Effects of Graphene Oxide Nanoparticles on the Immune System Biomarkers Produced by RAW 264.7

by

Hend Emhemed Algadi

Student Number: 3582417

Thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae

Department of Medical Bioscience
Faculty of Natural Sciences
University of the Western Cape
South Africa

Supervisor: Professor E. J. Pool

Co-supervisor: Professor M. M. Fidalgo

April 2019
KEY WORDS

Graphene oxide nanoparticles
Cytotoxicity
Cell-mediated immunity
Cytokines
Macrophage activations
Immunotoxicity
Engineering nanomaterials
Inflammation
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>2-D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3-D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>mg/l</td>
<td>milligram per litre</td>
</tr>
<tr>
<td>mg/ml</td>
<td>milligram per millilitre</td>
</tr>
<tr>
<td>n</td>
<td>sample size</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>µl</td>
<td>microliter</td>
</tr>
<tr>
<td>cells/ml</td>
<td>cells per milliliter</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CMI</td>
<td>Cell-mediated immunity</td>
</tr>
<tr>
<td>CNTs</td>
<td>Carbon nanotubes</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CXCL10/IP-10</td>
<td>Interferon gamma induced protein 10</td>
</tr>
<tr>
<td>CCL2/MCP-1/JE</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>CCL3/MIP-1α</td>
<td>Macrophage inflammatory protein 1 alpha</td>
</tr>
<tr>
<td>CCL4/MIP-1β</td>
<td>Macrophage inflammatory protein 1 beta</td>
</tr>
<tr>
<td>CCL5/RANTES T</td>
<td>Cell expressed and secreted</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay accelerating Factor</td>
</tr>
<tr>
<td>DAS</td>
<td>Double antigen sandwich</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DAS ELISA</td>
<td>Double antibody sandwich enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DWCNTs</td>
<td>Double walled carbon nanotubes</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ENMs</td>
<td>Engineered nanomaterials</td>
</tr>
<tr>
<td>FBS</td>
<td>Fatal bovine serum</td>
</tr>
<tr>
<td>FLG</td>
<td>Few layered graphene</td>
</tr>
<tr>
<td>G</td>
<td>Graphene</td>
</tr>
<tr>
<td>GO</td>
<td>Graphene Oxide</td>
</tr>
<tr>
<td>GONPs</td>
<td>Graphene oxide nanoparticles</td>
</tr>
<tr>
<td>GQDs</td>
<td>Graphene quantum dots</td>
</tr>
<tr>
<td>GFNs</td>
<td>Graphene family nanomaterials</td>
</tr>
<tr>
<td>GNPss</td>
<td>Graphene nanoplatelets</td>
</tr>
<tr>
<td>GNRs</td>
<td>Graphene nanoribbons</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>IFNα</td>
<td>Interferon-alpha</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulins</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin 12</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin 1 receptor</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL-27</td>
<td>Interleukin 27</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactase dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane Attack Complex</td>
</tr>
<tr>
<td>MASP</td>
<td>MBL-associated serine protease</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannan binding lectin pathway</td>
</tr>
<tr>
<td>MCNTs</td>
<td>Multi walled carbon nanotubes</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cells</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NM</td>
<td>Nanomaterials</td>
</tr>
<tr>
<td>NNPs</td>
<td>Naturally occurring nanoparticles</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyether imide</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>rGO</td>
<td>Reduced graphene oxide</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor 1</td>
</tr>
<tr>
<td>SWCNTs</td>
<td>Single walled carbon nanotubes</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UFPs</td>
<td>Ultrafine particles</td>
</tr>
<tr>
<td>WST-1</td>
<td>2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disflphenyl)-2H-tetrazolium</td>
</tr>
</tbody>
</table>
ABSTRACT

Graphene oxide (GO) is a single carbon layer, oxygen bearing graphene derivative, containing hydroxyl and carboxyl groups. Graphene oxide nanoparticles (GONPs) are promising nanomaterials for a variety of applications such as electrochemical analysis, adsorption of biomolecules, biosensors and drug and vaccine delivery systems. While these newly engineered nanoparticles hold great potential for developments in industry and medicine, the widespread use of these material will inevitably result in GO residues in the environment where they could possibly pose a risk to human and wildlife health. Interaction of the nanoparticles and biota can affect numerous biological processes. In humans they can affect any of the physiological systems such as the immune, endocrine, reproductive and cardiovascular systems. Although studies have indicated that GO exposure cause increased reactive oxygen species in cells, they mechanisms whereby GO act on the cell are still poorly understood. A few studies have investigated the effects of GONP and other graphene nanoparticle derivatives on the immune system.

The aim of this study was to investigate the in vitro effects of GONPs on the immune system by the exposure of the murine macrophage cell line, RAW 264.7, to different concentrations of GONPs. Selected immune system biomarkers were used to investigate the potential immunotoxicity of GONPs. A number of immune system biomarkers were monitored, such as cytotoxicity, inflammatory biomarkers and cytokine and chemokine proteome profiles. The inflammatory
biomarkers monitored were nitric oxide (NO), IL-6 and macrophage inflammatory proteins 1α, 1β and 2 (MIP-1α, MIP-1β, MIP-2).

Results show that GONPs were cytotoxic to RAW 264.7 cells at 500 µg/ml GONP. The addition of 15.6 µg/ml GONPs to the macrophages induced several inflammatory biomarkers. The activation of inflammatory biomarkers by GONPs was confirmed by proteome profile analysis. The GONPs induced inflammatory cytokines and chemokines such as NO, IL-6, TNF-α, MIPs (MIP-1α, MIP-1β, MIP-2) and RANTES.

From the results obtained it can be concluded that GONPs exposure can induce inflammatory responses. Although in vivo studies were not included in the current study, the in vitro data indicates that GONPs may pose a risk to individuals exposed through use of products containing this nanoparticle.
DECLARATION

1. Hend Algadi, declare that this thesis, Effects of Graphene Oxide Nanoparticles on the Immune System Biomarkers Produced by RAW 264.7, is my work and has not been submitted before for any degree or examination at any other university, and that all the sources of my information have been indicated and acknowledged by complete references.

Hend Algadi

April 2019

Signed

http://etd.uwc.ac.za/
ACKNOWLEDGMENT

In the name of God, the Most Beneficent, the Most Merciful

This study was performed in the Department of Medical Biosciences at the University of the Western Cape and was funded by the Libyan Embassy.

To my supervisor Prof. Pool and Co Supervisor, Prof. M. Fidalgo, a big Thank you for their valuable advice, sheer supervision and constant encouragement in pursuance of this work. Their association with this endeaver of mine, will remain a beacon light to me throughout my life.

Thank you to my husband Mr. Husam, and our daughters Hana and Fatima. Your patience, love and encouragement have upheld me, particularly during those many hours in which I spent more time with my computer than with you. My husband has always been my pillar, my joy and my guiding light, and I thank him.

My deepest gratitude goes to my family: my parents, Emhemed and Fatima, my sisters Sara and Marwa, and my brothers Munier, Moktar, Mostafa, Hussain and Mohamed. Thank you for all their support and understanding throughout my academic life. It is their love and constant encouragement that motivates me to do my best.

I am indebted to my late mother-in-law, Hania, for her care and love. Although she is no longer with us, she is forever remembered. I am sure she shares our joy and happiness in the heaven. Thank you to my father-in-law; he has never complained in spite of all the hardships in his life.

To my best friend, Thaera and my sister-in-law, Elham; Thank you for standing by me through thick and thin.

To my lab colleagues Kim, Jodi, Nurjahaan, Dewald and Yuekai: Thank you for always lending a helping hand in times when I needed it the most.

To Dr Abdalmonam Baleg Thank you for help and finalize my thesis.

I am eternally grateful to you all. Thank you for your support and encouragement.
# TABLE OF CONTENT

Title.......................................................................................................................... i  
KEY WORDS........................................................................................................... ii  
LIST OF ABBREVIATIONS ................................................................................. iii  
ABSTRACT ........................................................................................................... vii  
DECLARATION .................................................................................................... ix  
ACKNOWLEDGMENT .......................................................................................... x  
TABLE OF CONTENT ...................................................................................... xi  
LIST OF TABLES ................................................................................................. xiv  
LIST OF FIGURES ............................................................................................... xv  

CHAPTER 1 ........................................................................................................... 1  
1.1 PROBLEM STATEMENT AND AIM OF THE STUDY ............................... 1  

CHAPTER 2 ........................................................................................................... 3  
2.1 Background ................................................................................................... 3  
2.2 Organic nanoparticles .................................................................................. 4  
2.2.1 Naturally occurring nanoparticles ......................................................... 4  
2.2.2 Man-made nanoparticles ....................................................................... 5  
2.2.2.1 Graphene and Graphene Oxide Nanoparticles (GONPs) .................. 7  

CHAPTER 3 ........................................................................................................... 12  
3.1 Introduction .................................................................................................. 12  
3.2 The cells of the immune system .................................................................. 13  
3.3 Immune defences ....................................................................................... 15  
3.3.1 Innate immunity .................................................................................... 15  

http://etd.uwc.ac.za/
3.3.1.1 Anatomical barriers ................................................................. 15
3.3.1.2 Phagocytosis ........................................................................ 18
3.3.1.3 Complement system ............................................................... 21
  3.3.1.3.1 The classical pathway ......................................................... 22
  3.3.1.3.2 The lectin pathway ............................................................. 23
  3.3.1.3.3 The alternative pathway .................................................... 24
3.3.2 Adaptive immunity ................................................................. 25
  3.3.2.1 Activation of T helper lymphocytes ....................................... 25
  3.3.2.1.1 Humoral Immunity .............................................................. 26
  3.3.2.2 Cell-Mediated Immunity ....................................................... 28
3.4 Inflammation ............................................................................. 29
3.5 Interaction between GFNs and immune system components ............... 31

CHAPTER 4 .................................................................................. 37
MATERIALS AND METHODS ......................................................... 37
  4.1 Synthesis and characterization of graphene oxide nanoparticles (GONP) .. 37
  4.2 Preparation of GONP for cell cultures .......................................... 38
  4.3 Cell culture and exposure to GONP .............................................. 38
  4.4 Cytotoxicity Assay ..................................................................... 39
  4.5 Nitric Oxide (NO) assay ............................................................... 40
  4.6 Mouse IL-6 quantitation .............................................................. 40
  4.7 Macrophage inflammatory protein (MIP) quantitation ....................... 41
  4.8 Mouse Proteome Profiling .......................................................... 42
  4.9 Statistical analysis ..................................................................... 44

CHAPTER 5 .................................................................................. 45
RESULTS ................................................................................. 45
  5.1 The effects of GONPs on viability of RAW 264.7 cells ...................... 45
  5.2 Effect of GONP on inflammatory biomarkers produced by RAW 264.7 ... 46

http://etd.uwc.ac.za/
5.2.1 Nitric oxide (NO) ................................................................. 46
5.2.2 Interleukin 6 (IL-6) ............................................................. 47
5.2.3 The effects of GONPs on MIP-1α synthesis by RAW 264.7 cells .... 48
5.2.4 The effects of GONPs on MIP-1β synthesis by RAW 264.7 cells .... 49
5.2.5 The effects of GONPs on MIP-2 synthesis by RAW 264.7 cells .... 50
5.3 GONP modulation of cytokine and chemokine profiles of RAW 264.7 cells ........................................................................... 51
CHAPTER 6 ........................................................................................................ 53

