnO or TiO$_2$ NPs, crystalline silica (Min-U-Sil 5) particles or SLIGRL for 20 h. All NPs and ZnCl$_2$ did not induce type 1 (cellular) or type 2 (humoral) inflammatory responses, except for sZnO 200 and SLIGRL, which caused an increase in IL-6 production. Responses below 9 pg/mL were considered to be below the level of detection for this assay. Note: this kit did not contain the inflammatory cytokine IL-8.
4.3.2.2 Quantification of IL-6 and IL-8 release by ELISA

Whilst the multiplex was used as an initial screening method for cytokine profiling, the more accurate ELISA method was carried out to accurately examine the differences in cytokine expression of IL-6 and IL-8.

The A549 cells were exposed to three different concentrations of NPs, including sub toxic (10 µg/mL), cytotoxic (60 µg/mL) and highly cytotoxic (100 µg/mL) concentrations, to enable closer examination of the differences between cytokine profile expression among these closely-related ZnO particulates. All tested ZnO particulates and ZnCl$_2$ at 60 µg/mL had greater IL-8 expression, relative to the negative control (i.e. spontaneous release of cytokine) (Figure 4.6). However, TiO$_2$ NPs and crystalline silica particles did not significantly increase IL-8. When IL-8 release was normalized to cell viability, the smaller ZnO 30 NPs caused greater IL-8 production than the larger ZnO (200 nm) particulates. Figure 4.7 illustrates that untreated ZnO 30 NPs caused greater IL-8 release than surfactant-treated sZnO30 NPs, and then decreased in parallel with the loss of cell viability at 100 µg/mL. The kinetic IL-8 expression and cell viability data after 4, 8, 12 and 24 h showed that IL-8 release increased in the following order: sZnO 30 > ZnO 30 > ZnCl$_2$ > ZnO 200 > sZnO 200, respectively. The crystalline silica particles also caused a slightly increased expression of IL-8 (Figure 4.8). The peptide SLIGRL caused strong expression of both IL-8 and IL-6 cytokines and was therefore effective as a positive control for this test system.
Figure 4.6: The IL-8 release from A549 lung epithelial cells exposed to ZnO and TiO$_2$ NPs or crystalline silica (Min-U-Sil 5) particles after 20 h of incubation, and their effects on cell viability (percentile number under x-axis). The IL-8 spontaneous release from non-stimulated cells was used as a negative control, whereas SLIGRL-stimulated cells were used as a positive control. (Mean ± SEM of two experiments in triplicate).
Figure 4.7: Category line graph showing the different correlations between IL-8 expression (solid line) and cell viability (dashed line) in A549 lung epithelial cells exposed to sub-toxic, cytotoxic and highly cytotoxic doses of ZnO 30 or sZnO 30 NPs. Untreated ZnO 30 NPs caused higher IL-8 production than surfactant-treated sZnO 30 NPs.
Figure 4.8: Time course of IL-8 release (A) and cell viability (B) in A549 lung epithelial cells exposed to 60 µg/mL of ZnO NPs or crystalline silica (Min-U-Sil 5) particles over 24 h (Mean ± SEM of two experiments in triplicate).
All tested ZnO particulates, ZnCl₂, TiO₂ NPs and crystalline silica particles did not elicit significant increases of IL-6 production, except for sZnO 200 which had a significant increase in IL-6 production at concentrations of 60 and 100 µg/mL, compared to negative controls after 20 h of incubation (Figure 4.9). The decrease in IL-6 levels at concentrations of 60 and 100 µg/mL for ZnO 30, sZnO 30 and ZnCl₂ were due to the loss of cell viability at these toxic concentrations. The transient change in IL-6 over time was also examined after exposure the cells to NPs at different time points (4, 8, 12 and 24 h). Figure 4.10 demonstrates that sZnO 200 NPs was the only ZnO particulate that caused a sharp increase in IL-6 release between 8 and 12 h, and then a slightly elevated IL-6 expression from 12 to 24 h. Whereas, the crystalline silica particulate caused a significant increase in IL-6 expression throughout the duration.
Figure 4.9: The IL-6 release from A549 lung epithelial cells exposed to ZnO and TiO$_2$ NPs or crystalline silica (Min-U-Sil 5) particles after 20 h of incubation, along with their effects on cell viability (percentile number under x-axis). The IL-6 spontaneous release from non-stimulated cells was used as a negative control, whereas SLIGRL-stimulated cells were used as a positive control (Mean ± SEM of two experiments in triplicate).
Figure 4.10: Time course of IL-6 release (A) and cell viability (B) of A549 lung epithelial cells exposed to 60 µg/mL of ZnO NPs or crystalline silica (Min-U-Sil 5) particles over 24 h. IL-6 production was increased by exposure to sZnO 200 and silica particles. Cell viability was assessed by MTS assay over 24 h (B). Responses below 8 pg/mL were considered to be under the level of detection for this assay (Mean ± SEM of two experiments in triplicate).
To control for the possibility of NPs interfering with the assays, NPs without cells were incubated for 24 h at 37°C with IL-8 or IL-6 standard dilutions in RPMI-1640 cell culture media and compared to control standard curves (without NPs). All tested NPs at concentrations of 10, 60 and 100 µg/mL did not cause any alterations in the IL-8 and IL-6 standard curves, except sZnO 30 at 100 µg/mL, which appeared to slightly reduce IL-8 measurements but this was not significant (Figure 4.11 and 4.12).

The effects of ZnO, TiO$_2$ NPs and crystalline silica particles on IL-6 and TNF-α expression from LA4 mouse lung epithelial cells were examined after 24 h of incubation using the mouse IL-6 and TNF-α BD OptEIA kit -BD Systems. However, the productions of these cytokines were not detected (data not shown).

![Graph showing IL-8 levels in different conditions](image)

**Figure 4.11:** Controls for the possibility of ZnO or TiO$_2$ NPs binding or adsorbing IL-8 after incubation for 24 h at 37°C (Data represents mean ± SEM of two experiments in triplicate).
Figure 4.12: Controls for the possibility of ZnO or TiO₂ NPs binding or adsorbing IL-6 after incubation for 24 h at 37°C (Data represents mean ± SEM of two experiments in triplicate).
4.4 Discussion

In the present study, the *in vitro* cytotoxicity and immunomodulation of surfactant-treated (dispersed) and untreated (aggregated) ZnO NPs were investigated, in a concentration range from sub-toxic to highly cytotoxic, to examine the differential effects of the NPs on human lung epithelial cells. These ZnO NPs were treated with an industrial dispersant mix containing a hydrophilic polyacrylate copolymer (Orotan 731 DP). This dispersant formulation is inert and does not elicit cytotoxic or immunological effects on biological systems (as described by Feltis *et al.*, 2011). Crystalline silica particles (Min-U-Sil 5) were also used as a benchmark substance in this study. Crystalline silica was selected because it has well characterised *in vivo* respiratory and *in vitro* cytotoxicity and inflammatory effects (Sayes *et al.*, 2007).

