Liposomal Encapsulation of Silver Nanoparticles to Enhance Nanoparticle Cytotoxicity and Modulate Induced Inflammatory Responses in Vitro

Azeez Yusuf
Technological University Dublin

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Liposomal encapsulation of silver nanoparticles to enhance nanoparticle cytotoxicity and modulate induced inflammatory responses \textit{in vitro}

By

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MSc Molecular Medicine, BSc Biochemistry

A thesis submitted to Technological University Dublin

For the Doctor of Philosophy Degree

Supervised by

Dr Alan Casey

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January 2020
Abstract

High concentrations of silver nanoparticles (AgNP) are increasingly present as active ingredient in everyday consumable products for antibacterial purposes causing increased human exposure and high risk of adverse effect development. In this thesis, AgNP were encapsulated in dipalmitoyl phosphatidylcholine (DPPC)-based liposome (forming Lipo-AgNP), to enhance intracellular delivery and associated cytotoxicity, and suppress AgNP-induced inflammation.

It was noted that as a result of the encapsulation, Lipo-AgNP induced significantly reduced cell viability of THP1 monocytes and THP1 differentiated macrophages (TDM) at a notably lower dose than that of uncoated AgNP. The induced cytotoxicity was shown to result in an increased level of DNA fragmentation causing interruption at the S-phase of the cell cycle. In addition, Lipo-AgNP induced redox imbalance through suppression of both GSH levels and ROS levels. Possibly as a consequent and in addition to increased Bax to Bcl-2 ratio, it was found that the predominate form of cell death upon exposure to Lipo-AgNP was caspase-dependent apoptosis. It was also found that the encapsulation resulted in improved intracellular uptake of the nanoparticle changing the pharmacokinetics of uncoated AgNP.

It was found that AgNP induced release of IL-1β, IL-6, IL-8 and TNF-α in THP1 monocytes all of which were suppressed by Lipo-AgNP exposure. AgNP was also found to induce release of IL-6, IL-8 and TNF-α in TDMs while Lipo-AgNP suppressed these cytokine releases. However, both AgNP and Lipo-AgNP suppressed IL-1β and TNF-α release in LPS-stimulated THP1 monocytes and TDM respectively. AgNP-induce inflammation was found to be associated with induction of STAT3 protein expression in LPS-stimulated THP1 monocytes, and non-LPS- and LPS-stimulated TDMs. Whereas Lipo-AgNP may have suppressed AgNP-induced
inflammation through STAT3, as its exposure was associated with STAT3 protein expression levels comparable with the control untreated cells highlighting the potential of Lipo-AgNP in treatment of bacteria induced inflammatory diseases.

These findings showed that encapsulation of AgNP in DPPC liposome enhanced AgNP cytotoxicity by improving the intracellular delivery of the nanoparticle and also suppressed AgNP-induced inflammation which may be linked to the suppressed ROS generation. These processes resulted in redox imbalance within the cells causing induction of caspase dependent apoptosis.
Declaration

I certify that this thesis which I now submit for the final examination for the PhD degree, is entirely my own work and has not been taken from the work of others, safe and to the extent that such work has been duly cited and acknowledged here within.

This thesis has been prepared in accordance with the regulations of the postgraduate study by research of the Technological University Dublin and has not been submitted in whole or in part for another award in any other third level institution.

The work reported on in this thesis conforms to the principles and requirements of the TUDublin guidelines for ethics in research.

The Institute has the permission to keep or lend or copy this thesis in part or in whole, on the condition that any such use of the material or the thesis will be duly acknowledged.

Signature: ___________________________ Date: ___/___/_____

Azeez Yusuf
Dedication

This project is dedicated to almighty Allah (SWT), by whom I could not achieve anything. Also, to my Dad and my late Mom, you are wonderful, and I will always appreciate you for birthing me. To my wife Aminat and my son Abdul-dayyan, I am grateful for having you.


Acknowledgements

Firstly, my utmost and sincere appreciation goes to Dr Alan Casey for his unwavering support both technically and academically and for giving me the honour of being a student under his supervision. He was instrumental to my PhD achievement.

I would like to also appreciate the Fiosraigh Dean of Postgraduate Scholarship Scheme for giving me this opportunity and full support both material and monetary, to undertake my PhD research in the Technological University Dublin.

I want to appreciate my siblings for their moral support during the dark PhD years, it would have been difficult coping if not for you.

Lastly, I want to appreciate the Nanolab members in the FOCAS Research Institute, TUDublin for the humour and friendly environment, I appreciate you all.
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Abbreviations

µg  Microgram
AgNO₃  Silver nitrate
AgNP  Silver nanoparticles
ATP  Adenosine triphosphate
DLS  Dynamic light scattering
EDX  Energy dispersive X-ray
EE  Encapsulation efficiency
GSH  Reduced glutathione
IC₅₀  Inhibitory concentration with 50% effect
IL-  Interleukin-
Lipo-AgNP  liposomal AgNP
LPS  Lipopolysaccharide
mL  Millilitre
 mM  Millimolar
MOMP  Mitochondrial outer membrane permeabilisation
nm  Nanometre
PDI  Poly-dispersity index
PMA  Phorbol 12-myristate 13-acetate
ROS  Reactive oxygen species
SEM/STEM  Scanning (Transmission) electron microscopy
SSC  Side scatter
TAM  Tumour associated macrophages
TDM  THP-1 differentiated macrophages
THP1  Lymphocytic leukaemia cell line
TNF-α  Tumour necrosis –α
UV  Ultraviolet
XRD  X-ray diffraction
1 Nanotechnology: A brief introduction