DISCUSSION ................................................................................................. 53

6.1 The effect of GONPs on viability of RAW 264.7 cells ...................... 53
6.2 The effect of GONPs on inflammatory biomarkers produced by RAW 264.7 cells ................................................................. 54
6.3 Conclusion ......................................................................................... 55
CHAPTER 7 ..................................................................................................... 57

CONCLUSIONS AND FUTURE PERSPECTIVES .................................... 57

References .................................................................................................. 58
LIST OF TABLES

TABLE 3.1 THE MYELOID CELLS AND THEIR FUNCTIONS…………………………14

TABLE 3.2 THE LYMPHOCYTES AND THEIR FUNCTIONS…………………………14

TABLE 5.1 THE MODULATION OF CYTOKINES/CHEMOKINES SECRETED BY RAW 264.7 CELLS CULTURES AFTER EXPOSURE TO LPS AND 15.6 mg/ml GONPs COMPARED TO CELLS EXPOSED TO MEDIUM OF (0 mg/ml GONPs). DATA IS REPRESENTED AS NOT DETECTABLE (ND) OR VISIBLE (+). PROTEINS UPREGULATED BY CELLS DUE TO THE EXPOSURES TO LPS OR 15.6 mg/ml GONP COMPARED TO THE 0 mg/ml GONP EXPOSED CELLS ARE SHARED……………………………53
LIST OF FIGURES

FIGURE 2. 1 (A) Graphene as honeycomb lattice of carbon atoms. (B) Graphite as a stack of graphene layers. (C) Fullerene (C60) molecules of wrapped graphene through the introduction of pentagons on the hexagonal lattice. (D) SWCNTs as rolled-up cylinders of graphene sheets. (E) DWCNT as two concentric nanotubes. (F) MWCNTs as multi-concentric nanotubes (Hansora et al., 2015). .............................................................. 8

FIGURE 2. 2 Graphic of chemical structure of graphene and graphene oxide (GO) (Zhou and Liang, 2014). ............................................................. 9

FIGURE 3. 1 The origin of the cellular components of the immune system (King and Wills, 2005). ............................................................................. 13

FIGURE 3. 2 The processes of phagocytosis (https://za.pinterest.com/pin/6333255701547986/?lp=true). .................................................................................. 18

FIGURE 3. 3 Diagrammatic representation of the classical, lectin and alternative pathway complement cascades (Tegla et al., 2011). ..... 22

FIGURE 3. 4 An illustration of acute and chronic inflammation pathways (Serhan, 2010). ......................................................................................... 31

FIGURE 5. 1 The effects of GONP on RAW 264.7 cell viability. Cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. At the end of the incubation period the cell viability was determined using the WST-1 assay. Data presented are the mean ± standard deviation of 9 replicates. The * sign indicates that the result is significantly different compared to 0 µg/ml GONP (P < 0.01). ........................................... 45

FIGURE 5. 2 The effect of GONP on NO production by RAW 264.7 cells. Cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. Data
PRESENTED ARE THE MEAN ± STANDARD DEVIATION OF 9 REPLICATES. THE * SIGN INDICATES THAT THE RESULT IS SIGNIFICANTLY DIFFERENT COMPARED TO 0 µG/ML GONP (P < 0.01).

FIGURE 5.3 THE EFFECTS OF GONPS ON IL-6 PRODUCTION BY RAW 264.7 CELLS. CELLS WERE EXPOSED TO VARIOUS CONCENTRATIONS OF GONP IN CULTURE MEDIUM. CELLS WERE ALSO INCUBATED WITH A CONTROL CONSISTING OF CULTURE MEDIUM CONTAINING LPS AS DESCRIBED IN THE METHODS. DATA PRESENTED ARE THE MEAN ± STANDARD DEVIATION OF 9 REPLICATES. THE * SIGN INDICATES THAT THE RESULT IS SIGNIFICANTLY DIFFERENT COMPARED TO 0 µG/ML GONP (P < 0.01).

FIGURE 5.4 THE EFFECTS OF GONP ON THE SECRETION OF THE MIP-1α(ng/ML) BY RAW 264.7 CELLS. THE CELLS WERE EXPOSED TO VARIOUS CONCENTRATIONS OF GONP IN CULTURE MEDIUM. CELLS WERE ALSO INCUBATED WITH A CONTROL CONSISTING OF CULTURE MEDIUM CONTAINING LPS AS DESCRIBED IN THE METHODS. DATA PRESENTED ARE THE MEAN ± STANDARD DEVIATION OF 9 REPLICATES. THE * SIGN INDICATES THAT THE RESULT IS SIGNIFICANTLY DIFFERENT COMPARED TO 0 µG/ML GONP (P < 0.01).

FIGURE 5.5 THE EFFECTS OF GONP ON THE SECRETION OF THE MIP-1β(ng/ML) BY RAW 264.7 CELLS. THE CELLS WERE EXPOSED TO VARIOUS CONCENTRATIONS OF GONP IN CULTURE MEDIUM. CELLS WERE ALSO INCUBATED WITH A CONTROL CONSISTING OF CULTURE MEDIUM CONTAINING LPS AS DESCRIBED IN THE METHODS. DATA PRESENTED ARE THE MEAN ± STANDARD DEVIATION OF 9 REPLICATES. THE * SIGN INDICATES THAT THE RESULT IS SIGNIFICANTLY DIFFERENT COMPARED TO 0 µG/ML GONP (P < 0.01).

FIGURE 5.6 THE EFFECTS OF GONP ON THE SECRETION OF THE MIP-2(ng/ML) OF RAW 264.7 CELLS. THE CELLS WERE EXPOSED TO VARIOUS CONCENTRATIONS OF GONP IN CULTURE MEDIUM. CELLS WERE ALSO INCUBATED WITH A CONTROL CONSISTING OF CULTURE MEDIUM CONTAINING LPS AS DESCRIBED IN THE METHODS. DATA PRESENTED ARE THE MEAN ± STANDARD DEVIATION OF 9 REPLICATES. THE * SIGN INDICATES THAT THE RESULT IS SIGNIFICANTLY DIFFERENT COMPARED TO 0 µG/ML GONP (P < 0.01).
**Figure 5.7** The effect of GONPs on inflammatory biomarker secretion by RAW 264.7 cells. Cells were incubated with (I) medium only (negative control), (II) medium in the presence of LPS (positive control) and (III) medium containing 15.6 mg/ml GONPs not stimulated with (LPS). Supernatants were screened using a proteome profiler array. Cytokines/chemokines that were detected were allocated numbers: 1, 3, and 16 are reference spots; 2- IP-10; 4- G-CSF; 5- TNF-α; 6- GM-CSF; 7- IL-6; 8- JE; 9-sICAM-1; 10- MIP-1α; 11- MIP-1β; 12- IL-1β; 13- MIP-2; 14- IL-1RA; 15- RANTES; 17- IL-27; 18- SDF-1.
CHAPTER 1

1.1 PROBLEM STATEMENT AND AIM OF THE STUDY

Nanoparticles are particles with at least one dimension of the same size range as ultrafine particles (1-100 nm). Engineered nanoparticle development and production have increased very rapidly over the last few decades. This is because nanomaterials offer a new range of properties that are commercially exploitable. The physicochemical properties of nanomaterials differ from their bulk counterparts due to the size of the particle and more specifically due to the increased surface to volume ratios of nanoparticles compared to bulk chemicals. One of the major developments in nanoparticles over the last number of years has been the development of single atom thickness graphene nanoparticles (Liu et al., 2015; Zhu et al., 2013; Novoselov et al., 2004). Currently methods are available for the production of graphene and also several chemically modified graphene compounds such as graphene oxide (GONP). Although the graphene and graphene oxide nanoparticles are currently mainly used experimentally, the commercially exploitable properties of these compounds are so numerous that they may become common in products and pharmaceuticals in the near future. As with most chemicals, increased production and use increase the risk of pollution and possible environmental and human health risks of the chemical. For this reason, it is of the utmost importance that new nanoparticles be screened for potential risks before being allowed to be used commercially.

In mammals, adverse health effects can be due to interaction of the nanoparticle with any of the animal’s physiological functions or biochemical processes (Ray et al., 2009). Studies have shown that the immune system functions, such as inflammatory reactions, are very
common adverse risks posed by nanoparticles (Ray et al., 2009). Currently nanoparticle preparations are already being produced commercially and some of these are being used in the pharmaceutical industry. This has raised many questions about the long-term safety of these products.

Adverse health effects of nanoparticles depend on the exposed individual’s biological status and the properties of the nanoparticles. The individual’s biological status includes genetics, existing diseases, the level and duration of exposure, while nanoparticle properties include the chemistry, size, shape, agglomeration state and electromagnetic ions of the nanoparticle itself (Buzea et al., 2007; Wiesner et al., 2009). Although the biological effects of some nanomaterials have been assessed, mode of action studies remains insufficient.

The immunotoxicity of GONPs has not been adequately evaluated. Therefore, research must be conducted to determine if GONPs poses a risk to human and environmental health. The aim of this study was to investigate the in vitro effects of GONPs on the immune system. This was done by exposing the murine macrophage cell line, RAW 264.7 to GONPs. A number of immune system biomarkers were monitored such as cytotoxicity, inflammatory biomarkers, cytokines and chemokines and also the cytokine and chemokine proteome profile of cells cultured in the absence and presence of GONPs. Data from this study will give as indications if GONPs pose a risk to the immune system and our defences against disease causing pathogens.
2.1 Background

Nanoparticles are particles of the same size range as ultrafine particles (1-100 nm) (Taghavi et al., 2013). It is commonly accepted that the distinction between ultrafine particles and nanoparticles lies in their origin. Ultrafine particles (UFPs) are those particles that are produced and released as spurious emissions resulting from everyday life or industrial by products. In contrast, nanoparticles are engineered, but the distinction between these two groups is not always clear-cut. Thus, particles of nanometer scale can be loosely divided into three categories, those that are naturally occurring (natural UFPs), those that are produced inadvertently as a result of human activity (anthropogenic UFPs), and those that are deliberately engineered for specific uses (nanoparticles) (Chang, 2010).

Nanomaterials are often classified on the basis of their physicochemical characteristics or structure. There are four classes of materials from which nanoparticles are typically composed, namely carbon-based compounds, metals, and ceramics. The nanometer form of metals such as gold or silver and metal oxides such as titanium dioxide, are the most commonly used engineered nanomaterials. Nano-sized silica, silver, and natural clays are also common materials in use. The carbon nanotube is a unique nanomaterial being investigated for a wide range of applications (Raab et al., 2011; Schodek et al., 2009).
The potential adverse health effects of naturally occurring or anthropogenic ultrafine particles may be either immunologic or non-immunologic in nature. These health effects may or may not be explained by the ultrafine particle paradigm of oxidative stress and inflammation (Chang, 2010).

Nanomaterials are applied in the field of nanotechnology, and they display different physiochemical characteristics from normal chemicals. Many of these materials have increased strength or weight ratios, enhanced conductivities, and improved optical and magnetic properties. These new properties make nanomaterial development so interesting and promising for great economic potentials (Narasimha and Lall, 2016). However, the change in properties may potentially pose risks to consumers and the environment. For this reason, nanomaterial risks need to be screened for before embarking on large scale production of nanoparticles for commercial purposes.

### 2.2 Organic nanoparticles

#### 2.2.1 Naturally occurring nanoparticles

Naturally occurring nanoparticles (NNPs) are often present in all spheres of the earth, irrespective of human activities. Nature comprises of plants, algae, fungi, yeast, etc (Nowack and Bucheli, 2007). All living organisms are composed of biomolecules. Naturally occurring nanoparticles can be found in volcanic ash, ocean spray, fine sand and dust, and even in biological matter (e.g. viruses). These naturally occurring biomolecules play an active role in the formation of nanoparticles with distinct shapes and sizes, thereby act as a driving force for the
designing of greener, safe and environmentally benign protocols for the synthesis of nanoparticles (Sharma et al., 2015).