The MTS assay data (Figure 3.1 and 3.2) showed that a cytotoxic dose-response was observed for the ZnO NPs and ZnCl$_2$ in both A549 and LA4 cells. This was in agreement with previous findings for ZnO NPs using lung epithelial cell lines, including A549 human lung epithelial cells (Lin *et al.*, 2009; Karlsson *et al.*, 2008; Horie *et al.*, 2009), BEAS-2B human bronchial epithelial cells (Huang *et al.*, 2010), and L2 rat lung epithelial cells (Sayes *et al.*, 2007).

The surfactant-treated sZnO 30 (30 nm) NPs were equitoxic to undispersed ZnO 30 NPs in the A549 cells after 24 h. This result was in contrast to our previous findings with RBL-2H3 rat and BMMCs mast cells, which were more susceptible to sZnO 30 than untreated ZnO 30 (as described in section 3.3). Interestingly, not only were the LA4 cells four-fold more sensitive to the ZnO NPs than A549 cells,
but the LA4 cells were very sensitive to all ZnO particulates and ZnCl₂, which were almost equitoxic in these cells (Table 4.1).

The cell exposure systems used in this study have sought to specifically assess the cytotoxicity and immunomodulatory effects of NPs in the same culture media (RPMI-1640). This was firstly to avoid any differences in either composition or concentration of ingredients present in different culture media, such as phosphate and carbonate, which can result in different responses as previously illustrated in section 2.3, but secondly to also avoid changing the physical and chemical states and dispersion characteristics of the NPs.

The cytotoxicity of ZnO NPs in different cell lines has shown significant variability, and clearly illustrates that cytotoxicity is not solely a function of the size of NPs but depends on a complex relationship between NPs and cellular properties. These results were in agreement with a previous study by Lankoff et al. (2012), which revealed that the NP-induced cytotoxicity was due to the interaction between cells and NPs, and not only due to the physical size of the NPs. TiO₂ NPs and crystalline silica particles were less cytotoxic to both A549 and LA4 cells, which may also indicate that these particulates are less soluble than ZnO NPs (i.e. TiO₂ NPs do not release metal ions intracellularly), and thus they would require extremely high doses to observe any cytotoxic effects.

In the current study, cytokine secretion was evaluated in the supernatants of lung epithelial cells that were incubated with surfactant-dispersed and non-dispersed ZnO particulates, TiO₂ NPs and crystalline silica. The transient release of cytokines
was monitored for non-cytotoxic, cytotoxic and highly cytotoxic concentrations of NPs at 4, 8, 12, 20 and 24 h after exposure.

Cytokine profiling in A549 lung epithelial cells showed that IL-8 and IL-6 were the most prominent. No other specific type 1 or type 2 cytokine responses to the NPs were observed for the cytokines measured in this study. Both IL-8 and IL-6 are major proinflammatory cytokines in the lung. IL-8 is responsible for inflammatory cell recruitment, primarily neutrophils, to the site of inflammation. It is well established that when lung epithelial cells are exposed to particulates, IL-8 secretion is increased, leading to an inflammatory response (Singh et al., 2007). IL-6 is an essential trigger for the bone marrow, leading to synthesis and recruitment of leukocytes to the site of infection. It also stimulates the liver to synthesise acute phase proteins, such as C reactive protein or fibrinogen (van Eeden et al., 2001), which suggests it has a major role in lung fibrosis (Fries et al., 1994). The other important activities of the IL-6 cytokine are the induction of T cell proliferation and T and B cell differentiation, and shifting the innate immune responses to more prolonged adaptive immune responses (Bacon et al., 1990). Also, IL-6 may contribute to the pathogenesis of lung diseases, such as asthma (Doganci et al., 2005) and chronic obstructive pulmonary fibrosis (COPD) (Eddahibi et al., 2006).

The benchmark substance of crystalline silica in this study showed a significant increase in IL-6 production, but not in IL-8 level (Figure 4.10). These results are in line with the findings of Cho et al. (2007), who demonstrated that intranasal instillation of mice with silica NPs increased the levels of IL-6 in the lung. It is well known that prolonged exposure to crystalline silica particles has been associated with lung fibrosis (Hetland et al., 2001). Therefore, it is suggested that IL-6
production induced by silica particles from lung epithelial cells may contribute to lung fibrosis.

All tested ZnO NPs and ZnCl$_2$ at 60 µg/mL caused a significant increase ($p < 0.005$) in the proinflammatory cytokine IL-8, compared to untreated cells when normalized for cell viability. The elevation of IL-8 levels could be responsible for the transient fever (metal fume fever) observed in the workplace when workers are exposed to high levels of inhalable ZnO particles. More interestingly, the levels of IL-8 cytokine secretion varied with particle sizes. Small ZnO 30 NPs caused greater IL-8 release than large ZnO 200 particles. It may be that small ZnO 30 NPs in lysosomes release high levels of Zn$^{2+}$ ions, which induce inflammation. The Zn$^{2+}$ ions from ZnCl$_2$ exhibited similar behaviour to ZnO NPs (30 nm), with virtually equal proinflammatory IL-8 production to the ZnO NPs. This supports the conclusion that Zn$^{2+}$ ions contribute to the induction of inflammatory responses. This finding was consistent with a previous study, where Feltis et al. (2011) found that the size of ZnO NPs was one of the key factors that influenced IL-8 production in THP-1 human monocytic cells.

The surfactant-treated sZnO 30 NPs had reduced IL-8 release when compared to their untreated ZnO 30 NPs counterparts at the studied concentrations of 10, 60 and 100 µg/mL (Figure 4.7). This could be due to the possibility that surfactant-treated sZnO NPs may escape into the cytoplasm and other organelles, where interference with intracellular processes may reduce IL-8 release.

Surprisingly, only the surfactant-treated sZnO 200 particles (bulk) increased IL-6 production in the A549 cells. The kinetic profile of IL-6 release by sZnO 200
particles showed a slight rise between 12 and 24 h (Figure 4.11). This may be due
to release of preformed IL-6 that may be triggered by exposure to particles, which
could then be followed by production and release (at a lower apparent rate) from
that point on. Further studies may be carried out to analyse the mRNA expression
at different time points, in order to differentiate between preformed IL-6 release or
de novo production. Similarly, in vivo studies using nasal installation of NPs in mice
models would strengthen and further clarify our in vitro findings.