Nanotechnology is the intentional engineering and manipulation of particulate matter into a physical state of between 1 nm and 100 nm that can be rearranged or reassembled into nano-systems with improved function (Nasrollahzadeh et al., 2019). The emergence of nanotechnology and its application has put Ireland in the forefront of scientific research in the last decade (Doran and Ryan, 2019). Nanoparticles are the ultimate result of technological modification of matter and depending on their sizes, they are a few degrees larger than an atom consequent of molecular processing of matter. As they possess enhanced characteristics such as auto-reactive stability and self-reassembly, they are easily adaptable and can be modified to achieve a specific particle with required properties with a high surface area when compared to conventional substances (Cheng et al., 2012, Kango et al., 2013). Nanotechnology, as a new branch of science, has gained attention in the last two decades and is rapidly expanding from the academic arena into industry. Due to the possible advancements that can be achieved by nanotechnology, it has been estimated that nanotechnology will impact the global economy by about three trillion dollars by 2020, making the field highly viable economically speaking (Roco, 2017). This could be attributed to the unique physicochemical properties of nanoparticles at the interface of chemistry, medicine, physics and engineering. This introduction will briefly look at the contribution of nanotechnology in different fields with a focus in medicine and the concept of nanoparticle enabled drug delivery systems for disease treatment. In addition, the popularity of silver nanoparticles (AgNP) in medicine will be explored while the imminent harmful effects its wide application portends and possible ways by which AgNP can be modified for better application will be introduced as the main objective of this study.
1.1 Industrial application of Nanotechnology

1.1.1 Food industry

With the increasing awareness and demand for healthy food products, research has been devoted into devising tools for improving food shelf life and nutrient absorption. Nanotechnology as an enabling technology has been widely employed in achieving these fit in recent years for food preservation and delivery of nutraceuticals (Huang et al., 2010, Bajpai et al., 2018). Nanoparticles are added to packaging materials to act as barrier molecules or as antibacterial agents and have displayed great promise (Bajpai et al., 2018). One of the more widely utilised nanoparticle additives for this purpose is that of silver (AgNP) primarily due to silver’s innate antibacterial properties. AgNP can be added to food products in form of an edible biodegradable casing for food products such as fruits, meat and poultry or included as an active ingredient in the polymeric matrix of the packaging material (Carbone et al., 2016). In fact, there are studies that have investigated the preservative effect of AgNP containing packaging on asparagus (An et al., 2008), poultry meat (Banach et al., 2016), orange juice (Emamifar et al., 2011), and strawberries (Zhang et al., 2018) all of which improved shelf life by inhibiting the activities of pathogens such as *E. coli*, *S. aureus*, moulds and yeasts. In addition to AgNP, Zinc oxide (ZnO\textsubscript{2}) and titanium dioxide (TiO\textsubscript{2}) have been shown to be active against wide variety of food pathogens such as *S. aureus*, *Salmonella typhi* and *Klebsiella pneumoniae* (Venkatasubbu et al., 2016), while their used in preservation of food items like orange juice, strawberries, and liquid egg albumen have been documented (Bajpai et al., 2018).

Nano-encapsulation is a well-established technique used in retaining and enhancing the release of functional nutrients and flavour in food items. Typically these
encapsulations are carbohydrate based delivery systems made from starch, cellulose, chitosan and dextrin which have been modified (Fathi et al., 2014). For example, phosphatidylcholine based liposomes have been employed in delivery of vitamin C and this encapsulation is found to be more effective at maintaining bioavailability of the nutrient likely through controlled release of the content when compared with free supplements when administered orally (Davis et al., 2016). In general, nanotechnology in the food industry has made it possible to extend the shelf life of fresh food products in a cost effective and pragmatic manner. While nanoparticles used for improvement of nutraceutical delivery may be relatively non-toxic, nanoparticles used in packaging such as AgNP which may sometime leak into the main food product portend possible toxicity issue. While it has been estimated that up to 80 µg of AgNP can be consumed daily (Frohlich and Frohlich, 2016), there is limited and conflicting reports on toxic effect of ingested AgNP. There some reports considering AgNP to be safe for consumption without toxic effect and there are others that have reported significant toxicity upon AgNP ingestion (<125 mg/body weight) with accumulation of the nanoparticle within organs such as the liver, kidneys and small intestine (Kim et al., 2008, Shahare and Yashpal, 2013, Park et al., 2010b). While this may be a source of concern, it is worth considering if the level of AgNP trialled in these studies are achievable through diet.

1.2 Cosmetic industry
There is a considerable usage of nanotechnology in the cosmetic industry with cosmetic manufacturers now including nanomaterials in their product for variety of reasons. In the lucrative sun screen industry, nanoparticles of zinc oxide and titanium dioxide are routinely added to sunscreen as by virtue of their sizes, they act as efficient filters of UV radiation without serious health hazards (Morganti, 2010) or unsightly
“white streaking” when the cream is applied due to the reduction in particle size. Liposomes prepared from varying lipid formulations of synthetic or natural lipids are also widely used in cosmetics such as ethosomes and transferosomes that are used to improve transdermal delivery of active cosmetic ingredients. The primary justification for inclusion of liposomes in cosmetics is to enhance the transdermal delivery of cosmetic ingredients based on the ability of the liposomal lipid bilayer to fuse with cell membranes and alter the membrane fluidity for easy entry and delivery of liposomal content (Verma and Pathak, 2010). In addition to these, AgNP are important ingredients in many cosmetic products, having been shown to be effective antibacterial agents, as active ingredients in bathing products and because of AgNP activity against different yeast strains, they are also present in different dental products such as mouthwash and toothpaste (Abadi et al., 2013, Prabhu and Poulose, 2012). A practical example is the already commercialised Silvosept mouth rinse (https://product.statnano.com/product/4502) and Royal Denta Silver toothpaste/toothbrushes (http://www.royaldenta.com/en/), which all contain AgNP as main active ingredient. Although nanotechnology is commonplace in the cosmetic industry, just like any other industry, the use of nanoparticles in the cosmetic industry is not without safety concerns due to the general repeated use of many cosmetics.

1.3 Nanomedicine
Nanotechnology was first conceptualised in medicine by Dr. Richard P. Feynman in the late 1950s, when describing the creation of molecular machines with atomic precision for use in engineering and medicine. He described the use of molecular mechanical machines capable of carrying out surgery or even ones that can permanently reside in the body for the functional assistance of damaged organs (Feynman, 1960). Nanotechnology has strongly influenced the field of medicine,
influencing how diseases are treated particularly with use of advanced drug delivery systems from both natural and synthetic compounds. For instance, researchers in the Wyss institute of Harvard University developed a “nano-robot” that can specifically target cancer cells to deliver anticancer drugs (Douglas et al., 2012). Nano-robots that can treat cardiovascular diseases such as those that can engage in blood vessel repair by acting as artificial platelets (Trihirun et al., 2013), or those that an treat patient with coronary artery occlusion (Cavalcanti et al., 2006) are also in development. One of the most important applications of nanotechnology in medicine is in drug delivery systems, it is currently hypothesised that most conventional drugs have poor bioavailability and aqueous solubility limiting their absorption and retention within biological system (Khadka et al., 2014), as such significant efforts are being made to improve the efficiency of many traditional drugs such as but not limited to chemotherapeutics.