Naturally occurring nanoparticles can serve as a model for engineering nanoparticles (ENPs) in the environment and naturally occurring mineral NPs. Their behavior can point out important mechanisms in which NPs can move through environments and affect various environmental systems (Osaka et al., 2006). Once NPs are released into the environment from either natural or man-made sources, very little is known about their environmental fate, specifically potential changes in the nanoparticle’s physicochemical properties. Naturally occurring nanoparticles (NNPs) in the atmosphere have been studied in atmospheric sciences as particulate matters and carbon NPs from unprocessed fuel (Gustafsson et al., 2009). Nanoparticles are produced in many natural processes, including photochemical reactions, volcanic eruptions, forest fires, and simple erosion, and by plants and animals, e.g. shed skin and hair. With a diameter of one nanometer C60 buckminsterfullerene occurs in nature and in the air after a forest fire. The small particles suspended in the atmosphere, often known as aerosols, affect the earth’s energy balance by absorbing the radiation from the sun and scatter it back to space. About 90% of these particles are of natural origin, and the remaining 10% of the total is estimated to be produced by the activities of humans (Houghton, 2005; Taylor, 2002).

2.2.2 Man-made nanoparticles

Synthetic nanoparticles (sometimes called anthropogenic nanoparticles) fall into two general categories: “incidental” and “engineered” nanoparticles. Incidental
nanoparticles are by products of human activities and have poorly controlled sizes and shapes. Many of the processes that generate incidental nanoparticles are common every day activities such as running diesel engines, large-scale mining, and even starting a fire (Raab et al., 2011).

Industrial nanoparticle materials constitute a tiny but significant pollution source that is, so far, literally buried beneath much larger natural sources and nanoparticle pollution incidental to other human activities, particularly automobile exhaust soot (Bahadar et al., 2016).

Engineering nanomaterials (ENM) that are being manufactured currently include mainly metals, non-metals, metal oxides, lipids, and polymers, as well as various nanocomposites. Besides ENPs, nanoparticles can be formed naturally via processes occurring in all ‘‘spheres’’ of the Earth.

While some nanomaterials occur naturally, others occur as a result of human activities. The latter type is called engineered nanomaterials (ENMs). ENMs are used in many commercial products and processes and can be found in sunscreens, cosmetics, sporting goods, stain-resistant clothing, tires, electronics, etc. They are also used in medicine for purposes of diagnosis, imaging and drug delivery (Buzea et al., 2007).

Humans are frequently exposed to tiny particles via dust storms, volcanic ash, and other natural processes, but the human body systems are well adapted to defend us from any potential harmful intruders. The reticuloendothelial system in particular actively neutralizes and eliminates foreign elements in the body, including viruses and non-biological particles (Buzea et al., 2007).
It can be argued that particles originating from human activities (e.g. smoke from combustion and lint from garments) have existed for centuries, but the recent development of industry and combustion-based engine transportation has profoundly amplified anthropogenic particulate pollution (Buzea et al., 2007).

2.2.2.1 Graphene and Graphene Oxide Nanoparticles (GONPs)

Graphene is an engineered nanoparticle (Liu et al., 2015; Zhu et al., 2013). The word ‘‗graphene‘‘ derived from the Greek word graphein, which means ‘‗to write.‘‘ It was discovered in 2004 by Novoselov (Novoselov et al., 2004). Graphene is a 2-dimensional single sheet of carbon atoms arranged in a hexagonal network. It is an intriguing material for highly controlled systems, e.g., evolution from two-dimensional (2-D) to three-dimensional (3-D) topology having new properties. These 2-D materials can be modified by substituting the carbon atoms with selected heteroatoms or entire functional groups (Novoselov et al., 2004).

Figure 2.1 shown various carbon allotropes such as graphite, fullerenes, nanorings, nanobuds, single-walled carbon nanotubes (SWCNTs), double-walled carbon nanotubes (DWCNTs), and multiwalled carbon nanotubes (MWCNTs), (Novoselov et al., 2004).

Graphene can be composed of a single-layer hybrid nanosheet of sp\(^2\) or multiple layers of carbon atoms. These carbon atoms are densely packed into benzene rings stripped of their hydrogen atoms. This 2-D material has exceptional characteristics like electronic and high crystal quality. Its short history has already shown a cornucopia of new physics and potential applications.
Figure 2. 1 (a) Graphene as honeycomb lattice of carbon atoms. (b) Graphite as a stack of graphene layers. (c) Fullerene (C60) molecules of wrapped graphene through the introduction of pentagons on the hexagonal lattice. (d) SWCNTs as rolled-up cylinders of graphene sheets. (e) DWCNT as two concentric nanotubes. (f) MWCNTs as multi-concentric nanotubes (Hansora et al., 2015).

Graphene oxide (GO) has a sheet-like structure which looks like graphene. However, its preparation method and electronic properties are entirely different from those of graphene. The schematic chemical structure of graphene and GO are shown in Figure 2.2. Graphene consists solely of benzene rings made of carbon (C) atoms with C=C double bonds (sp2 bonds). In GO, many of the double bonds in graphene are scissor and linked to oxygen (O) to form C-O bonds, which yields C-C single bonds (sp3 bonds). Mobile π electrons that dominate the conductivity in graphene are lost at the oxidized bonds. Thus GO becomes an insulator (Furukawa and Ueno, 2013).
Graphene has attracted much attention from the scientific community due to its enormous potential in a myriad of fields, including medical sciences, agriculture, food safety, cancer research, and tissue engineering. Graphene and its derivatives, or what is scientifically known as graphene family nanomaterials (GFNs) have raised some safety concerns due to their potential for widespread human exposure (Singh, 2016).

Graphene family nanomaterials (GFNs) include single or few layered graphene (FLG), graphene nanoribbons (GNRs), graphene nanoplatelets (GNPs), graphene oxide (GO), reduced graphene oxide (rGO) and graphene quantum dots (GQDs). GFNs are applied in many fields, such as photonics/plasmonics (Bonaccorso et al., 2010; Grigorenko et al., 2012), electronics (Jang et al., 2016), sensors (Shao et al., 2010), catalysis (Machado and Serp, 2012), drug delivery (Liu et al., 2013), and DNA sequencing (Heerema and Dekker, 2016). GFNs can be prepared chemically and their properties can be changed by various synthesis techniques (Sun et al., 2011).
Their unique chemical and physical properties have found important places in their respective application fields. Although GFNs have many useful and unique application, some have cytotoxic and genotoxic effects. Since the discovery of graphene, studies have been conducted using different cell and animal models to find new uses and monitor toxic potential of the graphene family nanomaterials (GFNs). Some *in vitro* and *in vivo* studies showed no particular risks, while others have indicated that GFNs may have adverse effects on the health of exposed individual (Bianco, 2013).

Graphene and its derivatives are promising candidates for important biomedical applications due to their versatility. Because of the potential risk factors associated with the manufacture and use of graphene-related materials, the number of nano-toxicological studies of these compounds have increased rapidly over the past decade. These studies have investigated the effects of the molecular interactions between GFNs and different organizational levels of the living system, from biomolecules to animals (Seabra et al., 2014).

The applications of GFNs have developed rapidly in the past few years. However, the potential for widespread human exposure to these nanoparticles raises safety concerns about graphene and its derivatives, referred to as graphene family nanomaterials (Saleem et al., 2017). A comprehensive understanding of the interaction of GFNs with biota and their adverse effects *in vitro* and *in vivo* are essential for further development and safe use of graphene-based nanomaterials (Guo and Mei, 2014). For this reason, studies need to be conducted on the effects of the graphene family of nanoparticles on various organ systems and biochemical processes. The current study will focus on effects of nanoparticles on the immune
system and the next chapter will give an overview of the immune system and also interactions of nanoparticles with the immune system.
CHAPTER 3

THE IMMUNE SYSTEM

3.1 Introduction

The immune system serves to defend the body against harmful microorganisms and substances which have breached its defences. The immune system must initiate an effective and appropriate action to inactivate pathogens (Abbas et al., 2014; Palomäki, 2014). It does so by being able to identify self from foreign invaders. The immune system comprises of two utilitarian components which together form an intricate physiological network. These two components are the innate immune system and the adaptive immune system. These two components have mechanisms that work interactively to destroy foreign substances (Edgar, 2006; Abbas et al., 2014). The immune system is made up of an extensive variety of task-specific cells and secretory molecules which act synergistically to destroy harmful organisms and substances such as pathogens that enter the body. Some pathogens such as viruses, and some bacteria and parasites, can reproduce intracellularly, while others multiply outside the host’s cells. Due to the pathogen localization in the body, the immune system have developed mechanisms to protect the host against extracellular and intracellular pathogens (Mayer, 2009; Abbas et al., 2014; Yatim and Lakkis, 2015).
3.2 The cells of the immune system

The immune system consists of a variety of cell with unique roles in protecting the organism against infection. The origin of these cells is presented in Figure 3.1. Most of the cells of the immune system originate from stem cells that multiply inside the bone marrow, flow into the blood and migrate into stable tissues. Immune responses contain interactions among a number of those cells and/or their secreted productions.

Figure 3.1 The origin of the cellular components of the immune system (King and Wills, 2005).

The cells of the immune system have specific functions and these are depicted in Table 3.1. The cells of the immune system work synergistically and often secretory products produced by one cell type will attract other cells to the site of infection, and/or assist in the activation of another cell type to carry out a specific function.
**Table 3.1**: The myeloid cells and their functions (Todd, 2001; Abbas et al., 2014)

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes/macrophages</td>
<td>Phagocytosis, killing of pathogens, antigen presentation and inflammation.</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>Phagocytic, defenses against parasites, release vasoactive amines during allergic reactions.</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>Phagocytosis, killing of pathogens, inflammation.</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Involved in allergic reactions, release inflammatory mediators such as histamine.</td>
</tr>
<tr>
<td>Basophil</td>
<td>Involved in allergic reactions, release inflammatory mediators such as histamine.</td>
</tr>
</tbody>
</table>

The lymphocytes consist of B cells, T cells and NK (natural killer) cells **Table 3.2**. They arise from lymphoid progenitors in the bone marrow (Abbas et al., 2014).

**Table 3.2**: The lymphocytes and their functions (Abbas et al., 2014)

<table>
<thead>
<tr>
<th>Type of cells</th>
<th>Main functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>B- lymphocytes</td>
<td>Produce antibodies.</td>
</tr>
<tr>
<td>Cytotoxic T- lymphocytes (CD8+)</td>
<td>Kill cells infected with intracellular pathogens.</td>
</tr>
<tr>
<td>Helper T- lymphocytes (CD4+)</td>
<td>Produce cytokines that regulate B- and cytotoxic T- lymphocytes immune responses.</td>
</tr>
<tr>
<td>γδ T-lymphocytes</td>
<td>Play an important role in the downregulation of immune responses in infectious diseases to return the body to homeostasis.</td>
</tr>
<tr>
<td>Natural killer (NK) cells</td>
<td>Kill cells infected with intracellular pathogens.</td>
</tr>
</tbody>
</table>
3.3 Immune defences

Organisms are constantly being attacked by pathogens. The immune system defends the body against such as attacks by using various mechanisms. These mechanisms can be grouped under immune mechanisms that animals are born with, namely innate immunity, and immune mechanisms triggered after birth due to exposure to pathogens, namely the acquired immunity.