In this study, TiO$_2$ NPs did not induce any cytokine expression and seemed to be
inert. It’s probably because the TiO$_2$ doesn’t dissolve at the low pH within
lysosomes, unlike the more soluble ZnO NPs. Thus, it may be that much higher
concentrations of TiO$_2$ NPs are required (~1000 µg/mL) to induce biological
changes.

In conclusion, the results in this chapter show that NP size, composition,
concentration, time of exposure and the interaction of NPs with different cell types
are critical in determining the degree of cytotoxicity in in vitro studies. Moreover,
the most pronounced proinflammatory signal of the production and release of the
cytokine IL-8, was observed for the smallest ZnO NPs. The bulk ZnO particles and
ZnCl$_2$ also showed significant IL-8 proinflammatory responses. This work shows
that surfactant-treatment of sZnO 30 NPs appears to reduce the proinflammatory
IL-8 cytokine response to small ZnO NPs. Crystalline silica particles elevated IL-6
production only. However, it was observed that TiO$_2$ NPs had minimal cytotoxic
effects and did not induce immune responses toward A549 lung epithelial cells.
Chapter 5: Chitosan nanocapsules as a non-toxic and non-allergenic drug delivery vector

5.1 Introduction

Chitosan is a biocompatible and biodegradable polymer, derived by deacetylation of chitin, which is the main component found in the exoskeleton of arthropods and usually derived from crustacea. In addition to its good mechanical properties, chitosan has found a broad range of applications in the medical field because of its low immunogenicity and toxicity (Zeng & Ruckenstein, 1996). Furthermore, it can be broken down by lysosomes to the innocuous N-acetyl glucosamine (Gan & ang, 2007), making it a highly desirable material in drug delivery applications.

Although different approaches have been undertaken in the past to prepare chitosan-based nanoparticulate systems (Aydin & Akbūğa, 1996; Shu & Zhu, 2000), most of these methods produce solid chitosan particles restricting their use as nanocapsule-based drug delivery systems. In contrast, particle templating methods such as layer-by-layer (LbL) assembly have been widely employed to prepare polymer capsules of well-defined chemical and structural properties (Caruso et al., 1998; Wang et al., 2007), along with improved homogeneity and monodispersity (Caruso, 2000; Sivakumar et al., 2009).

Büchel et al. (1998) reported the fabrication of highly robust and monodispersed solid core/mesoporous shell (SC/MS) silica spheres, with a 420 nm solid core
surrounded by a 75 nm mesoporous shell. The employment of these SC/MS silica particles as templates for nanocapsule synthesis offers an advantage over the solid particle templates used in LbL techniques, as a mesoporous shell enables polymeric material of controllable quantities to infiltrate into its porous matrix in a single step, using simple charge interactions. The polymeric chains can then be cross-linked covalently using an appropriate agent, adding tuneable stability and degradability characteristics to the capsules formed after the removal of the sacrificial silica template.

Curcumin is a hydrophobic anticancer drug, naturally found in the yellow curry spice turmeric (Curcuma longa), which has shown growth inhibition of a number of human cancer cell lines (Ammon & Wahl, 1991). Curcumin is a potent anti-oxidant and possesses anti-proliferative activities against tumor cells in vitro, but has limited applicability in vivo due to its instability in the aqueous environment (Mehta et al., 1997).

The aims of the study described in this chapter were to evaluate the role of different structural properties (size, wall thickness and porosity) of chitosan nanocapsules in drug delivery. Also the effects of curcumin-loaded chitosan nanocapsules and pristine chitosan nanocapsules on cellular uptake, cell viability and allergenic responses in mast cells (using the P815 mouse mastocytoma cell line) were investigated.
5.2 Materials and Methods

5.2.1 Chitosan nanocapsules

Chitosan nanocapsules in this study were provided by Dr. Vipul Bansal and Emma Goethals (RMIT University, Melbourne, Australia). Our group demonstrates for the first time that the monodispersed SC/MS silica templating approach can be extended to polysaccharides, thereby resulting in chitosan capsules of controllable size, wall thickness and porosity by tuning relevant features of the sacrificial SC/MS template. The approach presented here combines the versatility of solid core particles and the high loading capacity of mesoporous shells to prepare nearly monodispersed chitosan capsules of three different diameters (220, 270 and 440 nm), three different wall thickness (28, 45 and 56 nm), and three different porosity levels (corresponding to a template pore size of 2.9, 3.68 and 4.85 nm). This led to seven different types of chitosan capsules, amongst which one capsule type was common with the intermediate features of 270 nm capsule size, 45 nm wall thickness and 3.68 nm template pore size (Figure 5.1). The following chitosan nanocapsules were characterised by TEM (Figure 5.2): medium-size medium-shell medium-porosity (CapCSP); small-size medium-shell medium-porosity (CapC1SP); large-size medium-shell medium-porosity (CapC2SP); medium-size small-shell medium-porosity (CapCS1P); medium-size large-shell medium-porosity (CapCS2P); medium-size medium-shell high-porosity (CapCSP1); and medium-size medium-shell low-porosity (CapCSP2). The physico-chemical properties of the seven different types of chitosan nanocapsules, and the amount of curcumin loaded/capsule, are illustrated in Table 5.1.
Figure 5.1: Representation of the solid core/mesoporous shell (SC/MS) approach employed to fabricate seven different types of chitosan nanocapsules with different structural features. As shown in the schematic, the first step is the synthesis of solid silica particles (central core), which was followed by synthesis of a mesoporous shell around solid core particles, leading to SC/MS templates of different structural characteristics (middle layer). The SC/MS templates thus obtained varied in three different sizes, three different shell thicknesses and three different porosities, with one common type of SC/MS template in each category (highlighted by an arching triangle). SC/MS template synthesis was followed by infiltration of chitosan within the mesoporous shells of SC/MS silica templates, cross linking of infiltrated chitosan chains, and etching of SC/MS template, thereby resulting in chitosan nanocapsules of seven different structural characteristics (outermost layer) (from Emma Goethals, unpublished data).
Figure 5.2: TEM images of different chitosan nanocapsules (A-L). TEM images of chitosan nanocapsules of three different sizes: (A) CapC₁SP (220 nm), (B) CapCSP (270 nm) and (C) CapC₂SP (440 nm); scale bars correspond to 500 nm. Chitosan nanocapsules of three different wall thickness (D-I): (D) CapCS₁P, (E) CapCSP and (F) CapCS₂P; (G-I) show TEM images of ultramicrotomed chitosan nanocapsules shown in D-F, respectively, scale bars correspond to 200 nm. Chitosan nanocapsules of three different porosity levels: (J) CapCSP₁, (K) CapCSP and (L) CapCSP₂, scale bars correspond to 200 nm (from Emma Goethals, unpublished data).
**Table 5.1:** Physico-chemical properties of different types of chitosan capsules used in this study, wherein ‘Cap’ corresponds to the capsule, ‘C’ corresponds to the capsule diameter, ‘S’ corresponds to capsule shell thickness and ‘P’ corresponds to the capsule porosity (from Emma Goethals, unpublished data).