Many nanoparticles are thought to have improved pharmacokinetic properties due to their physical nature and reduced size, they can target specific cells for selective action dependent on the particle type. These particles can easily penetrate target cells and accumulate into subcellular structures to modify cellular processes which may be beneficial in the treatment of lifelong diseases such as diabetes, cancer and kidney diseases (Barua and Mitragotri, 2014). As such many of these nanoparticles have already been approved by the Federal Drug Administration in the United States for clinical use (Table 1-1). Nanoparticles that are popularly used in research for therapeutic purposes include encapsulated mRNA (siRNA) or DNA (in gene therapy), inorganic metal and metal complexes or chemotherapeutic agents with pharmacologic abilities. However, some of these nanoparticles do not easily traverse the cell membrane, requiring delivery systems to alleviate such difficulties. Thus, different
nanoparticle delivery systems have been developed, some of which include liposomes, micelles, chitosan and synthetic dendrimers (Park et al., 2008, Wang et al., 2013a, Sanyakamdhorn et al., 2013, Hasan et al., 2014). The entrapment of both hydrophobic and hydrophilic drugs into liposomes is possible and this helps to bypass the toxicity associated with anticancer drugs (Koudelka et al., 2010). In particular, nanoparticles enabled delivery systems like liposomes are well established for disease treatment such as in DoxilTM (liposomal doxorubicin) which is approved by the FDA for treatment of Kaposi sarcoma and ovarian cancer (Table 1-1). As such, liposomal encapsulation represents an effective route which enhances drug therapeutic effect. In addition, modification of liposomes allows for a passive or active tumour targeting (Figure 1-1). This effect facilitates an efficient drug payload into the malignant tumour cells, while the non-malignant cells become minimally impacted. Encapsulation of doxorubicin within the DPPC based liposome enhances the cytotoxicity of the drug and at the same time suppress the toxic side effects, thus improving the antitumoural therapeutic efficacy in comparison to conventional doxorubicin (Barenholz, 2012).

Table 1-1. List of nanodrugs approved by the FDA for clinical application (Adapted from (Bobo et al., 2016, Ventola, 2017)