3.3.1 Innate immunity

The innate system is a general, broadly-specific system and its purpose is to eliminate diverse pathogens and contaminants penetrating the body (Murphy 2012). This system does not possess an immunological memory, but it responds quickly and it has efficient mechanisms for the destruction of harmful microorganisms and substances which enter the body, and it has the ability to stimulate the adaptive immune system into the defence processes (Abbas et al., 2014). Even though innate immunity operates in all organs and tissues, it is more active in regions that are in proximity to the body's external membranes. These are regions like the gastrointestinal tract, the skin, the genitourinary tract and respiratory tract (Abbas et al., 2014; Yatim and Lakkis, 2015). The innate immune system consists of anatomical barriers and processes such as phagocytosis and the complement pathways.

3.3.1.1 Anatomical barriers

Anatomical barriers are formed by a variety of penetration-resistant membranes, each emitting their own variety of biochemical substances that further enhance the
effectiveness of the barrier. Examples of such anatomical barriers are the internal epithelial layers of the respiratory, gastro-intestinal and urinary tract, and the skin (Saladin, 1998).

The first line of defence of the innate immune system is provided by the various external membranes. These membranes create an obstruction to entry for pathogens, and can even eradicate them after penetration, or wash them out before they can entrench themselves in the soft underlying tissues (Saladin, 1998). Although fairly basic, external membranes are an effective and essential component of the body’s protection armoury. These membranes are not reactive to penetration attempts but are permanently in place to ward off or eliminate a wide spectrum of intruding pathogens (Trinchieri and Sher, 2007; Saladin, 1998). There are diverse ways in which the various external body surfaces prevent the ingress of pathogens. The skin is enveloped by a thin layer of dead cells, which are constantly being shed. During shedding various microorganisms and substances lodged in the dead skin, are also shed off. Also, skin secretions like sweat and other discharges from the skin can lower the pH, contain salt and noxious lipids that acts together to inhibit the growth of most microbes. Furthermore, these secretions physically flush away microorganisms and substances (Saladin, 1998). The saliva formed by glands in the mouth contains a high concentration of lysozyme, an enzyme that breaks down bacterial cell walls. The prevailing acidic condition in the stomach also creates an effective barrier, because the resulting environment is deadly for a multitude of pathogens. Furthermore, both microorganisms and substances are trapped by the mucous secreted by the membranes of the respiratory tract, the eyes, the ears, the nose, the
gastrointestinal tract, and the reproductive tract. Mucous membranes produce viscous mucus. This mucus is capable of capturing particles. Microorganisms or substances trapped by the mucus are swept out of the body by various mechanisms (Turvey and Broide, 2010; Saladin, 1998).

Another mechanism for ejecting unwanted microorganisms and substances from the body is flushing. Examples of such flushing mediums are urine, vomit, saliva and tears. The total effect of these various measures by which the immune system obstructs, destroys and evicts threatening elements, makes it hard for microorganisms and substances to penetrate and establish in the tissue material below the epithelial membranes (Gennery and Cant, 2006).

Commensal microbiota comprises microorganisms that live on the epithelial membranes of the body and the skin (Tlaskalová-Hogenová et al., 2004). The typical microbiota of the gastrointestinal tract and the skin hamper the proliferation of pathogens by competing for nutrients, attachment space on the body surface, and by the secretion of antimicrobial substances which retard pathogen growth and reproduction (Mayer, 2009). These counter measures make it difficult for pathogens to penetrate and establish themselves in the body of a host (Helbert, 2006). If pathogens manage to cross the barriers presented by the body’s epithelial membranes, they attempt to establish themselves in the underlying tissue. Specific cells of the immune system and substances emitted by such cells, will try to destroy these pathogens by phagocytosis. Additionally, proteins of the complement system will be activated and tasked with the eradication of invading pathogens (King and Wills, 2005; Turvey et al., 2010).
3.3.1.2 Phagocytosis


The phagocytes and epithelial cells express a wide range of receptors and signalling molecules on their cell surface membranes which facilitate phagocytosis (Gordon, 2016). Figure 3.2 is a graphic representation of the phagocytosis process.

Phagocytosis is initiated by the binding of opsonins (i.e. complement or antibody) on the pathogen surface and/or specific molecules named pathogen-associated molecular pathogens (PAMPs) on the pathogen to cell surface receptors on the phagocyte. This results in receptor clustering, thus stimulating phagocytosis (Kumar et al., 2011). Phagocytes have an assortment of pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000; Kumar et al., 2011). Pattern recognition receptors and opsonin receptors are diverse groups of receptors. The pattern recognition
receptors include Toll-like receptors, lectin and scavenger receptors. Toll-like receptors (TLR) are a part of the (PRRs) family and are capable of recognizing molecules which are shared by pathogens but recognized as different from host molecules. TLRs are homologues to the Drosophila Receptor Toll and are able to recognize PAMPs such as peptidoglycan and lipopolysaccharide (Kumar et al., 2011). Stimulation of TLRs induces a cascade of effector adaptive responses. TLRs are cellular surface transmembrane proteins and presently, 10 human TLRs are recognized. TLR4 recognizes lipopolysaccharide (LPS) while in aggregate with CD 14, while TLR2 recognizes peptidoglycan of Gram-positive bacteria and lipoproteins. TLR9 recognizes unmethylated cytosine-guanine motifs in pathogens (Medzhitov and Janeway, 2000; Kumar et al., 2011). Contact of these TLRs with pathogen-associated molecular patterns (PAMPs) activate intracellular signalling cascades leading to the nuclear translocation and liberation of the transcriptional factor nuclear factor-kappa Beta (NF-κB) from IkB. NF-κB induce the gene expression of a wide variety of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, IFN-γ etc, and enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX). The iNOS and COX are responsible for synthesizing potent inflammatory molecules such as nitric oxide (NO) and prostaglandins respectively.

Opsonin receptors include Fc receptors and complement receptors (Takeda et al., 2003). Fc receptors bind to the Fc part of immunoglobulins that are bound to pathogen. Cells express Fc receptors (FcR) for the different antibody classes such as FcRγ for IgG, FcRα for IgA, FcRε for IgE, FcRδ for IgD and FcRμ for IgM (Medzhitov and Janeway, 2000).
According to their capability to internalise microbes during phagocytosis, the receptors are divided into Cis and Trans groups. Trans receptors detect a variety of infections through recognising the released ligands of a pathogen from a distance. They also stimulate a generalised inflammatory reaction. However, they are not able to facilitate the internalisation and killing of the pathogen. Trans receptors include Toll-like receptors and C5a receptors. Cis receptors, also named phagocytic receptors, have a low detecting range where they can only recognise the physical presence of a pathogen. Hence direct interaction of receptors with a pathogen stimulates a killing activity of the phagocytes. Underhill and Ozinsky, 2002, reported that the most characterised phagocytic receptors are the Fcγ receptors. However, other cis receptors such as the complement receptors CR1, CR3 and CR4 and the lectin receptor, Dectin-1 can also assist with the initiation of phagocytosis. (Underhill and Ozinsky, 2002). Direct contact with the pathogen leads to induction of molecular structural changes that prepare the cellular components of the innate immunity to fight invasive pathogens.

The PRRs and PAMPs binding stimulate the cytoskeletal contractions of the phagocytic cells leading to the formation of a pseudopods around the microbes (Tosi, 2005). The pseudopodia engulf or ingest the pathogen and this membrane bound vesicle containing the pathogen is called a phagosome. After the pathogen is engulfed by a phagocyte, it then fuses with lysosomes to become a phagolysosome. Within the phagolysosome, reactive oxygen, nitrogen intermediates and toxic peptides degrade the invading pathogen. The killing of the pathogen can happen by two mechanisms namely oxygen dependent and oxygen independent killing. Oxygen dependent killing are due to the formation of toxic
Oxygen metabolites such as singlet oxygen, hydrogen peroxide, hydroxyl radicals and superoxide anion that degrade the pathogen. The oxygen independent response employs enzymes and toxic proteins, that are found in primary and secondary granules of the lysosome, to kill the microbe (Parkin and Cohen, 2001). A typical example of oxygen independent killing of pathogens is via the enzyme lysozyme. Lysozyme cleaves linkages in the peptidoglycan layer of bacterial cell walls. This results in the disintegration of the cell wall which increase protein permeability across the cell wall, allowing the microbial proteins and nutrients to leach out of the microbe thereby causing the death of the microbe (Tosi, 2005). Defensins are cationic proteins that also play a role in oxygen independent killing of microbes. Defensins, upon binding to the microbial cell wall, change the charge of the cell wall, and disrupts transport of molecules across the microbe membrane (Tosi, 2005).

3.3.1.3 Complement system

The complement system is a crucial element of the innate immune system (Nesargikar et al., 2012; Fujita et al., 2004). Complement are proteins found in the blood plasma and are mainly produced by cells of the hematopoietic system and the liver. The complement system comprises in excess of 30 complement proteins (Mayer, 2009; Magcwebeba, 2008; Edgar, 2006). The complement system is an effector mechanism for both innate and adaptive immunity, and it can be mobilized via a number of different routes (King and Wills, 2005). Activation of the complement cascade results in the development of its primary effector functions such as recruiting phagocytes, bacteriolysis and microbial
opsonization. When the protein molecules of the complement system come into contact with invading pathogens, they are able to destroy these pathogens by direct or indirect means. Direct killing is achieved by following either the classical complement pathway, the mannan-binding lectin pathway, or the alternative complement pathway that ultimately result in the formation of membrane attack complex that kills pathogens (King and Wills, 2005; Tosi, 2005).

**Figure 3.** Diagrammatic representation of the classical, lectin and alternative pathway complement cascades (Tegla et al., 2011).

### 3.3.1.3.1 The classical pathway

The classical complement pathway is triggered by IgG or IgM antigen-antibody complexes which bind to and activate C1. C1 is a multimeric molecule consisting of C1q, C1r and C1s proteins. The antibody-antigen complex bind and activates
C1q, which results in this activation of C1r and subsequently C1s (Arumugam et al., 2006; Janeway et al., 2001). The C1s causes both C4 and C2 to be split, which results in C3 convertase (C4b2a) being formed. Subsequently, C4b2a splits C3 into C3a and C3b. The C3a acts as an anaphylatoxin (inflammatory mediator), while C3b binds with the C4b2a complex to form the C5 convertase (C4b2a3b).

The C5 convertase begins the formation of the membrane attack complex (MAC), that drills tiny pores in the pathogen membrane, resulting in lysis of microbes (Nesargikar., 2012; Edgar, 2006). The C5 convertase split C5 to C5a and C5b. The C5a is a potent inflammatory mediator, while the C5b then collect and recruits C6, C7, C8 and numerous C9 molecules to assemble the MAC. The MAC then kills the invading pathogen (Xiong et al., 2003; Tosi, 2005; Nesargikar et al., 2012).

The classical pathway can be activated by the other triggers like apoptotic cells, viral proteins, C-reactive proteins, amyloids and polyanions (Gasque, 2004; Padilla et al., 2007; Ehrnthal et al., 2011).