<table>
<thead>
<tr>
<th>Capsule Type</th>
<th>Capsule Description</th>
<th>Capsule Diameter (nm)</th>
<th>Capsule Shell Thickness (nm)</th>
<th>Chitosan Loading (mg/g of SC/MS)</th>
<th>Curcumin Loading (fg/Capsule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CapCSP</td>
<td>Medium size Medium shell Medium porosity</td>
<td>270</td>
<td>45</td>
<td>76.35</td>
<td>2.155</td>
</tr>
<tr>
<td>CapCSP1</td>
<td>Small size Medium shell Medium porosity</td>
<td>220</td>
<td>47</td>
<td>97.73</td>
<td>0.163</td>
</tr>
<tr>
<td>CapCSP2</td>
<td>Large size Medium shell Medium porosity</td>
<td>440</td>
<td>42</td>
<td>73.36</td>
<td>23.492</td>
</tr>
<tr>
<td>CapCS1P</td>
<td>Medium size Small shell Medium porosity</td>
<td>235</td>
<td>28</td>
<td>65.84</td>
<td>0.748</td>
</tr>
<tr>
<td>CapCS2P</td>
<td>Medium size Large shell Medium porosity</td>
<td>325</td>
<td>56</td>
<td>83.25</td>
<td>6.870</td>
</tr>
<tr>
<td>CapCSP1</td>
<td>Medium size Medium shell High porosity</td>
<td>269</td>
<td>40</td>
<td>47.11</td>
<td>0.851</td>
</tr>
<tr>
<td>CapCSP2</td>
<td>Medium size Medium shell Low porosity</td>
<td>283</td>
<td>42</td>
<td>81.26</td>
<td>4.507</td>
</tr>
</tbody>
</table>
5.2.2 Preparation of nanoparticles for cell culture

Following the synthesis of the seven different types (various size, wall thickness and porosity) of chitosan nanocapsules, these capsules were dehydrated in ethanol and dispersed in curcumin/oleic acid mixture (0.01 mg curcumin per mL of oleic acid) to allow infiltration of the oil phase through the semi-permeable walls of chitosan nanocapsules over 12 hour period. The drug loading technique used here is similar to that previously demonstrated for the anticancer chemotherapeutic drugs doxorubicin and 5-fluorouracil into poly (methacrylic acid) capsules (Sivakuma et al., 2009). Curcumin in oleic acid was employed as a model hydrophobic drug-in-oil. Chitosan nanocapsules were centrifuged, the supernatant removed and the capsules washed twice with hexane to remove any extraneous curcumin/oleic acid. The capsules were washed and resuspended in milli Q water to make a stock solution $10^{12}$ nanocapsules/mL. The same procedure was done to non-loaded chitosan nanocapsules (negative control). The stock solution was vortexed, inverted for 10-20 times and resuspended by pipetting up and down 3-5 times. The desired volume of stock solution was added to the RPMI-1640 media to make the working solution.

Dilutions were made by inverting the working solution 3-5 times until no particles were stuck to the bottom of the tube, and pipetting up and down 5 times, then the desired volume of the working solution was added to the RPMI-1640 media. A 100 µL aliquot of each dilution (ranging from $10^{10}$ to $10^7$ nanocapsules/mL) were added to the 100 µL of volume in the control and cell-containing wells, to provide a total volume of 200 µL/well.
5.2.3 Cellular uptake method

Mouse P815 mastocytoma cells (Manassas, VA, USA) were used to study both the uptake and cytotoxicity of the chitosan nanocapsules. These cells were cultured in RPMI-1640 media (Sigma) containing 10% FBS, gentamycin, glucose, pyruvate, 2-mercaptoethanol and L-glutamine supplementation, and maintained in a humidified incubator at 37°C and 5% CO₂. Phagocytic uptake of large chitosan nanocapsules was evaluated by confocal laser scanning microscopy (CLSM; Nikon, A1 Confocal Microscope). After 24 h, culture cells were harvested, centrifuged and washed 3 times with phosphate-buffered saline (PBS) and the cells seeded onto glass slides for imaging. Chitosan nanocapsules are highly fluorescent by nature and therefore do not require labeling with a fluorescent dye.

5.2.4 Cellular cytotoxicity assay

The P815 mast cell viability was determined using a soluble tetrazolium based colourimetric assay (Promega MTS CellTiter 96® AQueous kit). A 100 µL aliquot of 10⁶ cells/mL in cell culture medium was seeded with equal density into each well of 96-well plates (10⁵ cells per well). A 100 µL aliquot of (i) chitosan nanocapsules, or (ii) chitosan nanocapsules loaded with curcumin suspended in RPMI-1640 media, were added to provide a final concentration range of 10⁶-9 nanocapsules/mL (10⁵-8 nanocapsules per well). Untreated cells served as the control group. Cells were subsequently incubated for 20 h at 37°C before MTS was added to each well. The plates were incubated for an additional 4 h before being measured at 490 nm using a microplate reader (Perkin Elmer, USA) (see details for MTS assay in Chapter 2.2.6). Each treatment was performed in quadruplicate in 3-4 series of experiments.
5.2.5 ELISA measurement of IL-4

The P815 cells were seeded onto 96-well plates at $10^5$ cells/well, and various concentrations of NPs ($10^6$-8 nanocapsules/well) were added to the cells, which were then stimulated using 1 µM ionomycin and 20 nM PMA while being incubated at 37°C for 24 h. Before the MTS reagent addition step, supernatant was collected from each well at the 20 h time point for storage at -80°C for the IL-4 cytokine analysis. An enzyme-linked immunosorbant assay (ELISA; BD OptEIA kit, BD Systems, Franklin Lakes, NJ, USA) was used to measure IL-4 in thawed cell supernatants. The absorbance at 450 nm was obtained using a microplate reader (Perkin Elmer EnSpire®, Waltham, MA, USA) as per the manufacturer’s instructions. The same procedure was followed for wells containing unstimulated cells, but without the addition of the stimulants ionomycin plus PMA.