<table>
<thead>
<tr>
<th>Trade Name (Manufacturer)</th>
<th>Component</th>
<th>Delivery method</th>
<th>Indication(s)</th>
<th>Nanoparticle benefit compared to conventional formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomal nanoparticle</td>
<td>AmBisome (Gilead Sciences)</td>
<td>Liposomal amphotericin B</td>
<td>Intravenous infusion</td>
<td>Fungal/protozoal infections</td>
</tr>
<tr>
<td></td>
<td>DepoDur (Pacira Pharmaceuticals)</td>
<td>Liposomal morphine sulphate</td>
<td>Epidural administration</td>
<td>Postoperative analgesia</td>
</tr>
<tr>
<td></td>
<td>Doxil (Janssen)</td>
<td>Doxorubicin HCl liposome injection</td>
<td>Intravenous infusion</td>
<td>Kaposi’s sarcoma, ovarian cancer, multiple myeloma</td>
</tr>
<tr>
<td></td>
<td>Marqibo (Spectrum Pharmaceuticals)</td>
<td>Liposomal vincristine</td>
<td>Intravenous injection</td>
<td>ALL</td>
</tr>
<tr>
<td>Product Name</td>
<td>Formulation</td>
<td>Route of Administration</td>
<td>Indication</td>
<td>Benefits</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>----------------------</td>
<td>-------------------------</td>
<td>----------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Onivyde (Ipsen Biopharmaceuticals)</td>
<td>Liposomal irinotecan</td>
<td>Intravenous injection</td>
<td>Pancreatic cancer</td>
<td>Increased delivery to tumor site, decreased systemic toxicity</td>
</tr>
<tr>
<td>Visudyne (Bausch and Lomb)</td>
<td>Liposomal verteporfin</td>
<td>Intravenous injection</td>
<td>Wet AMD, ocular histoplasmosis, myopia</td>
<td>Increased delivery to site of diseased vessels, photosensitive release</td>
</tr>
<tr>
<td>Vyxeos (Jazz Pharmaceuticals)</td>
<td>Liposomal daunorubicin and cytarabine</td>
<td>Intramuscular, intrathecal, or subcutaneous injection</td>
<td>AML, AML with myelodysplasia-related changes</td>
<td>Increased efficacy through synergistic delivery of co-encapsulated agents</td>
</tr>
<tr>
<td>Adagen (Leadiant Biosciences)</td>
<td>Pegademase bovine</td>
<td>Intramuscular injection</td>
<td>SCID</td>
<td>Longer circulation time, decreased immunogenicity</td>
</tr>
<tr>
<td>Cimzia (UCB)</td>
<td>Certolizumab pegol</td>
<td>Pills or intravenous injection</td>
<td>Crohn’s disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis</td>
<td>Longer circulation time, greater stability in vivo</td>
</tr>
<tr>
<td>Copaxone (Teva)</td>
<td>Glatimer acetate</td>
<td>Subcutaneous injection</td>
<td>Multiple sclerosis</td>
<td>Controlled clearance</td>
</tr>
<tr>
<td>Eligard (Tolmar)</td>
<td>Leuprolide acetate and polymer</td>
<td>Subcutaneous injection</td>
<td>Prostate cancer</td>
<td>Longer circulation time, controlled payload delivery</td>
</tr>
<tr>
<td>Macugen (Bausch and Lomb)</td>
<td>Pegaptinib</td>
<td>Intravitreal injections</td>
<td>Neovascular AMD</td>
<td>Greater apatmer stability</td>
</tr>
<tr>
<td>Neulasta (Amgen)</td>
<td>Pegfilgrastim</td>
<td>On-body injection</td>
<td>Chemotherapy-induced neutropenia</td>
<td>Greater protein stability</td>
</tr>
<tr>
<td>Pegasys (Genentech)</td>
<td>Pegylated IFN alpha-2a</td>
<td>Subcutaneous injection</td>
<td>Hepatitis B, hepatitis C</td>
<td>Greater protein stability</td>
</tr>
<tr>
<td>PegIntron (Merck)</td>
<td>Pegylated IFN alpha-2b</td>
<td>Subcutaneous injection</td>
<td>Hepatitis C</td>
<td>Greater protein stability</td>
</tr>
<tr>
<td>Plegridy (Biogen)</td>
<td>Pegylated IFN beta-1a</td>
<td>Subcutaneous injection</td>
<td>Multiple sclerosis</td>
<td>Greater protein stability</td>
</tr>
<tr>
<td>Rebinyn (Novo Nordisk)</td>
<td>Coagulation factor IX (recombinant), glycopegylated</td>
<td>Intravenous injection</td>
<td>Hemophilia B</td>
<td>Longer half-life, greater drug levels between infusions</td>
</tr>
<tr>
<td>Somavert (Pfizer)</td>
<td>Pegvisomant</td>
<td>Subcutaneous injection</td>
<td>Acromegaly</td>
<td>Greater protein stability</td>
</tr>
<tr>
<td>Zilretta (Flexion Therapeutics)</td>
<td>Triamcinolone acetonide ER injectable suspension</td>
<td>Intra-articular injection</td>
<td>Osteoarthritis knee pain</td>
<td>Extended release</td>
</tr>
<tr>
<td>Micellar NPs</td>
<td>Estrasorb (Novavax)</td>
<td>Micellar estradiol</td>
<td>Topical application of emulsion</td>
<td>Vasomotor symptoms in menopause</td>
</tr>
<tr>
<td>Nanocystal nanoparticles</td>
<td>Avinza (Pfizer)</td>
<td>Morphine sulfate</td>
<td>Oral administration</td>
<td>Psychostimulant</td>
</tr>
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<td></td>
<td>Emend (Merck)</td>
<td>Aprepitant</td>
<td>Oral administration</td>
<td>Antiemetic</td>
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<td></td>
<td>Focalin (Novartis)</td>
<td>Dexamethylphenidate HCl</td>
<td>Intravenous injection</td>
<td>Psychostimulant</td>
</tr>
<tr>
<td></td>
<td>Invega Sustenna (Janssen)</td>
<td>Paliperidone palmitate</td>
<td>Intramuscular injection</td>
<td>Schizophrenia, schizoaffective disorder</td>
</tr>
</tbody>
</table>
NanOss (RTI Surgical) | Hydroxyapatite | Injectable paste | Bone substitute | Mimics bone structure
---|---|---|---|---
Ostim (Heraeus Kulzer) | Hydroxyapatite | Injectable paste | Bone substitute | Mimics bone structure
OsSatura (IsoTis Orthobiologics) | Hydroxyapatite | Injectable paste | Bone substitute | Mimics bone structure
Rapamune (Wyeth Pharmaceuticals) | Sirolimus | Oral administration | Immunosuppressant | Greater bioavailability
Ritalin LA (Novartis) | Methylphenidate HCl | Oral administration | Psychostimulant | Greater drug loading and bioavailability
Tricor (AbbVie) | Fenofibrate | Oral administration | Hyperlipidemia | Greater bioavailability simplifies administration
Zanaflex (Acorda) | Tizanidine HCl | Oral administration | Muscle relaxant | Greater drug loading and bioavailability

| Inorganic nanoparticles | | | | |
|---|---|---|---|
| Dexferrum (American Regent) | Iron dextran | Intravenous injection | Iron deficiency in CKD | Increased dose
| Feraheme (AMAG Pharmaceuticals) | Ferumoxytol | Intravenous infusion | Iron deficiency in CKD | Prolonged, steady release with less frequent dosing
| Ferrlecit (Sanofi-Aventis) | Sodium ferric gluconate complex in sucrose injection | Intravenous infusion | Iron deficiency in CKD | Increased dose
| Venofer (American Regent) | Iron sucrose | Intravenous injection | Iron deficiency in CKD | Increased dose

AMD- Age related macular degeneration, ALL- Acute lymphoblastic leukaemia, CKD- Chronic kidney disease

Nanoparticles have also found favour in the medical and diagnostic imaging of internal organs and tissues due to their interactions with mammalian cells owing to their modifiable physicochemical characteristics such as size, shape, optical, magnetic and electronic properties (Nam et al., 2013). Iron oxide and silica based nanoparticles have been used to develop multifunctional imaging platforms such as MRI/optical dual modal imaging, which possess several advantages over existing positron emission tomography (PET) and computed tomography (CT) both of which have radiation related concerns (Lee et al., 2015, Kim et al., 2007, Louie, 2010). Iron oxide is a magneto-responsive metal that is also biocompatible due to its degradable nature within biological systems. This in addition to its optical properties makes it a good imaging material for MRI. Kim et al. (2007) described a silica based nanoparticle with paramagnetic shell containing a luminescent core offering the possibility of using the
magnetic field of the MRI and the optical feature of the nanoparticle core for the multimodal imaging. The paramagnetic shell can also be functionalised by peptides or moieties of interest offering the possibility of specifically targeting cancer cells. These techniques, through the magnetic/optical properties of the nanoparticles and the magnetic field of the MRI thus offer a way of detecting and monitoring changes in a living tissue for diagnostic purposes without the need for using radioactive tracers used during a PET or CT scan. In addition to all these, nanoparticles, in particular AgNP, are used as coating materials in medical garments, wound bandages, medical implants and devices as antibacterial (Chaloupka et al., 2010). Conventional disinfection only exerts bactericidal effect that may not be effective after disinfection. On the contrary, AgNP coatings on medical devices and clothing materials remain effective against wide range of bacterial strains a long as the nanoparticle is retained on the material surface. Nanotechnology seem to be playing a prominent role in medicine from use as therapeutic agent based on the bactericidal properties to imaging and diagnostic purposes. These innovations are possible due to the properties that are peculiar to nanoparticles (see section 1.4) and increased nanotechnology research in the medical field does not show any sign of slowing down.