3.3.1.3.2 The lectin pathway

The lectin pathway is activated by binding of mannan binding lectin (MBL) to mannan containing surface membrane proteins on the pathogen surface (Petersen et al., 2001). This causes the activation of MBL-associated serine proteases (MASPs). The MASPs have similar physical and operating characteristics to activated C1 and acts on C2 and C4 to form the C3 convertase, C4b2a. The rest of the lectin pathway proceeds identically to the classic complement pathway (Petersen et al., 2001; Janeway et al., 2001).
3.3.1.3.3 The alternative pathway

Low-grade activation of C3 via spontaneous cleavage of C3 to C3(H_2O) happens continuously in plasma. The C3(H_2O) resembles C3b (Mathern and Heeger, 2015). The C3(H_2O) can bind covalently to pathogen surfaces, and this covalent binding of C3(H_2O) molecules to pathogens triggers the alternative pathway. In the absence of pathogens, C3(H_2O) is inactivated. Upon forming the C3(H_2O)-pathogen complex, the C3(H_2O) binds to factor B. Factor D then acts on factor B to give C3(H_2O)Bb an intermediate C3 convertase and Ba. Properdin, another serum protein, binds to and stabilise the intermediate C3 convertase. Complement factor C3 molecules in serum are split by C3 convertase, to produce C3a and C3b. While the new-formed C3b molecules have various tasks, C3a behaves as an anaphylotoxin. C3b binds to factor B, after which Factor D acts on C3bB to give C3bBb, a C3 convertase and Ba. Properdin, another serum protein, binds to and stabilise the C3 convertase. C3b can also bind C3bBb to produce C3bBbC3b, which is a C5 convertase. C3b also facilitate phagocytosis by opsonizing pathogens. Complement factors H and I, as well as the decay-accelerating factor (DAF), are deployed to inhibit chronic stimulation of the alternative complement pathway (Duncan et al., 2008; Nesargikar et al., 2012). When the C5 molecule is split by the C5 convertase, it forms C5a and C5b. Complement C5a is a potent inflammatory mediator, while C5b act as an opsonin. The C5b binds to the pathogen membrane and recruits C6, C7, C8 and C9 molecules to the form membrane attack complex (MAC). The MAC causes pores in the pathogen membrane that results in killing of the pathogen (Xiong et al., 2003; Tosi, 2005; Nesargikar et al., 2012).
3.3.2 Adaptive immunity

Adaptive immunity reacts to infections using cytotoxic T cells and antibodies (Ab) which kill off the pathogen (Takeda and Akira, 2004). The adaptive immune system can invoke one of two types of immune responses namely a humoral immune response facilitated by antibodies originating from B lymphocytes, or a cell-mediated immune response driven by T cytotoxic lymphocytes (Bonilla and Oettgen, 2010; Norbert et al., 2008). The response elicited is linked and specific for the particular microorganisms or substances causing the infection, and generally, this response is sufficient to eradicate the infection. The adaptive immune system has the ability to recognize pathogens that were previously encountered and can accordingly effect an enhanced response from memory when the same type of pathogen invades the body again. This result in a faster, more efficient response to subsequent attacks. Therefore, as observed by Norbert et al. (2008), adaptive immunity is characterized by two fundamental traits namely memory and specificity. The gene families encoding the specific antigen-recognition molecules are the result of mutations, recombination and duplications of genes producing histocompatibility complex (MHC) proteins, antibodies, and T cell receptors (Nairn and Helbert, 2005).

3.3.2.1 Activation of T helper lymphocytes

Helper T cells are important cells in adaptive immunity (Alberts et al., 2002). Helper T cells regulate and direct specific immune responses by producing cytokines to promote B and T cytotoxic cell responses, and are also involved with stimulating mononuclear phagocytes (Rabb, 2002). T cell antigen receptors can
only recognize antigens when these are presented on the surface of another cell and attached to MHC molecules. The T cells that recognizes antigen peptides on MHC class II are known as T helper (Th) cells (Helbert, 2006; Albert et al., 2002). When Th cells are activated, they produce a range of cytokines. The major types of Th cells are known as the Th1 and Th2 cells. Each of these two type cells produces different types of cytokines. Th1 cells produce pro-inflammatory cytokines that include the IFN-γ and IL-12 family (IL-18, IL-23, and IL-27). IL-18, IL-23 and IL-27 assist IL-12 to increase production of IFN-γ. IFN-γ is produced by activated CD4 Th cells that were stimulated by IL-12 through receptor signalling that involves activation and phosphorylation of Jak2/Tyk2 and STAT4 (Szabo et al., 2003). Cytokines from Th1 cells activate T cytotoxic cell mediated responses and also activate NK cells and mononuclear phagocytes to kill cells infected with intracellular pathogens such as mycobacteria and viruses (Zhu et al., 2006). The Th2 cells produce various cytokines such as IL-4, IL-5, IL-10 and IL-13. Their development is initiated by IL-4 and the transcription factor GATA-3. Cytokines produced by Th2 cells promote antibody production, and are responsible for defences against extracellular infections (Zhu et al., 2006).

3.3.2.1 Humoral Immunity

Humoral immunity defends the body against extracellular pathogens, like bacteria. In humoral immunity, B lymphocytes fulfil a crucial function by producing antibodies (Janeway et al., 2001). Humoral immunity is reliant on B cells for the creation of antigen-specific antibodies. To produce antibodies require aligned interaction of B cells, Th2 cells and antigen presenting cells.
B cells function as antigen presenting cells by expressing MHC class II molecules and peptides on their surface (Dorner et al., 2009). These peptides are formed during the process in which the antigens are enveloped by the B cell, after being bound to its immunoglobulin receptors. When B cells are stimulated, there are two possible options; either they form a germinal centre after entering a follicle, or they differentiate into short-lived plasma cells. B cells start off producing IgM and IgD, but then in the germinal centre, they can the antibody class to produce IgA, IgE or IgG. This change in the type of antibodies produced by B cells is referred to as class-switching or isotype switching. It can occur through a process of gene rearrangement, similar to the gene segment rearrangement process for T and B cell receptors (Bonilla and Oettgen, 2010).

Following the processing of the MHC class II molecules/peptide complexes and its presentation on the cell membrane, Th0 cells binds MHC class II molecules/peptide complexes to developed into Th2 in the presence of Interleukin 4 and 10 (IL-4 and IL-10). The cytokines, IL-4 and IL-10 stimulate B-lymphocytes to produce antibodies (effector cells) and memory cells from B-lymphocytes (Franchimont et al., 2000; Chung, 2001).

There are similarities between the functioning of macrophages and B cells — both cell types devour antigens, and subsequently present remnants of these antigens on their external surface as MHC class II-antigen complexes. The extracellular display of antigen debris attracts T helper cells, which bind to the B cell surface. Upon binding cytokines secreted by the Th2 cells, the B cells are stimulated to proliferate and produce plasma B-cells that secrete antibodies and memory cells.
While the plasma cells discharge a huge number of antigen-specific antibodies, the memory cells are stored to act immediately should a subsequent invasion with the same pathogen occur (Bonilla and Oettgen, 2010).

3.3.2.2 Cell-Mediated Immunity

Cell-mediated immunity (CMI) is the part of the immune system that defends us against intracellular pathogens. Cell mediated immunity can function in the absence of antibodies. Cells responsible for CMI are the T cytotoxic cells, memory T cells, Th1 cells, antigen presenting cells (APCs) and phagocytes (Norbert et al., 2008).

CMI is tasked with the identification and destruction of intracellular pathogens, for example mycobacteria or viruses that cross the cell membrane and live and replicate inside cells. Antigens from intracellular pathogens are attached to MHC Class I molecules in the infected cells. MHC Class I-antigen complexes are presented to the immune system on the membrane of the infected cell, while MHC Class II-antigen complexes are presented to the immune system on the membrane of APCs. When Th1 cells are stimulated by MHC Class II-antigen complexes they produce cytokines such as IL-12 and IFN-γ. T cytotoxic cells are activated when they bind MHC Class I-antigen complexes through their T cell receptor. The Th1 cell cytokines then stimulate the T cytotoxic cells to produce IFN-γ and various other lymphokines (Murphy, 1996). IFN-γ slows down viral replication within the affected cells. This cytokine also activates macrophages, resulting in the killing of infected cells by macrophage secretory products such as perforin (Szabo et al., 2003; Spellberg and Edwards, 2001).
3.4 Inflammation

Inflammation is an intricate, very structured, chain of actions that are initiated by an array of triggers, among which are autoimmune reactions, destructive chemical and mechanical processes, as well as pathogens. Inflammatory triggers mentioned in the previous sentence cause the release of inflammatory mediators. The inflammatory mediators will be discussed in more detail later in this section. The sequence of events that follow inflammatory molecule secretion is perceived by the affected individual by symptoms such as pain, heat, swelling, and redness. An inflammatory reaction takes place in the vascularized connective tissue, which includes extracellular and cellular components, circulating cells, plasma, and blood vessels.

This inflammatory response coincides with the liberation of inflammatory mediators. The inflammatory mediators cause various physiological and biochemical reacts such as the recruitment of leukocytes, dilation of microvasculature, and increased vascular permeability (Fujiwara and Kobayashi, 2005). The main mechanism utilised by the body to repair damaged tissue and fights infections occur via inflammatory processes. In the normal physiological situation, a regulated reaction from the immune system protects the body from injury and clears away impaired tissue. If unchecked however, inflammation can lead to the destruction of tissue, and result in organ malfunction (Beckmann et al., 2009).

Inflammation can either be acute, or chronic. Acute inflammation occurs over a comparatively short time, typically ranging from minutes to a couple of days. Its primary traits are oedema caused by the excretion of plasma proteins and fluid
from the vasculature, and the migration of leukocytes, mainly neutrophils, from the circulatory system to the locality of injury. Chronic inflammation lasts longer than acute inflammation and, in terms of histology, it is coupled to the advent of fibrosis, tissue necrosis, and tissue infiltration by lymphocytes and macrophages. During the course of both acute and chronic inflammation, there are numerous histological factors and features which play a part. During inflammation, the three main contributions of macrophages are: phagocytosis, the presentation of antigens, and immunomodulation, which is achieved by producing a variety of growth factors and cytokines (Kasahara and Matsushima, 2001). Macrophages secrete cytokines such as tumor necrosis factor (TNF), IL-1, IL-6, IL-8, and IL-12, after macrophages are exposed to inflammatory stimuli. Even though macrophages and monocytes are the main sources of these cytokines, they are also produced by stimulated endothelial cells, lymphocytes, and fibroblasts. Macrophages also produce chemokines, leukotrienes, prostaglandins, and complement factors that stimulate inflammation (Arango and Descoteaux, 2014). It must be noted that inflammation is not only caused by infection, but that tissue injury can also cause inflammation. Debris from damaged cells at the injury site is sufficient to elicit the response. Phagocytic cells are summoned to the injury site by the inflammatory reaction and are tasked with the removal cellular debris. This process prepares the injury site for the repair of the injury, which is induced by the activation of complement (Serhan, 2010).
3.5 Interaction between GFNs and immune system components

Research into the interaction between the graphene family nanoparticles (GFNs) and biotic systems has increased rapidly (Zhao et al., 2015). The biocompatibility of GFN is becoming important for biomedical applications such as drug and gene delivery, tissue engineering, biosensing and imaging. In this regard, it is crucial to understand the process of interaction of GFN with the immune system (Saleem et al., 2017; Seabra et al., 2014). The immune system recognizes foreign particles. By manipulating the chemical substitution on a nanoparticle, new particles can be produced that do not trigger an immune reaction, making it safer for use in medical applications (Ricklin et al., 2010).

Some chemicals have the ability to modulate the immune function. These immunomodulatory chemicals can be environmental contaminants, chemicals in the occupational environment (e.g. pesticides), heavy metals, pharmaceutical and
direct and indirect food additives. The field of immunotoxicology has experienced rapid developments during the past two decades and hence numerous studies have been conducted on the immune system as a target organ. This prompted many federal agencies and international organizations to prepare guidelines for conducting immunotoxicity studies. Furthermore, various immunotoxicity testing approaches have been proposed by researchers in the field (Palomäki, 2014).