The NPs alone were titrated against the IL-4 standard diluted in RPMI-1640 media to investigate the possibility of IL-4 cytokine absorption or binding onto the chitosan nanocapsules after 24 h incubation at 37°C. The IL-4 standard in RPMI-1640 without NPs was used as a control.

5.2.6 Statistical analysis

Statistics were performed using the same methods described in the section 2.2.13.
5.3 Results

5.3.1 Chitosan nanocapsule uptake by P815 mast cells

The cellular uptake of chitosan nanocapsules was imaged using confocal laser scanning microscopy (CLSM), as shown in Figure 5.3. Confocal fluorescent images of the P815 mast cells exposed to chitosan nanocapsules for 24 h confirmed that chitosan nanocapsules were present and visible within the cytoplasm (Figure 5.3 C). Whereas, P815 mast cells alone (negative control) did not exhibit fluorescence (Figure 5.3 B).

5.3.2 Effect of chitosan nanocapsules on the viability of P815 mast cells

The cell viability was investigated to determine the cytotoxicity of both curcumin-loaded and non-loaded chitosan nanocapsules after incubation with P815 mast cells for 24 h (Figure 5.4). The cytotoxicity of non-loaded chitosan nanocapsules (Control Cap) was found to be negligible, with >90% cell viability occurring during exposure to 1000 capsules per cell (Figure 5.4A). However, all types of curcumin-loaded capsules caused 95-100% cell death on exposure to less than 1000 capsules. The minimum number of capsules required to cause >95 % cell death varied with the type of capsule used (Figure 5.4A), wherein capsule size was found to have the most influential effect on cytotoxicity, followed by porosity and wall thickness, respectively. The cytotoxicity profile related to the number of capsules per cell showed that the largest capsules loaded with curcumin (CapC2SP) had the highest cytotoxicity, while the smallest capsules (CapC1SP) showed the lowest cytotoxicity, and all other capsule types had intermediate effects (in decreasing order of potency): CapCSP2 > CapCS2P > CapCSP > CapCS1P > CapCSP1. However, if one looks at the cytotoxic efficiency of the capsules with regards to the
amount of curcumin available to the cells during the exposure (Figure 5.4B), then a different cytotoxicity profile is seen, i.e. capsule size and wall thickness were found to be very influential on cytotoxicity, but porosity had minimal effect. Nonetheless, all of the curcumin-loaded chitosan nanocapsules showed higher cytotoxic efficiency (per curcumin load) over free curcumin, except for the largest capsules (CapC<sub>2</sub>SP) (Figure 5.4B).

**Figure 5.3:** Confocal microscopy images of the cellular uptake of chitosan nanocapsules by P815 mast cells. A & B – cells without chitosan; C & D – cells with chitosan. A & C – overlays of fluorescent and phase contrast images; B & D – fluorescence image only; Scale bars correspond to 20 µm.
Figure 5.4: Cytotoxicity response of P815 mouse mastocytoma cells on exposure to seven different types of chitosan nanocapsules. (A) Compares the cytotoxicity profile on exposure to equivalent numbers of different types of capsules per cell, and (B) depicts the cytotoxic efficiency of different types of capsules with regards to the amount of curcumin present in the specific capsule type (Mean ± SEM of three experiments in quadruplicate).
5.3.3 The effect of chitosan nanocapsules on IL-4 production from P815 mast cells

The inhibitory effects on IL-4 release by small (CapC₁SP) and large (CapC₂SP) chitosan nanocapsules, without drug loading, were also evaluated in both unstimulated and ionomycin plus PMA-stimulated P815 mast cells. The IL-4 cytokine is central in the allergic immune response, and down regulation of IL-4 release might therefore have a beneficial anti-allergic effect.

IL-4 production from stimulated P815 mast cells was significantly inhibited by both small and large chitosan nanocapsules compared to the stimulated cells, (p < 0.05) at the lowest investigated dose of 100 and 10 capsules/cell, respectively. Both small and large chitosan nanocapsules showed a dose-dependent decrease in IL-4 production. Large nanocapsules caused more inhibition of IL-4 production than small nanocapsules at the same dose of 100 capsules/cell, which is comparable to an added chitosan concentration of 24 and 173 pg/cell, respectively (Figure 5.5).

The effects of chitosan nanocapsules on spontaneous IL-4 production from P815 mast cells without the addition of the stimulant, also confirmed that IL-4 was inhibited by both small and large chitosan nanocapsules in a dose-dependent manner (Figure 5.6). This effect was significant (p < 0.05) at the intermediate dose of 500 and 50 capsules/cell, respectively. The pristine chitosan capsules (curcumin-free) were found to have 100% cell viability from 1 to 700 chitosan nanocapsules/ cells in these experiments. Therefore, the diminished IL-4 production was not due to a decrease in cell number caused by cytotoxicity at these concentrations doses (10, 50, 100 and 500 nanocapsules/ cells). In addition, chitosan nanocapsules alone were titrated against IL-4 standard curves to control
for the possibility of cytokine absorption onto the nanocapsules. The nanocapsule dose range employed in this study did not cause any alteration in the IL-4 concentration standard curve (Figure 5.7).

Figure 5.5: The inhibitory effects of small and large pristine chitosan nanocapsules on IL-4 release from PMA + ionomycin-activated P815 cells after 24 h of exposure. Data represents mean ± SEM of two experiments in triplicate.
Figure 5.6: Inhibitory effect of small and large pristine chitosan nanocapsules on IL-4 release from unstimulated P815 cells after 24 h of exposure. Data represents mean ± SEM of two experiments in triplicate.
**Figure 5.7:** Controls for the possibility of small and large chitosan nanocapsules binding or adsorbing IL-4 after incubation for 24 h at 37°C (Data represents mean ± SEM of two experiments in triplicate).
To the best of my knowledge, the present study was the first to investigate the cytotoxicity and anti-allergic responses of semi-permeable chitosan nanocapsules of various size, wall thickness and porosity.

Seven different types of chitosan nanocapsules (of various size, wall thickness and porosity) were used in this study, and these capsules were loaded with curcumin/oleic acid mixture. Curcumin was used as a model lipophilic drug due to its well-established anticancer properties. To investigate the effect of different types of chitosan nanocapsules on their chemotherapeutic efficiency, the uptake of the chitosan capsules by P815 mouse mastocytoma cells (a tumour cell line) was first investigated. This was followed by the assessment of their cytotoxic profiles, and finally by an investigation of their immune inhibitory effect on IL-4 production, which may indicate the anti-allergenic potential of the pristine chitosan nanocapsules.