1.4 Physiochemical properties of nanoparticles in medicine

Nanoparticles have various properties that facilitate enhanced pharmacologic behaviour when compared with larger molecules. As such, significant efforts are being made in research modifying the nanoparticles size, shape, surface area and surface chemistry to maximise their benefits for medical purposes.

Different nanoparticles such as gold nanoshells, liposomes and micelles are synthesised in various ways and the sizes and shapes of these nanoparticles can be controlled during the synthesis process based on the intended functionality.
Nanoparticles can agglomerate into larger sized particles during synthesis which may enhance or indeed suppress the nanoparticle cytotoxicity depending on composition. The surface chemistry of nanoparticles can be modified by adding reactive groups or molecules such as antibodies to surfaces in targeted drug delivery systems (Figure 1-1).

**Figure 1-1. Physicochemical properties of nanoparticles:**
Nanoparticles have different physicochemical properties such as charged surfaces, ability to agglomerate and conjugate other groups to the surfaces or controlled synthesis that facilitate specific shapes and sizes to be obtained. These properties allow nanoparticles to possess a more reactive nature in comparison to conventional particles within the biological environment.

1.4.1 **Size and surface area**
As stated, nanoparticles are small particles with sizes ranging between 1 nm and 100 nm giving them a high surface area to volume ratio. By virtue of this property,
nanoparticles have a high surface area of interaction per mass unit compared with more bulky particles, making some particles that are otherwise inert such as gold, be reactive in the nanometre range (Nel et al., 2006). A nanoparticle’s small size which is controllable also allow them to easily infiltrate body tissues and fluids which are otherwise hindered when in the bulk form. In essence the size and surface area of these particles contribute to rate at which these nanoparticles are endocytosed, distributed, retained and eliminated within biological systems (Powers et al., 2007). As nanoparticles do not simply diffuse through the cell membrane, the extensive research into nanoparticles movements into normal and cancer cell lines have shown they are internalised by endocytotic means in a size-dependent fashion (Tsai et al., 2012, Jiang et al., 2008). Nanoparticles <200 nm are known to be internalised by clathrin coated vesicles while larger nanoparticles, usually 500 nm are known to be internalised by caveolae mediated endocytosis (Rejman et al., 2004). In immune cells such as macrophages however, nanoparticles are prone to phagocytosis and indeed research has shown that nanoparticles less than 500 nm in size enter immune cells through the phagocytotic pathway while particles with larger particle sizes of between 2 and 3 µm, approximately around the size of bacteria cells, exhibit maximal phagocytotic uptake. Smaller nanoparticles such as liposomes can now be engineered for maximal uptake by mammalian cells based on their size (Chithrani et al., 2010). For example, different lipid formulations can be used to prepare liposomes of specific sizes that can be more easily internalised by mammalian cells. There are studies that have used extrusion methods for instance, with a polycarbonate membrane of predetermined size to make liposomes of suitable sizes that can be easily internalised by mammalian cells. Such production methods of liposomes have been shown to improve the activity of
chemotherapeutic drug due to improved drug uptake by the cells (Gosangari and Watkin, 2012).

In addition to uptake, intracellular localisation of nanoparticles has also been shown to be size dependent. Oh et al., (2011) showed that gold nanoparticles (AuNP) that were 2.4 nm in size were localised in the nucleus while those larger than that up to 89 nm were localised in the cytoplasm after internalisation. Findings like these form the basis of how to modify nanoparticles size to evade or harness the immune system and how to localise nanoparticles in subcellular organelles of interest to maximise their effect.

1.4.2 Surface chemistry

The surface chemistry of nanoparticles such as charge or attached chemical groups is an important factor that determines their reactivity and ultimately can control their function. Many nanoparticles have been modified to change their surface chemistry to suit specific purposes. Rod shaped gold nanoparticles (AuNP) and DNA because of its charge, cannot easily permeate or enter the cell. Both the AuNP and DNA have had their surfaces modified by coating them with lipid layers while DNA has also been electrostatically conjugated to cationic liposomes to facilitate their transport into the cell which resulted in improved uptake (Chithrani et al., 2010, Fillion et al., 2001, Dichello et al., 2017, Ewert et al., 2016). As liposomes and micelles have lipid layers that can interact and fuse with the cell membrane through hydrophobic interactions resulting in improved uptake, they can be used to deliver higher concentrations of nanoparticles intracellularly. Silicon nanoparticle’s (SiNP) are important semiconductors that are used in optoelectronics, but its hydrophobicity hinders its application in biomedicine such as applications of internal imaging of tissues since the
biological system is aqueous and SiNP are not stable in aqueous environments. Pan et al., (2013) described a method of modifying the SiNP surface through coating it with silicon dioxide (SiO\(_2\)) to make it more hydrophilic allowing for more biocompatible applications. ZnO\(_2\) nanoparticles are widely used in sunscreen, due to its UV protection properties, but some studies have indicated potential cytotoxicity of the nanoparticles making its application in cosmetic products worrisome. In order to negate this, some researchers alter the surface properties and indeed a study has shown that by surface coating ZnO\(_2\) with poly methyl acrylic acid (PMAA), the cytotoxicity was reduced the nanoparticles retained their UV protection characteristics (Yin et al., 2010).

Liposomes are made up of phospholipids which mimics the lipid bilayer of the plasma membrane. The phospholipids component of the liposomes is amphiphilic with a polar head and hydrophobic tail (Figure 1-2). The polar head is comprised of phosphate group and glycerol both containing oxygen that can form hydrogen bond in aqueous environment. The hydrophobic tail on the other hand is made up of long chain fatty acid which aligns with the hydrophobic tail of another adjacent phospholipid, creating a hydrophobic core that can hold non-polar hydrophobic drugs in the bilayer so formed. The compatibility of liposomal surface chemistry with that of plasma membrane allows adsorption of liposome to the cell membrane where the liposome is internalised via receptor mediated endocytosis or through fusion with the plasma membrane inducing membrane invagination and internalisation (Bozzuto and Molinari, 2015).