Most research on the toxicology of nanomaterials has focused on the effects of nanoparticles that penetrate the body accidently or involuntarily. There is a lack of studies on the toxicology of nanoparticles that are used for biomedical applications and which are deliberately introduced into the body, such as nanoparticles, drug delivery or imaging. Moreover, there are no harmonized standards for assessing the immunotoxicity of nanoparticles to the immune system (immunotoxicity) (Dobrovolskaia and McNeil, 2007).

Nanomaterials, depending on their characteristics and compositions, can interact with the immune system in several ways and they either up- or down-regulate immune system function (Elsabahy And Wooley, 2013). Cytokines are proteins that do pleiotropic functions and play a key role in regulating and mediating the immune response. Cytokines are generally recognized as biomarkers of immunotoxicity (Elsabahy and Wooley, 2013). While the specificity and validity of certain cytokines as markers of adverse immune responses has been established for chemicals, small and macromolecular drugs, research on their applicability for predicting and monitoring the immunotoxicity of engineered nanomaterials are still scarce (Elsabahy and Wooley, 2013). The exact mechanism of nanoparticle immunotoxicity and correlation with the endocytic pathways have not been
clarified yet. Variable results for nanoparticles made from the same chemical can often be found in the literature. The variabilities are normally due to nanoparticle structure, cell type used, cell cycle phase, animal model, disease status, etc (Stebbings et al., 2007). In addition, most studies utilize the various markers to predict the possible immunomodulatory effects of nanomaterials and they do not investigate the exact mechanisms behind the immunotoxic effects and/or the differences in immune responses to nanoparticles of different size, shape, surface chemistry and composition. Sometimes the induction of both proinflammatory cytokines (e.g. IL-6 and TNF-α) and anti-inflammatory cytokines (e.g. IL-10) due to unregulated innate immune responses (i.e. cytokine storm) makes it difficult to understand the underlying mechanisms of immunotoxicity (Stebbings et al., 2007; Suntharalingam et al., 2006).

Modulation of the immune function by nanomaterials can either be useful or detrimental, depending on the intended use (Zolnik et al., 2010). Nanoparticles can serve as immunomodulatory agents (e.g. vaccine adjuvants, anti-inflammatory, immunosuppressive drugs). However, concerns are raised when an engineered nanomaterial not intended for interaction with the immune system, changes its function. It has been found that certain nanomaterials can be immunotoxic. No standardized immunotoxicity testing protocol, specific for a particular size of nanoparticle shape/size, has been described so far (Dobrovolskaia and McNeil, 2007; Chang, 2010). In general, the same set of immunological tests are routinely used to assess immunotoxicity of chemicals, medical devices and drugs can be applied to engineered nanomaterials (Dobrovolskaia et al., 2009).
Engineering nanomaterials (ENM) may increase the production of reactive oxygen species (ROS), resulting in oxidative stress capable of activating innate immune responses. ENM may also induce the release of pro-inflammatory cytokines independently of ROS production (Hornung et al., 2008; Lu and Liu, 2009).

Some nanomaterials interact with the complement system in a variety of ways (Thomas et al., 2014). However, only a few studies have shed light on the interaction of graphene with the complement system. It has been demonstrated that single walled CNTs can trigger the classical pathway, while double walled CNT trigger both the classical and alternative complement pathways (Salvador-Morales et al., 2006).

Nanomaterials can activate macrophages responsible for local and systemic inflammation. Differentiated monocytes and professional phagocytes assist the body to eliminate pathogen or dead cells. When inflammation is activated through reactive oxygen species (ROS) or primed by antigens, macrophages trigger an innate immune response (Nicolete et al., 2011).

Adding functional groups on graphene sometimes minimize the harmful effect of graphene derivatives on macrophages. Zhi et al. (2013) stated that PVP modification reduced the phagocytosis of GO by macrophages and it showed less cytotoxicity as compared to pristine GO (Zhi et al., 2013). In another study, Luo et al. (2015) used graphene oxide (GO) and engineered the surface with polyethylene glycol (PEG), bovine serum albumin (BSA), and polyether imide (PEI) to examine their interaction with macrophages. The study found that PEG and BSA functionalized GO decreased endocytosis of the particle. They
concluded that the PEG and BSA were more biocompatible than PEI-GO because PEI GO, being positively charged, interacts strongly with the plasma membrane and thus it is rapidly internalized (Luo et al., 2015).

Macrophages are one of the most important effector cells of the innate immune system, and play a significant role in the response to graphene exposure (Zhou et al., 2012). Macrophages are a major producer of many cytokines and chemokines involved in immune responses. These cytokines can modulate most macrophage functions (autocrine effects) and the expression of cell surface markers, while chemokines contribute to the recruitment of circulating monocytes (paracrine and endocrine effects) within tissues (Zhou et al., 2012).

The majority of the research performed on GFNs in vitro was carried out using the murine macrophage cell line RAW 264.7, due to high experimental reproducibility, it is ease of culture, the cell is good transfection host and it is suitable for nanomaterial-based conjugate uptake assays. Also, a possible reason for a lot of studies is that this cell type can be used for long term studies, compared to primary immune system cells that only have a finite culture life-span of a few days to weeks (Orecchioni et al., 2014).

In the context of graphene, the number of publications related to the interaction of this material and the immune system are increasing rapidly. Studies have shown that pristine graphene can induce cytotoxicity on macrophage through the depletion of the mitochondrial membrane potential and the increase of intracellular reactive oxygen species (Li et al., 2012). Graphene (G) and its derivatives also interact with macrophages. A study has reported that phagocytes were capable of internalizing graphene oxide (Yue et al., 2012). Graphene, bound
to platelets, induce inflammatory responses \textit{in vivo} and \textit{in vitro} (Schinwald et al., 2012).

Many studies examined how macrophages respond to GO treatment. Chen et al. (2012) found that RAW264.7 cells exposed to GO caused autophagy of the cells in a concentration-dependent manner. The treatment yielded autophagic vacuoles and the activation of autophagic marker proteins (Chen et al., 2012; Qu et al., 2013). GO induced autophagy was observed in various other cell lines and in macrophage treated with GO of different sizes. Strikingly, GO treatment of macrophages induced the toll like receptor (TLR) signalling cascades and triggered ensuing cytokine responses. Molecular analysis showed that TLR4 and TLR9 and their downstream signalling mediators MyD88, TRAF6 and NFkB played a significant role in the GO induced responses (Chen et al. 2012).

Although graphene (G) has unique properties that make it useful for various medical, agricultural and industrial applications, large scale production and use of this chemical pose potential adverse human and environmental health effects. It is essential that investigations into potential chronic effects posed by this and other nanoparticles be conducted before such nanoparticles are used and produced in large quantities. Therefore more \textit{in vitro} and \textit{in vivo} studies on graphene and GO are required to fill the gaps in our current knowledge and give us indications of potential adverse effects posed by these new chemicals (Li et al., 2013). The current study will investigate the inflammatory effects of GO.
CHAPTER 4

MATERIALS AND METHODS

4.1 Synthesis and characterization of graphene oxide nanoparticles (GONP)

Graphene oxide nanoparticles (GONP) were synthesized using the modified Hummer’s method (Hummers Jr et al., 1958). In brief, 1 g of graphite and 1 g of sodium nitrate (Sigma-Aldrich) were mixed together in an ice-bath followed by adding 46 ml of concentrated sulfuric acid (Fisher Scientific). This was followed by the addition of 6 g of KMnO$_4$ (Flinn Scientific) to the mixture. After stirring for 1 h at 35°C, 80 ml of distilled water (dH$_2$O) was added to the mixture and the temperature was increased to 90°C for 30 min. Hydrogen peroxide (H$_2$O$_2$) (Fisher Scientific) at 30% v/v (200 ml) was then added to the reaction mixture. The precipitate was washed with dH$_2$O until the pH reached 4-5. The final GO was obtained using exfoliation under ultrasonication at 42 kHz for 45 min using a Branson 2510 sonicator.

Graphene oxide sheets were visualized via transmission electron microscopy (TEM, JEOL 1400). At size 540 nm further characterization revealed the particles had a maximum absorbance at 227 nm using ultraviolet-visible spectrophotometry (UV-vis) (Lab Tech, UV 8100B, USA), with a scanning range 200-600 nm. A number of surface functional groups (i.e. hydroxyl and carboxyl groups) were detected with Fourier-transform infrared spectroscopy (FT-IR) using a Nicolet 4700 FT-IR spectrophotometer (Thermoscientific). The spectrum was collected over a wave number range from 400 to 4000 cm$^{-1}$. Zeta potential ($\zeta$-potential) was
measured with a ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, U.K.) and indicated a surface potential of -49.2 mV at pH 7.

4.2 Preparation of GONP for cell cultures

Stock GONP (10 mg/ml) was prepared in distilled water. The GONP was sonicated (QSonica, LLC. Misonix sonicators, XL-200 Series) in short bursts, on ice for a total of 90 min. Aliquots of the stock solution were frozen at -80 °C until use. Before use in experiments, nanoparticles were defrosted and further sonicated in short bursts on ice for 5 min.

4.3 Cell culture and exposure to GONP

The murine macrophage cell line, RAW 264.7, was obtained from American Type Culture Collection (ATCC TIB-71). The macrophages were grown in 75 cm² tissue culture flasks (Sigma-aldrich). Cells were maintained in complete media comprised of Dulbecco’s Modified Eagle’s Medium (DMEM) (Lonza) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone), 1% antibiotic/ antifungal mixture (Sigma-aldrich), 0.5% gentamycin (Sigma-aldrich), and 1% glutamax (Sigma-aldrich). Cells were incubated in a humidified atmosphere of 5% CO₂ at 37 °C for 48 hours until the cells reached 80-90% confluence.

The Raw 264.7 cells were cultured in complete medium at a concentration of (1 x 10⁵ cells/ml) and seeded at 400 µl/well in cell culture treated 24 well plates (Nunc). After a 48-hour incubation period, media was removed and replaced with 1000 µl/well medium containing 1% FBS. The subsequent procedures occurred in serum free media. Various concentrations of the sonicated GONP
stock diluted in serum free medium. Replicates of each GONP concentration were added a column on the culture tray (1000 µl/well). A column containing no GONP received 1000 µl/well of serum free medium only, while a column representing a positive inflammation control received 1000 µl/well of serum free medium containing 1 µl/ml lipopolysaccharide (LPS). The final concentration of FBS/well was 0.5% v/v and the final volume per well was 2 ml. Cultures were incubated overnight (18 hours) under standard tissue culture conditions. Following the incubation, 100 µl supernatant were removed from each well and assayed for nitric oxide (NO) and Interleukin 6 (IL-6). The rest of the spent culture supernatants were collected and stored in aliquots at -80°C.