Fluorescent microscopy clearly demonstrated that the chitosan capsules could be taken by the P815 mouse mastocytoma cells (Figure 5.3), justifying the subsequent investigation of the efficacy of the cytotoxic effects of chitosan capsules loaded with an anticancer agent (Figure 5.4). The pristine curcumin-free chitosan capsules were found to be highly biocompatible, showing negligible cytotoxicity. Conversely, all types of curcumin-loaded capsules caused 95-100% cell death on exposure to less than 1000 capsules/cell. Our investigations suggest that the minimum number of capsules required to cause >95 % cell death varied with the type of capsule used (Figure 5.4A), wherein capsule size (load of curcumin) was found to show the most influential effect on toxicity, followed by porosity and wall thickness, respectively.
The cytotoxicity data corresponding to number of capsules per cell (shown in Figure 5.4A) can be used to compare the ability of a single capsule of certain type towards causing cytotoxicity, while Figure 5.4B compares the chemotherapeutic efficiency of a particular capsule type with regards to the amount of curcumin available to cells. Figure 5.4A indicates that, if chemotherapeutic drugs are administered based on the lowest number of capsules required to cause cytotoxicity, the order of decreasing potency is: largest capsules (CapC\textsubscript{2SP}) > CapC\textsubscript{SP\textsubscript{2}} > CapC\textsubscript{SP} > CapC\textsubscript{SP1} > smallest capsule (CapC\textsubscript{1SP}). For instance, comparison of capsule size revealed that while exposure to only 100 of the largest size capsules (CapC\textsubscript{2SP}) per cell is required to cause 100% cell death, it will require a ten-fold higher number of the smallest size capsules per cell to cause similar cytotoxicity (Figure 5.4A). This is not surprising considering that a large capsule will have a markedly higher amount of curcumin loading in comparison with a smaller capsule (Table 1).

However, when we compare the overall efficiency of these nanocapsule systems to evaluate their therapeutic effect as a drug carrier (Figure 5.4B), it becomes evident that the capsules with the smallest diameter are likely to be the most efficient for therapeutic applications. This is notable because the overall amount of curcumin present in a dose of small capsules to achieve total cell death is significantly lower (0.16 pg) than that present in a dose of larger capsules (2.34 pg). This indicates that a 14.4-fold lower amount of drug is required to achieve similar cytotoxic effects if smaller size capsules are used for the drug delivery.

Interestingly, if one compares the efficiency of free curcumin molecules with that of smallest capsules loaded with curcumin, the latter requires one-sixth of the
curcumin to achieve 95-100% cell death. In fact, all but one of the curcumin-loaded nanocapsular systems showed higher cytotoxic efficiency over free curcumin (Figure 5.4B). We believe that the higher efficiency of curcumin after encapsulation may be due to an increased stability of drug molecules in the biological environment, since it is well known that curcumin and several other chemotherapeutic drugs are unstable in aqueous environments (Oetari et al., 1996; Wang et al., 1997). The encapsulation of such drug molecules provides a significant opportunity to achieve higher efficiency of existing chemotherapeutic drugs. Similarly, it has been previously demonstrated that loading doxorubicin inside nanocarriers significantly enhances its efficiency by allowing a greater cellular uptake of drug over that seen with free drug molecules (Wang et al., 2008). This emphasizes the important role that small nanocarriers can play for drug delivery applications.

The next most influential factor (after capsule size) in controlling chemotherapeutic efficiency was found to be the thickness of the capsule wall. This is evident from Figure 5.4B, where thinner capsules (CapCS1P) were found to outperform capsules of medium thickness (CapCSP) and thick walls (CapCS2P), respectively. In comparison with the capsule size and wall thickness, the porosity of the capsule was found to have minimal effect on the chemotherapeutic efficiency of curcumin. This was demonstrated by capsules of different porosities (CapCSP1, CapCSP and CapCSP2) showing very similar chemotherapeutic efficiencies (Figure 5.4B).

In the current study, the impact of pristine (non-loaded) chitosan nanocapsules on cytokine release by a mast cell line, and thereby indirectly on the allergic immune response, was also evaluated. Mast cells produce Th2 type cytokines such as IL-4,
which can shift immune responses towards allergy (Galli et al., 2010). It has been reported that chitin significantly inhibited the allergen-stimulated Th2 cytokines (IL-4, IL-5 and IL-10) produced by cultured spleen cells (Shibata et al., 2000). In the present study, both small and large chitosan nanocapsules strongly inhibited IL-4 production from both non-activated and ionomycin plus PMA-stimulated P815 mouse mastocytoma cells. Large chitosan nanocapsules had a greater IL-4 inhibitory effect (p < 0.01) than smaller capsules at the same dose of 100 capsules/cell. Furthermore, the IL-4 inhibitory effect of chitosan nanocapsules was dose dependent (Figure 5.5 and Figure 5.6). The inhibitory mechanism of chitosan capsules may be due to the inhibition of cell signalling, such as via nuclear factor kappaB (NF-KB). It has been found that chitosan can inhibit IL-6 production by LPS stimulated human umbilical vein endothelial cells (HUVECs) via regulation of cells signalling pathways such as p38 MAPK pathway independent of NF-KB activation and ERK1/2 pathway dependent on NF-KB activation (Liu et al., 2009). Moreover, Tahara et al. (2011) reported that chitosan NPs (338 nm) inhibited histamine release from IgE activated RBL-2H3 through impairing the exocytosis mechanism that involved in the release of histamine.

In conclusion, the experiments conducted in this chapter showed that the loading of seven types of chitosan capsules possessing different structural features (size, wall thickness and porosity) with the lipophilic anticancer drug curcumin, enabled us to correlate the effect of these structural features with the efficiency of delivering an anticancer drug. In addition, pristine chitosan nanocapsules themselves were found to be highly biocompatible with negligible cytotoxicity, and also possessing anti-allergic potential by reducing IL-4 release from a mouse mast cell line.
Chapter 6: Summary and Conclusion

6.1 Summary and Discussion

The development of NPs for commercial products is undergoing a dramatic expansion. Nanomaterials have been used in many commercial products, such as sunscreens, cosmetics, antimicrobial agents, vaccines and drug delivery systems (Choksi et al., 2010). Recently, various sunscreen formulations containing ZnO and TiO$_2$ NPs have been made commercially available; these materials are effective at filtering broad-spectrum UV from sunlight (Nohynek et al., 2007). However, there are currently aspects of the potential cytotoxic and immune allergy effects resulting from exposure to these NPs, that are poorly characterised, and this has prompted the work described in this thesis.