The pH of the environment where the particles are delivered can also affect the function of the nanoparticle based on its surface chemistry and this phenomenon has been utilised to trigger drug release in the tumour microenvironment that is
characterised with acidic pH. For example, carrageenan oligosaccharide capped AuNP have been recently shown to significantly release epirubicin in an acidic pH inducing cell death in HCT-116 colorectal cancer cells (Chen et al., 2019). The surface of nanoparticles can alter their movement within aqueous biological systems and subsequently affect their reactivity or delivery. Such surface properties facilitate their use in variety of ways such as in biomedical sensors, coatings of medical implants and drug delivery systems. For example, a AgNP functionalised titanium implant surface was developed to prevent postoperative infection due to resistant strains of *Staphylococcus epidermidis* and *Staphylococcus aureus* due to the antimicrobial properties of AgNP (Wang et al., 2016).

**Figure 1-2. Liposomal modification for drug delivery:**
Liposome consists of lipid bilayer and an aqueous core which can both be used for drug transport in disease treatment. Cholesterol is often added to the recipe for preparing the liposome to restrict the fluidity of the phospholipid as in the plasma membrane.
As stated earlier, nanoparticles like AuNP and even conventional drugs are often coated with lipid layers as in liposomes to enable compatibility with the mammalian cell membrane, improving intracellular delivery. The liposome offers other functional benefits due to the active phospholipid heads that can be conjugated to variety of compounds for targeted delivery. For instance, conjugation of polyethylene glycol (PEG) to surface of liposome has been used to improve their bioavailability by making them undetectable to phagocytes, which eliminate them from the system (Milla et al., 2012). PEGylation or the use of other linkers also facilitate the addition of active groups such as folate and monoclonal antibodies to the liposomal surface for selectively targeting specific cells (Error! Reference source not found.). Folate is often used because of the high expression of folate receptor on cancer cells surfaces which, the cancer cells utilise to bind folate within the body for their uncontrolled proliferation (Marchetti et al., 2014, Carron et al., 2018). On the contrary, monoclonal antibodies represent a more flexible approach for targeted delivery due to countless number of unique receptors or surface antigens against which antibodies can be developed. Conjugation of these active surface agents to nanoparticle surfaces facilitates delivery of the nanoparticles or drugs to the tumour cells for selective cancer cell eradication (Steichen et al., 2013).

1.4.3 Shape

As stated previously nanomaterials have tuneable sizes but their shape is also controllable during their synthesis. The shapes of nanoparticles can be altered during the last synthesis stage and typically involves nucleation of the nanoparticles from seed. The nucleation process involves the fusion of nanoparticle nuclei known as the seeds forming a template on which the nanoparticle crystals grow. Just like the size, the shape of a nanoparticle is paramount to its biological function and reactivity.
Generally, nanoparticles that are round or spherical in shape are easily endocytosed in comparison to rod or tube shaped nanoparticles (Champion and Mitragotri, 2006). This is because the shape affects endocytosis by interfering with the manner in which the membrane wraps over the nano-construct during contact. As such, the reduced endocytosis of nano-rods or other shapes is most likely due to the inability of the cell to initiate the necessary actin-dependent membrane kinetics required for endocytosis. This reason may explain why most nanoparticles of biomedical importance are spherical in nature. On the contrary, there are reports from new studies on nanoparticles of different shapes with potential application in drug delivery. Zhao et al, (2017) reported that in addition to their ability to encapsulate more particles, long rod nanoparticles have prolonged bioavailability when compared to both spherical and short rod nanoparticles. Other shapes such as nanoflowers and nanoprisms do exist but these structures may not be as active as nanorods and nanospheres primarily due to their unique shapes (Wozniak et al., 2017).

1.5 Nanoparticle cytotoxicity

With the advent of nanotechnology and its growing application in almost all facets of everyday living comes the concern on possible hazards resulting from increased human exposure. Significant research into the toxic effect or toxicity of nanoparticle exposure gave rise to the field of nanotoxicology. In recent years, this field has identified that the properties of nanoparticles that confer them with suitable pharmacologic behaviour are also responsible for their toxicity (Gatoo et al., 2014).

Several studies have investigated the cytotoxicity of different nanoparticles using different cell lines and experimental conditions. For instance, toxicity of carbon nanotubes have been shown to affect the diversity of soil bacteria, (Kerfahi et al.,
2015), inhibit the growth of *Daphnia magna*, *Chlorella vulgaris* and *Oryzias latipes* (Sohn et al., 2015) and result in oxidative stress, membrane damage and inflammation in human A549 lung carcinoma cell line (Choi et al., 2009). Different findings have shown that the mechanism of nanoparticles size dependent cytotoxicity is due to their ability to infiltrate body tissues and subsequently enter cells to modify crucial cellular functions, one of which is to rupture membrane of subcellular structures and induce the overproduction of reactive oxygen species (ROS) (Fu et al., 2014). The presence of elevated levels of ROS induces oxidative stress that affects the normal physiological processes of the cell subsequently resulting in DNA damage, dysregulation of cell signalling and ultimately cell death.

Nanoparticles are often surface-modified to enhance their functions. This may inadvertently result in increased cytotoxicity of the nanoparticle due to the influence of nanoparticle surface chemistry on its inherent toxicity. Based on localisation within the biological system, nanoparticles with reactive surface moieties are able to react with different intracellular or extracellular biomolecules therefore disturbing the normal processes needed to maintain tissue or cellular homeostasis. For instance, charged AuNP’s have been shown to be more cytotoxic compared to that of neutral AuNP’s as they induce higher levels oxidative stress resulting in reduced mitochondrial function and increased expression of DNA damage related genes (Schaeublin et al., 2011). Anionic cyanoacrylic nanoparticles are known to be more cytotoxic to macrophages compared with the cationic forms (Tomita et al., 2011). These differences may be as a result of macrophage’s phagocytotic affinity towards the bacterial cell membrane, which demonstrates an overall negative charge due to the Lipid A molecule of the LPS component of the bacterial cell membrane. Contrary to this, aminated iron oxide nanoparticles with an overall positive charge have been
shown in a Chinese Hamster Ovary (CHO-K1) cell line to be efficiently internalised and hence with induce higher cytotoxicity when compared to a PEGylated form (Hanot et al., 2015). Often, nanoparticles are PEGylated to increase their bioavailability and to reduce immunogenicity for a prolonged effect in vivo (Suk et al., 2016). The retention of PEGylated particles in addition to the stability it confers them with, may be a result of the slower uptake by cells resulting in the substantial reduction in their cytotoxicity. 