4.4 Cytotoxicity Assay

After the removal of the supernatants, cells were washed with Dulbecco’s Phosphate Buffered Saline (DPBS) (Lonza), supplemented with glutamax, antibiotic/antimycotic mixture solution. Cytotoxicity was measured using the 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4 disulfophenyl)-2H-tetrazolium sodium salt (WST-1) (Roche). The WST-1 reagent (1:10 in serum free medium) was added to each well. This assay is based on the cleavage of the water soluble tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases and the level of formazan formed is directly proportional to cell viability. The plate was read immediately after WST-1 addition and again after a 1 hour incubation at 37 °C in 5% CO₂ at 450nm using a plate spectrophotometer (Multiskan Ex, Thermo Electron Corporation).
4.5 Nitric Oxide (NO) assay

The NO produced was determined using the Griess assay performed in a 96 well flat bottom plate (Greiner bio-one) at room temperature. Each well received 50μl of the respective supernatant or nitrite standard (doubling dilution range starting at 100 μM). Each well then received 100μl of Griess reagent consisting of a 1:1 v/v of 1% Sulphanilamide (Sigma-aldrich) and 0.1% naphylethylenediamine-dihydrochloride (Sigma-aldrich) in 2.5% phosphoric acid. The plate was then incubated at ambient temperature for 15 minutes, after which, the absorbance was read at 540nm using a microplate reader (Multiskan Ex, Thermo Electron Corporation). A standard curve was constructed using Excel and the NO in the culture supernatants were read from this curve.

4.6 Mouse IL-6 quantitation

The IL-6 in the cell culture supernatants were assayed using a double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). The mouse IL-6 ELISA kits used (e-Bioscience, Ready-Set-Go) contain all reagents required for the assay and was used as per the manufacturer’s instructions. Briefly, coat a 96 well Nunc Maxisorb™ plates with 50 μl/well of mouse IL-6 capturing antibody (Ab) in coating buffer and incubated at 4°C overnight. The plate was then washed 5 times (5X) with wash buffer (PBS containing 0.1% Tween 20) after which the non-specific binding sites were blocked with 200 μl/well assay diluent for an hour. The plate was then washed 5X with wash buffer after which 50 μl/well culture supernatant or standard were added to the plate. The LPS stimulated inflammation control was assayed at a 1/40 v/v dilution in assay diluent, while other samples
were assayed at 1/5 v/v in assay diluent. Standards were prepared by doubling dilutions of the kit’s 2000pg/ml IL-6 standard in diluent. The plate was then and sealed incubated for 2 hours at room temperature. The plate was then washed with wash buffer, after which 50μl/well of biotin conjugated anti-mouse IL-6 detecting Ab was added. The plate was then incubated for 1 hour. The plate was again washed 5X, after which 50 μl/well of streptavidin-horseradish peroxidase conjugate was added to each well. The plate was then incubated for 30 minutes. The plate was again washed 7X, after which 50 μl/well of 3, 3’, 5, 5’-Tetramethylbenzidine (TMB) ELISA substrate solution was added. Following a 15 minutes’ incubation in a dark, 50 μl/well of stop solution (0.18 M sulphuric acid) was added. The optical densities of the colour reaction were read at 450 nm using spectrophotometer (Multiskan Ex, Thermo Electron Corporation).

4.7 Macrophage inflammatory protein (MIP) quantitation

Commercially available DAS ELISA kits (R&D Systems) were used to quantitate MIP-1α, MIP-1β and MIP-2 in the cell culture supernatants. Assays were done according the manufacturer’s instructions and all reagents required for the assay were supplied by the manufacturer. Briefly, a 96 well Maxisorb™ plate (Thermo Fisher, Denmark) was coated with 50 μl/well anti-mouse MIP capture antibodies overnight at 4°C, after which the plate was washed 5X with wash buffer (PBS containing 0.1% Tween 20). Nonspecific binding sites were blocked with 2% m/v of human serum albumin (HSA) in PBS for an hour. Plates were washed 5X with wash buffer. The cell culture supernatants (samples) were diluted in assay diluent (1% m/v HSA in PBS). The MIP-1α samples were assayed at 1/270 v/v dilution and the inflammation positive control was assayed at 1/10000 v/v. For the MIP-1β
ELISA, the samples were assayed at 1/20 v/v and the inflammation positive control was assayed at 1/5000 v/v. The MIP-2 ELISA samples were assayed at 1/100 v/v and the inflammation positive control was assayed at 1/1000 v/v. The ELISA standards were prepared according to the manufacturer's instructions namely doubling dilutions of the supplied standard in assay diluent. The plate was sealed and incubated for 2 hours at room temperature. The plate was then washed 5X with wash buffer after which 50 μl/well of biotinylated anti-mouse MIP detecting Ab was added. The plate was then incubated for 2 hours. The plate was washed 5X after which 50 μl/well of streptavidin-horseradish peroxidase conjugate was added to each well and then incubated for 20 minutes. The plate was washed 7X after which 50μl/well of 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) ELISA substrate solution was added. This was followed by a 15 minute incubation in the dark. After the incubation period 50 μl/well of stop solution (0.18 M sulphuric acid) was added. Absorbances were read at 450 nm (Multiskan Ex, Thermo Electron Corporation). Cytokine concentrations were reported in pg/ml of MIP in the culture supernatant. The results were analysed by Microsoft Excel.

4.8 Mouse Proteome Profiling

Mouse cytokines in cell culture supernatants were measured by proteome profiling using the mouse cytokine antibody array kit panel A (R&D Systems) according to the manufacturer’s instructions. This cytokine and chemokine antibody array was used to determine the effects of GONP exposure on cytokine and chemokine synthesis by RAW 264.7 macrophage cells. The kit contains all
reagents required for the assays and were done according to the manufacturer’s instructions. The assay is done on a nitrocellulose membrane with 40 capturing antibodies, for specific cytokines and chemokines, spotted on it in duplicate. In brief, a membrane was removed from the kit for each cell culture supernatant sample to be analysed and placed in individual wells of a 4 well plate. The membranes were incubated with blocking buffer at room temperature for 1 h. During this step, cell culture supernatants (500 μl/sample) obtained after the various exposures were mixed with a biotinylated detection antibody cocktail at room temperature for 1 hour after which the mixtures were added to their respective well containing the blotting membranes. The dishes were incubated overnight at 4°C. The membranes were then washed four times for 15 min each and subsequently incubated with horseradish peroxidase-conjugated streptavidin for 30 min at room temperature. The membrane was then washed 7X for 15 min each, after which, the lower edge of the membrane was blotted onto a paper towel. The membrane was then placed back into the 4 well multi-dishes. Following the addition of 1 ml of insoluble 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate per well the multi-dish was incubated on a platform shaker at room temperature for 5-6 min. The reaction was stopped by decanting the substrate and adding distilled water to the respective wells. Positive proteins appear as blue/black spots on the membrane. Photographs of the membrane were taken. Membranes were inspected visually.
4.9 Statistical analysis

All experiments were performed triplicate and the data was calculated using Microsoft Excel. Data was presented as mean ± standard deviation (SD) of at least three triplicate experiments, each containing 4 replicates of all data points (i.e. at least 12 individual assays per data point). Data was statistically analysed via one-way analysis of variance (ANOVA) using Sigma Plot 12.0 to assess statistical differences. P values ≤ 0.01 are regarded as statistically significant.
CHAPTER 5

RESULTS

5.1 The effects of GONPs on viability of RAW 264.7 cells

RAW 264.7 cells exposed to various concentrations of GONPs were analysed for viability using the WST-1 assay kit. A positive inflammation control (1 µg/ml LPS in medium) was included in the assay. GONP concentrations \( \leq 250 \) µg/ml had no effect on cell viability (Figure 5.1). However, cell viability decreased significantly at 500 µg/ml GONPs (\( P < 0.01 \)). At 500 µg/ml GONPs the viability of cells was less than 60% when compared to the culture incubated at 0 µg/ml GONPs.

Figure 5.1 The effects of GONP on RAW 264.7 cell viability. Cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. At the end of the incubation period the cell viability was determined using the WST-1 Assay. Data presented are the mean ± standard deviation of 9 replicates. The * sign indicates that the result is significantly different compared to 0 µg/ml GONP (\( P < 0.01 \)).
5.2 Effect of GONP on inflammatory biomarkers produced by RAW 264.7

Several biomarkers were used to evaluate the inflammatory activity of GONPs. The biomarkers investigated were nitric oxide (NO), interleukin 6 (IL-6) and the macrophage inflammatory proteins 1α, 1β and 2 (MIP1α, MIP1β and MIP2).

5.2.1 Nitric oxide (NO)

None of the GONP concentrations investigated had an effect on NO synthesis by RAW 264.7 cells (Figure 5.2). A LPS stimulated inflammation positive control (Pos) was included in the results to verify that cells can produce NO. The Pos sample produced significantly higher \((p \leq 0.01)\) NO than the 0 \(\mu g/ml\) GONP exposed cells (17 ± 3 \(\mu M\) and 4 ± 1 \(\mu M\) respectively). All the GONP concentrations investigated induced similar \((p \geq 0.01)\) NO levels to the 0 \(\mu g/ml\) GONP exposed cells.

[Diagram showing NO production by RAW 264.7 cells exposed to various concentrations of GONP.]

**Figure 5.2** The effect of GONP on NO production by RAW 264.7 cells. Cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. Data presented are the mean ± standard deviation of 9 replicates. The * sign indicates that the result is significantly different compared to 0 \(\mu g/ml\) GONP \((P < 0.01)\).
5.2.2 Interleukin 6 (IL-6)

Interleukin 6 was used as a biomarker to determine the inflammatory response induced by GONP (Figure 5.3). The LPS stimulated positive inflammation control induced significantly higher IL-6 ($p \leq 0.01$) than the 0 µg/ml GONP exposed cells (not reflected in Figure 5.3, as IL-6 produced was too high at 80862 ± 24175 µg/ml). Data showed that GONPs at 15.6 and 31.25 µg/ml induce statistically significant higher levels of IL-6 ($p < 0.001$) compared to the 0 µg/ml GONP exposed cells. There was no significant difference in IL-6 produced by cells exposed to GONPs concentrations ≥ 62.5 µg/ml compared to the 0 µg/ml GONP exposed cells.

![Figure 5.3](http://etd.uwc.ac.za/)

**Figure 5.3** The effects of GONPs on IL-6 production by RAW 264.7 cells. Cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. Data presented are the mean ± standard deviation of 9 replicates. The * sign indicates that the result is significantly different compared to 0 µg/ml GONP ($P < 0.01$).
5.2.3. The effects of GONPs on MIP-1α synthesis by RAW 264.7 cells

The level of macrophage inflammatory protein (MIP-1α) produced by the cell cultures were measured by ELISA (Figure 5.4). A LPS positive inflammation control was included in the assay. MIP-1α secretion by the LPS positive control was significantly higher ($p \leq 0.001$) compared to MIP-1α produced by 0 µg/ml GONP exposed cells (803 ± 354 and 39 ± 8 ng/ml MIP-1α respectively). These data are not reflected in as the concentration for the positive control was too high.

Cell cultures exposed to GONPs at 15.6, 31.25 and 62.5 µg/ml secreted significantly higher MIP-1α than the 0 µg/ml GONP exposed cell culture, while cultures exposed to 125, 250 and 500 µg/ml GONP produced significantly less MIP-1α than the 0 µg/ml GONP exposed cell culture.

![Figure 5.4](http://etd.uwc.ac.za/)

**Figure 5.4** The effects of GONP on the secretion of the MIP-1α(ng/ml) by RAW 264.7 cells. The cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. Data presented are the mean ± standard deviation of 9 replicates. The * sign indicates that the result is significantly different compared to 0 µg/ml GONP ($P < 0.01$).
5.2.4 The effects of GONPs on MIP-1β synthesis by RAW 264.7 cells

The amount of macrophage inflammatory protein (MIP-1β) secreted by RAW 264.7 cells were measured by a DAS ELISA (Figure 5.5). GONP concentrations at 15.6 and 31.25 μg/ml produced significantly higher (P < 0.001) MIP-1β compared to the culture exposed to 0 μg/ml GONP. On the other hand, GONP concentrations of 62.5, 125, 250 and 500 μg/ml, produced significantly lower (P < 0.001) MIP-1β compared to the culture exposed to 0 μg/ml GONP. A positive inflammation control containing LPS was included in the experiment, but data is not reflected in Figure 5.5 as the MIP-1β produced was too high (1127 ± 469 ng/ml MIP-1β). The LPS positive inflammation control produced significantly higher (P < 0.001) MIP-1β than the 0 μg/ml GONP exposed cells.