The cytotoxicity and inflammatory responses to ZnO and TiO$_2$ NPs were studied \textit{in vitro} using mast cells and lung epithelial cells, to address the possible health risks resulting from the exposure of living tissue to these NPs. This study also investigated the relationship of structural properties to biological activity; i.e. the size, wall thickness and porosity of chitosan nanocapsules in drug delivery were examined. Chitosan nanocapsules were infiltrated with curcumin, which was used as a model lipophilic drug due to its well-established anti-cancer properties. The effects of chitosan nanocapsules on cellular uptake, cell viability and allergic responses in P815 mast cells were also studied.
Mast cells are present in all the tissue sites that NPs are likely to encounter first upon exposure of the body. Mast cells play essential roles in both activation and inhibition of innate immunity, adaptive immunity and allergic responses. Therefore, mast cells provide a very good model for studying the interaction between NPs and cells of immunity and how these interactions might alter the regulation of allergic immune responses. Lung epithelium cells were also used to study the potential cytotoxic and inflammatory effects of inhaled NPs.

In the present study, the *in vitro* assessment of the cytotoxicity and immune responses caused by surfactant-treated and untreated ZnO NPs and pristine TiO$_2$ NPs were investigated using different cell types, including the immune cells P815, RLB-2H3 and BMMC mast cells, and the lung epithelial A549 and LA4 cell lines. Of these cell types, RBL-2H3, A549 and LA4 cells were adherent cells, whereas P815 and BMMC cells were non-adherent cells. These cells also included the cancer cells P815, RLB-2H3, A549 and LA4, and the primary (non-cancer) BMMC cells.

The experiments showed that there was a dose-dependent cytotoxicity of ZnO NPs in all exposed cell types. The cytotoxicity profile of ZnO NPs varied with cell type, with LA4 cells being highly susceptible whilst BMMC were the most tolerant cells (Table 6.1). The treatment of these NPs with a surfactant dispersant (sZnO 30) retained their cytotoxicity in all cell types, with the surfactant-treated ZnO NPs being equitoxic in P815, A549 and LA4 cells. However this surfactant, which was not cytotoxic by itself, further increased the cytotoxicity of ZnO 30 NPs in RBL-2H3 cells, compared to the effects of the untreated NP counterpart. Furthermore, the cytotoxicity was increased by decreasing the size of the ZnO NPs. The cell culture media also had an impact on ZnO NPs cytotoxicity, as the faster growing P815.
cells in the richer RPMI-1640 medium were more susceptible to ZnO NP exposure than those growing more slowly in the sparse DMEM medium. The current studies also showed that ZnO NPs have selective cytotoxicity toward cancerous cells (RBL-2H3) but not to normal mast cells (BMMCs).

The dissolved ion control of ZnCl$_2$ exhibited an equitoxic effect to the ZnO NPs and had higher cytotoxicity than bulk particulates. The viability of P815 cells exposed to Zn$^{2+}$ dialysate from ZnO NPs confirmed that the cytotoxicity of ZnO NPs was not due to partial dissolution into the media producing extracellular Zn$^{2+}$ ions, but was instead mainly due to the NPs being internalised within the cells. Thus the cytotoxicity occurs after the ZnO NPs are taken up by the cells. Inside the lysosomes, where the pH is low, ZnO NPs are highly soluble and produce a bolus of Zn$^{2+}$ ions that elevate intracellular Zn$^{2+}$ levels, as detected by the zinquin ethyl ester fluorescent probe. The high level of Zn$^{2+}$ ions inside cells may damage the cell membrane and other cell components. Furthermore, some of these small ZnO NPs (30 nm) may also escape from lysosomes into the cytoplasm and cause further damage to cellular organelles, such as the mitochondria and nucleus. The damage of vital components of the cells may lead to the generation of ROS. The intracellular ROS data in the present study showed that ZnO NPs caused ROS generation in a concentration and dose-dependent manner, with smaller ZnO NPs causing more ROS production than larger NPs. In contrast, TiO$_2$ NPs showed low cytotoxic effects and did not induce ROS generation (section 2.4). This may be because the TiO$_2$ NPs are highly insoluble and do not dissolve at the low pH within lysosomes like the ZnO NPs to liberate metal ions, and therefore required a very high exposure concentration to induce biological changes in this *in vitro* system.
Table 6.1: Summary of EC50 values for ZnO NPs (µg/mL) in all cells used throughout the thesis, after 24 h of exposure.

<table>
<thead>
<tr>
<th>EC50/24 h</th>
<th>ZnO 30</th>
<th>sZnO 30</th>
<th>ZnO 200</th>
<th>sZnO 200</th>
<th>ZnCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>P815 mast cells (Mean ± SEM)</td>
<td>25 ± 2</td>
<td>28 ± 1</td>
<td>N/A</td>
<td>N/A</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>RBL-2H3 mast cells (Mean ± SEM)</td>
<td>187 ± 4</td>
<td>57 ± 4</td>
<td>N/A</td>
<td>N/A</td>
<td>114 ± 4</td>
</tr>
<tr>
<td>BMMC mast cells (Mean ± SEM)</td>
<td>N/A</td>
<td>122 ± 1</td>
<td>N/A</td>
<td>N/A</td>
<td>142 ± 1</td>
</tr>
<tr>
<td>A549 cells (Mean ± SEM)</td>
<td>55 ± 2</td>
<td>56 ± 2</td>
<td>N/A</td>
<td>N/A</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>LA4 cells (Mean ± SEM)</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
<td>13 ± 1</td>
<td>25 ± 1</td>
<td>13 ± 0</td>
</tr>
</tbody>
</table>

N/A = Non-cytotoxic

The immune responses of cells after NP exposure were also affected by surfactant dispersion and the specific size of NPs. The RBL-2H3 and BMMC mast cells were sensitized with TIB-142 IgE mAb in the presence and absence of ZnO NPs, TiO₂ NPs and ZnCl₂ and then challenged with TNP-BSA hapten to stimulate degranulation. Release of β-hexosaminidase and histamine were markedly inhibited by ZnO NPs, but very weakly inhibited by bulk ZnO particulates. Surfactant-treated sZnO 30 showed the greatest inhibition of the degranulation markers, β-hexosaminidase and histamine. ZnO NPs also inhibited spontaneous β-hexosaminidase release from non-activated mast cells (section 3.4), and caused more IL-4 inhibitory release than bulk ZnO in PMA and ionomycin-stimulated mouse P815 mast cells (section 2.4.6).