Coupled with the size, the shape and aspect ratio of nanoparticles play crucial role in their cytotoxic effects in vivo. It is believed that the higher aspect ratio of nanoparticles correlates with increased cytotoxicity due to a reduced clearance and an increased bioavailability of nanoparticles (Shukla et al., 2015). Higher aspect ratio nanoparticles often have cytotoxicity profile similar to that of asbestos. Such particles can induce macrophage cell death during phagocytosis and, as was the case with asbestos fibres, have the potential to promote cancer development (Fubini et al., 2011). In support of this, Wozniak et al. (2017) showed that gold nano-spheres and nano-rods both of which were under 50 nm were more cytotoxic on HeLa and HEK293T cell lines than their nano-star, nano-flower and nano-prism counterparts that were all above 200 nm in size. It was postulated that this was because of a more efficient internalisation of these nanoparticles by the cells coupled with their optimum surface area for interaction with intracellular molecules.

1.6 Nanoparticle drug delivery systems (DDS) in disease treatment

Nanoparticles used in drug delivery range from 10 to 1000 nm in size with at least one dimension being below 100 nm in size. The small sizes of nanoparticles as well as their surface chemistry are known to offer pharmaceutically beneficial attributes but may also contribute to their toxic effects as discussed earlier. Smaller nanoparticles
more effectively enter cells when compared with larger molecules but the administration of nanoparticles with a reduced clearance may result in some of the particles being retained within the body. In the case of a more active or cytotoxic nanoparticle being retained rather than a bulk of the drug being eliminated during the first pass effect, this may result in harmful effects in the targeted site due to unwanted retention. Systemic administration of cytotoxic drugs may cause the drugs to exert their cytotoxicity on tissues during the first pass effect before they reach the intended tissues. 70% of globally synthesised drugs have poor aqueous solubility and hence poor pharmacokinetic properties in vivo (Khadka et al., 2014). As a solution to this, nanoparticle drug delivery systems (DDS) have been developed to achieve targeted and more efficient delivery of the therapeutic substance, which would prevent damage to surrounding organs from the effect of administered drugs that will otherwise arise if the drugs were in the free form. Over the past few decades, research efforts into DDS have advanced significantly with various DDS already being investigated and developed for treatment of diseases such as cancer and neurodegenerative diseases (Gunay et al., 2016, Iwamoto, 2013). Some of the typical nanoparticle DDS currently in under study are discussed below.

1.6.1 Lipid based DDS

DDS made from lipids vary in formulation and sizes and are mainly of two types, namely micelles and liposomes. Micelles are formed through the self-assembly of a monolayer of lipid molecules in an aqueous environment into a nano-vesicle of between 5 and 50 nm (Wang et al., 2005). They are used to successfully transport hydrophobic molecules, trapped in the hydrophobic core, at concentrations above their inherent water solubility. This is possible because the hydrophilic phospholipids are
exposed to the aqueous environment while the hydrophobic tails form the core that can interact with the drug.

Unlike micelles, liposomes are bilayer nano-vesicles similar to the cell membrane with sizes ranging from 10 nm to several microns. The hydrophilic phospholipids of the outer layer are exposed to the aqueous environment while that of the inner layer encloses the aqueous core (section 1.4.2). Consequently, the hydrophobic tails of the bilayer lie above each other and are often used to trap hydrophobic drugs while the aqueous core is used to entrap hydrophilic drugs (Bozzuto and Molinari, 2015). Liposomes have been one of the most useful tools in drug delivery in cancer treatment due to their ability to transport both water soluble and insoluble drugs (Rau et al., 2015, Casagrande et al., 2014, Guo and Huang, 2014). Conventional drugs, which are often small molecular drugs have poor selectivity for tumour cells are not retained within the tumour microenvironment as they diffuse back into the circulation system causing cytotoxic side effects to normal cells. Liposomes however can improve the delivery of such drug to the tumour microenvironment, which have tight junctions with gaps between 100 nm and 800 nm unlike normal epithelial junctions which are 5 nm to 10 nm, via an enhanced permeability and retention (EPR) effect. Using the EPR effect, liposomes accumulate at the tight junctions of tumour cells and extravasate the blood vessels through to the tumour microenvironment for delivery of the encapsulated drugs (Deshpande et al., 2013).

Liposomes generally have short half-life but advancements in drug delivery research such as PEGylation of liposomes has allowed the development increased half-life (Milla et al., 2012). In forming DSPE-PEG for instance, PEG is conjugated to phosphatidyl ethanolamine of DSPE via covalent linking of the amide group of DSPE to the carboxyl end of PEG (Marques-Gallego and de Kroon, 2014). Through PEG
linker on the liposome surface, several other moieties can be conjugated to the liposome as in targeted drug delivery. For example, click chemistry can be used to couple an azide functionalised antibody to a Dibenzocyclooctyne-amine (DBCO)-PEG functionalised liposome in an azide-alkyne cycloaddition reaction (Gai et al., 2020). Thus, liposomes can now improve both the pharmacodynamic and pharmacokinetic properties of water soluble and insoluble drugs by increasing their delivery to target cells with potentially controlled release, thus lowering their cytotoxic effect on surrounding cells. For instance, liposomal doxorubicin coated with PEG (Doxil®) has been used to reduce doxorubicin cytotoxicity, improve its bioavailability and to enhance the delivery of the anticancer drug in different cancer types (O'Brien et al., 2004, Udhrain et al., 2007). While PEGylation of liposome result in prolonged bioavailability by increasing the liposome hydrophilicity and reduced glomerular filtration/excretion, it often result in reduced uptake of the liposome by cells and degradation for drug release by the endo-lysosomal pathway (Mishra et al., 2016). Liposomes are widely used in targeted delivery due to the flexibility of their surface chemistry, which allows conjugation of targeting biomolecules such as peptides and antibodies that bond with specific cell surface receptors. This feature makes it possible to specifically target cells such as cancer/tumour cells that express or overexpress specific receptors that recognises such molecules on the liposomes for targeted drug delivery (Error! Reference source not found. and Figure 1-3) (Accardo et al., 2012).