![Graph showing the effects of GONP on MIP-1β secretion by RAW 264.7 cells.](http://etd.uwc.ac.za/)

**Figure 5.5** The effects of GONP on the secretion of the MIP-1β(ng/ml) by RAW 264.7 cells. The cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. Data presented are the mean ± standard deviation of 9 replicates. The * sign indicates that the result is significantly different compared to 0 μg/ml GONP (P < 0.01).
5.2.5 The effects of GONPs on MIP-2 synthesis by RAW 264.7 cells

The concentrations of MIP-2 secreted by the RAW 267.4 cells were measured by DAS ELISA (Figure 5.6). Cultures exposed to 15.6, 31.25 and 62.5 μg/ml GONPs produced significantly higher MIP-2 (p < 0.001) compared to cells exposed to μg/ml GONP. However, 125, 25 and 500 μg/ml GONP levels did not affect the MIP-2 synthesis level. The positive inflammation control containing LPS secreted 307 ± 172 ng/ml MIP-2) is not reflecting in the Figure 5.6 as the MIP-2 was too high.

![Figure 5.6](http://etd.uwc.ac.za/)

**Figure 5.6** The effects of GONP on the secretion of the MIP-2(ng/ml) of RAW 264.7 cells. The cells were exposed to various concentrations of GONP in culture medium. Cells were also incubated with a control consisting of culture medium containing LPS as described in the methods. Data presented are the mean ± standard deviation of 9 replicates. The * sign indicates that the result is significantly different compared to 0 μg/ml GONP (P < 0.01).
5.3 GONP modulation of cytokine and chemokine profiles of RAW 264.7 cells

The proteome profile of the culture supernatants obtained from the LPS positive inflammation control, and the 0 and 15.6 μg/ml GONPs exposed cultures are presented in Figure 5.7. The photographs of the membranes show clear differences in chemokine/cytokine synthesis between the various cell treatments and confirm that the proteome profiles are modulated by the various exposure conditions.

![Image of membranes with labeled spots]

**Figure 5.7** The effect of GONPs on inflammatory biomarker secretion by RAW 264.7 cells. Cells were incubated with (i) medium only (negative control), (ii) medium in the presence of LPS (positive control) and (iii) medium containing 15.6 μg/ml GONPs not stimulated with (LPS). Supernatants were screened using a proteome profiler array. Cytokines/chemokines that were detected were allocated numbers: 1, 3, and 16 are reference spots; 2- IP-10; 4- G-CSF; 5- TNF-α; 6- GM-CSF; 7- IL-6; 8- JE; 9-sICAM-1; 10- MIP-1α; 11- MIP-1β; 12- IL-1β; 13- MIP-2; 14- IL-1ra; 15- RANTES; 17- IL-27; 18-SDF-1.

Visual inspection of proteome profiles presented in Figure 5.7 indicate that the proteins that are modulated include IFNγ-inducible protein 10 (IP-10);
granulocyte colony stimulating factor (G-CSF); granulocyte-macrophage colony-stimulating factor (GM-CSF); IL-6; monocyte chemoattractant protein 1 (JE); interleukin 27 (IL-27); macrophage inflammatory protein 2 (MIP-2); interleukin 1 receptor antagonist (IL-1ra) and IL-1β (Table 5.1). RAW 264.7 cells exposed to 0 μg/ml GONP secrete TNF-α, sICAM-1, MIP-1 α, MIP-1β, RANTES and SDF-1. The 15.6 μg/ml GONP exposed cells secrete all the cytokines produced by the 0 μg/ml GONP exposed cells and also three additional proteins namely G-CSF, JE and MIP-2. The LPS inflammation positive control secrete all the cytokines produced by the 0 μg/ml GONP exposed cells and also nine additional proteins namely IP-10, G-CSF, GM-CSF, IL-6, JE, IL-1 β, MIP-2, IL-1ra and IL-27.

Table 5.1 The modulation of cytokines/chemokines secreted by RAW 264.7 cells cultures after exposure to LPS and 15.6 μg/ml GONPs compared to cells exposed to medium of (0 μg/ml GONPs). Data is represented as not detectable (ND) or visible (+). Proteins upregulated by cells due to the exposures to LPS or 15.6 μg/ml GONP compared to the 0 μg/ml GONP exposed cells are shaded

<table>
<thead>
<tr>
<th>Cytokines / Chemokines</th>
<th>Allocated numbers</th>
<th>0 μg/ml GONPs</th>
<th>LPS Control</th>
<th>15.6 (μg/ml) GONPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Spots</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>2</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>G-CSF</td>
<td>4</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>6</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>IL-6</td>
<td>7</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>JE</td>
<td>8</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>11</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1β</td>
<td>12</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>MIP-2</td>
<td>13</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>14</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>RANTES</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-27</td>
<td>17</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>SDF-1</td>
<td>18</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
CHAPTER 6

DISCUSSION

6.1 The effect of GONPs on viability of RAW 264.7 cells

Carbon based nanomaterials have unique thermal, physical, and chemical properties that are potentially useful in biomedicine applications, such as drug and gene delivery (Cho et al., 2016). Their wide range of applications has necessitated investigations into their potential toxic effects on biological systems (Wan et al., 2013). However, very little is known about the effects of these nanoparticles on the immune system.

The current study shows that GONPs at concentrations of 250 μg/ml and lower were not cytotoxic to RAW 264.7 cells. However, 500 μg/ml GONPs induced cytotoxicity in RAW 264.7 cultures. Previous studies using RAW 264.7 cells showed similar effects for pristine graphene (Li et al., 2012). Lu et al., using HEK293T cells as model, found that GONP exposure decreased cell viability (Lu et al., 2017). Orecchioni demonstrated consistent cytotoxic effects against macrophages and monocytes regardless of cell models used for assays (Orecchioni et al., 2016). However, Zhang et al. reported that the decrease in cell viability could also be attributed to the higher capacity of macrophages to internalize GO and this could have affected the assembly of actin within the cell.
6.2 The effect of GONPs on inflammatory biomarkers produced by RAW 264.7 cells

Macrophages are major producers of many cytokines and chemokines involved in immune responses (Zhou et al., 2012). Due to the known immune responses of macrophages, they are the ideal cells for in vitro tests to monitor the effects of chemicals on the immune system. Previous studies indicated that graphene and graphene oxide nanoparticles activate macrophages and several other cell types, resulting in the modulation of biomarkers associated with metabolism, inflammation and apoptosis (Zhang et al., 2016). Due to the cytotoxic effects of GONP at high concentrations on RAW 264.7 macrophages, the current study embarked on further investigations to find if GONPs modulate biomarkers of inflammation. The specific immune biomarkers that were investigated are nitric oxide (NO) production, and secretion of interleukin 6, macrophage inflammatory protein (MIP) 1α, MIP-1β and MIP-2. The data obtained showed that although GONPs did not activate NO synthesis, the levels of IL-6, MIP-1α, MIP-1β and MIP-2 were upregulated at low concentrations of GONP. The upregulation of the biomarkers occurs at concentrations much lower (15.6 to 62.5 µg/ml) than the GONP concentration causing cytotoxicity (500 µg/ml). The data obtained in the current investigation is similar to those obtained by previous studies (Zhou et al., 2012; Riley et al., 1999). These authors found that GONPs significantly stimulate the secretion of Th1/Th2 cytokines such as IL-1α, IL-6, IL-10, TNF-a, and GM-CSF, as well as the chemokines such as MCP-1, MIP-1a, MIP-1β, and RANTES in both primary and immortalized macrophages (Zhou et al., 2012; Riley et al., 1999).
To obtain a more comprehensive picture of GNOP effects on RAW 264.7 cell, proteome profiling of RAW 264.7 cell culture supernatants after GONP treatment were conducted. This study indicated that RAW 264.7 cells cultured in the absence of GONPs secrete TNF-α, sICAM-1, MIP-1α, MIP-1β, RANTES and SDF-1. Treatment of RAW 264.7 cells with GONP secrete all the markers produced by the RAW cells not treated with GONPs, as well as G-CSF, JE and MIP-2. These results suggest activation of murine macrophages cells by GONPs and can possibly be attributed to the interaction of GONPs with the toll-like receptors (TLR) (Janssens and Beyaert, 2003; Chen et al., 2013). Feito et al. indicated that RAW-264.7 murine macrophages exposed to (polyethylene glycol-amine) (PEG) functionalized GO upregulated TNF-α under both basal and stimulated situations (+LPS) (Feito et al., 2014). The result of TNF-α inflammation that was achieved by Feito et al. is consistent with what was found in this study using proteome profiling assay (Feito et al., 2014).

6.3 Conclusion

Our research indicates that exposure to GONPs can potentially have adverse effects on health and the environment. The extent to which a person is exposed to nanoparticles would be affected, by the characteristics of the nanoparticles, as well as the biological condition of the person involved. When considering a person’s biological condition, the account is taken of the intensity and the period of exposure, the genetics of the individual, and any prior or current diseases. Amongst the characteristics of nanoparticles are chemical composition, the shape
and size of the particles, the degree of agglomeration, as well as its electromagnetic ions (Ali et al., 2015).

How the immune system is impacted by a GONP has to date, not been sufficiently assessed. Research into the risks that GONPs present to the health of individuals and the environment are therefore required. Despite the biological influence of a number of nanomaterials having been formally evaluated, knowledge of the processes of their toxicity is still inadequate. Therefore, in vitro testing plays a key role in the surveillance of specific biomarkers of the immune system, both in the absence and in the presence of endotoxins.

The focus of further research should be on the link between specific characteristics of a nanoparticle and the effects of such characteristics. Such research would entail an increase in the quantity of in vitro and in vivo research and would also require detailed characterisation of the nanoparticles. Also, to comprehend why specific immune functions are activated and others are subdued, when in contact with the same nanoparticles, more research on the interaction processes between the various parts of the immune system and the particular nanoparticles, is required. Should new information about these interaction processes come to light, it will result in safer and improved nanotechnological products.
CHAPTER 7

CONCLUSIONS AND FUTURE PERSPECTIVES

Nanoparticle production and use have increased exponentially over the last two decades specifically due to their unique properties, and industrial and medical applications. A major concern however is adverse effects that might be posed by nanoparticles to the environment and also human health. Due to this it is imperative that testing of nanoparticle toxicity must be done prior to using it commercially to prevent or minimize potential adverse effects due to exposure. It is important that research must be done to elucidate effects of nanoparticles earmarked for commercial applications may have on all the physiological systems. The current study was done to investigate the effects of graphene oxide nanoparticles (GONPs) on immune system biomarkers using an in vitro cell culture system. The results obtained indicate that exposure to GONPs have major effects on immune system biomarkers, specifically the upregulation biomarkers of inflammation. The inflammatory response is caused at much lower concentrations of GONPs than those required to decrease cell viability.

The current study only investigated GONP induced effects in vitro. Future studies using in vivo models should be undertaken to confirm the in vitro results.

http://etd.uwc.ac.za/
References


Magcwebeba, T., 2008. An in vitro study on the immunotoxicity of sewage effluents discharged into the Eerste River-Kuils River water catchment system. Western Cape:UWC.


http://etd.uwc.ac.za/