Overall, these findings comprise the first comparison of the cytotoxicity and allergic inhibitory effects of ZnO NPs in their agglomerated and dispersed states, and have direct implications for the proven effectiveness of topical zinc oxide preparations in rashes and other skin conditions.
Generally, ZnCl\textsubscript{2} showed similar effects to the smaller ZnO NPs, including inhibition of mast cell degranulation markers (β-hexosaminidase and histamine), which were stronger than with exposure to the larger ZnO 200 particulates. In all cases, TiO\textsubscript{2} NPs did not demonstrate any inhibition of immune function, but instead caused a significant increase in β-hexosaminidase release from mast cells.

The findings of the current study show that NP size, composition, concentration, time of exposure and the interaction of NPs with different lung epithelial cell types are critical in determining the degree of cytotoxicity in \textit{in vitro} studies. ZnO NPs caused lung epithelial cells to produce the proinflammatory cytokine IL-8, but did not stimulate other specific type 1 or type 2 cytokine responses. The levels of IL-8 cytokine secretion increased with decreased ZnO NP size. The increase of proinflammatory IL-8 release may be due to the damage of epithelial cells caused by ZnO NPs themselves or due to releasing of high levels of Zn\textsuperscript{2+} ions from ZnO NPs inside the cells (section 4.4).

Biopolymer based, chitosan nanocapsules were found to be highly biocompatible, showing negligible cytotoxicity and low immunogenicity. Seven types of chitosan capsules possessing different structural features, e.g. nanocapsule diameters ranging from 220 to 440 nm, nanocapsule shell thickness ranging from 31 to 55 nm, and pore diameter varying from 2.9 to 5.1 nm, were loaded with curcumin, a lipophilic anticancer drug, which enabled the correlation of the effect of these structural features of different chitosan nanocapsules with anticancer efficiency against P815 mouse mastocytoma cells, the size of the nanocapsules is likely to
play the most important role in dictating the chemotherapeutic efficiency in this drug delivery system (section 5.4).

6.2 Future directions

Preliminary work was done in this thesis using the Australian Synchrotron x-ray fluorescence beamline, which was used to detect the distribution of intracellular ZnO and TiO$_2$ NPs in P815 mast cells (section 2.3.8). Further mapping of the distribution of NPs inside the cell could be used to determine whether NPs are able to penetrate the nucleus. Limitations on this early work prevented accurate assessment of the precise intracellular position of the NPs, and techniques such as x-ray tomography or focussed ion-beam ablation will be necessary to confirm the NP localisation within the cytoplasm or nucleus.

As ZnO NPs appeared to inhibit the allergic immune response, including the mast cells degranulation markers and IL-4 release, as well as the surfactant-treated sZnO 30 NPs having the strongest impact on the Akt signalling phosphorylation to induce apoptosis, future studies could further evaluate these allergic inhibitory and apoptotic mechanisms by investigating the effect of ZnO NPs on cell signalling pathways, e.g. PI3K / Akt signaling, NF-kB signaling and MAPK signaling in IgE/antigen-activated mast cells.

In vivo studies are also required to evaluate the safety of nanomaterials. Therefore, it would be necessary to investigate the effect of surfactant-dispersed ZnO NPs on cytotoxicity and inflammatory responses using an intra-nasal mouse model.
Moreover, the allergic inhibitory effects of ZnO NPs could be further examined using an ovalbumin intra-nasal sensitized mice model.

In the current study, we have demonstrated the non-cytotoxic nature of chitosan nanocapsules, as well as their biodegradability and their down-regulation of IL-4 Th2 allergic responses in PMA and ionomycin-activated mast cells. Thus, it would be expected that chitosan nanocapsules are applicable as a safe and efficient drug carrier, and as an anti-allergic drug delivery system. Therefore, we suggest a further study to examine chitosan nanocapsules as a suitable delivery system for therapeutics, such as heparin, used in asthma treatment.

6.3 Conclusion

This project has provided a significant contribution to the existing knowledge about cytotoxicity, immune inhibitory and immune stimulatory effects of NPs in their dispersed and aggregated states in several cell types using an in vitro exposure system. Dispersed ZnO NPs exhibited the stronger immune inhibitory effects in vitro; therefore, the ultimate health outcomes need to be investigated in vivo to evaluate the true risks of using formulations of NPs containing surfactants that prevent the agglomeration of ZnO and TiO₂ NPs. Furthermore, it has also been demonstrated that non-cytotoxic chitosan nanocapsules are efficient as a drug carrier and can be exploited for further medical applications.
References


role of particle surface area and internalized amount. Toxicology 260, 142-149.


Appendix 1: Protein gel reagents

Separating gel (12.5%):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris pH 8.8 (Sigma-Aldrich, USA)</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>10% SDS (sodium dodecyl sulfate) (BDH, UK)</td>
<td>100 µL</td>
</tr>
<tr>
<td>40% (w/v) Bisacrylamide (Amresco)</td>
<td>3.124 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.275 mL</td>
</tr>
<tr>
<td>15% (w/v) Ammonium persulfate (Sigma-Aldrich)</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED (BioRad Laboratories, USA)</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Stacking gel (4%):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M Tris pH 8.8</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>40 µL</td>
</tr>
<tr>
<td>40% (w/v) Bisacrylamide</td>
<td>375 mL</td>
</tr>
<tr>
<td>H₂O</td>
<td>2.585 mL</td>
</tr>
<tr>
<td>15% (w/v) Ammonium persulfate</td>
<td>20 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

5x SDS sample buffer: 60 mM Tris Cl (pH 6.8), (Sigma), 25% (v/v) glycerol (Sigma), 2% (w/v) SDS (BDH), 14.4 mM mercaptoethanol (Biorad), 0.1% (w/v) bromophenol blue (BDH), (stored at -20 °C).

10x SDS Electrophoresis buffer (Tris – glycine): 30.2 g Tris, 144.0g Glycine and 10.0 g SDS made up to 1 litre with milli Q water, pH 8.3.

Coomassie Stain: 0.1% (w/v) Coomassie R 250 (Biorad), 50% (v/v) methanol (lab scan), 10% (v/v) acetic acid (Scharlau, Germany).
**Destaining solution:** 10% (v/v) ethanol (Lab scan), 10% (v/v) acetic acid (Scharlau).

**Bradford reagent:** 100 mg Coomassie Brilliant G-250 dye (Sigma), 50ml ethanol (BDH), 100ml 85% phosphoric acid (BDH), per litter of milli Q water, filtered through 0.45 µm filter and stored at 4°C.

**x10 Tris Buffer Saline (TBS):** 12.11 g Tris. (Sigma), 87.8 g NaCl per litre of milli Q water.