Liposomes can be used to transport hydrophobic drugs in the lipid bilayer via hydrophobic interactions with the fatty acid tail of the phospholipids while hydrophilic molecules, such as DNA or crystalline drugs, can be encapsulated within the aqueous core. Surface modifications are now possible on to the surface of the liposomes allowing enhanced bioavailability as occurs with PEG. Surface coating of drugs via
electrostatic or ionic interactions or conjugation of antibodies, chemotherapeutic agents, peptides and other proteins can prove useful for targeted delivery are routinely done with the aid of different linkers such as avidin-biotin complexes, PEG or peptide linkers that are chemically conjugated to the phospholipid head and to the drug or protein of choice (Error! Reference source not found. and Figure 1-3).
In targeted drug delivery, liposomes are surface modified by conjugating them with different groups using linkers such as PEG or chemically such as the “Click Chemistry” (see section 1.6.1). Such groups include drugs, peptides or antibodies specific for cell surface receptors that are overexpressed by cancer cells for example, which facilitate binding of such groups to the receptors and prevent access to normal cells. The liposome can be internalised through invagination of the cell membrane as endosomes or b receptor mediated endocytosis in which case the receptor is recycled and returned to the cell membrane. Drugs encapsulated in the liposome are released to subcellular structures after degradation of the endosome and the liposome by the lysozymes in the endo-lysosomal pathway.

1.6.2 Polymeric DDS

Polymer based nanoparticle DDS are made up of a repeating unit of specific polymers and have been widely investigated for medical purposes in recent years (Nicolas et al., 2013, Liu et al., 2014c). Some of the known polymeric DDS are PEG, chitosan, poly-(lactic-co-glycolic acid) (PLGA) and polylactic acid (PLA) but PEG, PLGA and PLA are the more widely studied while chitosan research is beginning to gain more attention due to its biocompatibility, low immunogenicity and low toxicity (Cheung et al., 2015). Several PEGylated drugs have been approved by FDA for clinical used making it the most commercialised polymeric DDS (Table 1-1). PLGA and PLA are however known to be characterised with an initial burst release of the encapsulated drug (within 24 h) irrespective of the drug localisation and this may result in high delivery of drugs at unwanted sites, reducing drug benefits (Bouissou et al., 2006). This has led to development of polymeric DDS with different triggers for release of entrapped drugs. For example, some polymer-based DDS are modified to respond to subtle change in pH or ROS generation within the biological system. The pH or ROS level within a
tissue may signal its physiological condition, since ischemic or tumour tissue sites have higher pH or ROS levels when compared to normal tissue. Self-assembling and pH-sensitive poly-amine have been demonstrated to possess flexible delivery capabilities. Doxorubicin entrapped polyamine coated with folate and ligands for HIV transcriptional transactivator (TAT) were shown to be successful in treatment of multidrug resistant cancer cell lines (Lee et al., 2008, Lee et al., 2005). These polyamines are pH sensitive and release the entrapped doxorubicin in an acidic pH environment. Since the tumour microenvironment is mostly acidic and without the need for specific targeting molecules, this system can effectively target the tumour microenvironment. Dextran nanoparticles containing linked arylboronic esters that are degradable by ROS have also been used to deliver ovalbumin to murine dendritic cells to enhance their antigen presenting abilities (Broaders et al., 2011). Because of the varying conditions that ensues in the tumour microenvironment, polymer-based DDS seem to be flexible and less technical way of targeting the tumour microenvironment.

1.6.3 Peptide nanoparticle DDS

Linear and cyclic peptides that are either synthesised or derived from existing fragments of naturally occurring proteins are also important contributors to the nanoparticle DDS that are currently available. Peptides are often used as the targets for cell surface receptors since most proteins that bind to such receptors do so via a specific fragment in their peptide sequence. These coupled with their ease of synthesis and low immunogenicity makes peptides a useful tool as potential DDS. Several peptides have been used alone or indeed as part of a surface modification to other nanoparticles for improved drug delivery. Kim et al. (2012) used an encapsulated peptidomimetic of epidermal growth factor receptor (EGFR) ligand in a cationic liposome to aid in the inhibition of EGFR signalling in a lung cancer cell line.
Somatostatins, a group of peptide hormones that are ligands highly expressed in different cancer cells, have also been conjugated to different anticancer drugs such as doxorubicin, methotrexate and paclitaxel to enable selective targeting of cancer cells (Sun and Coy, 2011). RIPL peptide is a short peptide that was developed to bind hepsin, a serine protease, that is highly expressed on the surface of hepatoma and prostate cancer cells. Kang et al. (2014) developed a RIPL conjugated liposome to selectively target a panel of cancer cells that overexpresses hepsin, yielding an increased selectivity and cellular uptake compared to conventional liposomes without the targeting peptide.

### 1.6.4 Background to the project

In a bid to improve on the activities of conventional drugs where possible, nanoparticles have been a subject of different research in recent years due to their potential pharmacologic properties (Ventola, 2012). Nanoparticles have shown promise in a variety of treatment options ranging from treatment of cancer, kidney disease, neurodegenerative diseases and even medical imaging for diagnostic purposes (Murthy, 2007) and in some instances surpass the performances of conventional treatment methods. It is now known that the use of nanoparticles alone or by incorporating them into nanocomposites offer improved potential for targeted drug delivery and potentially offer more effective disease treatment (Bao et al., 2013).

Nanoparticles extensively researched for their medical applications include AgNP, AuNP, silicon/silicon oxides and iron oxide nanoparticles. Of these, AgNP have been extensively researched for medical applications and in fact, AgNP is the most commercialised nanoparticle at present as an active ingredient in everyday consumable product driven by nanotechnology (Vance et al., 2015), especially in high concentration. These diverse applications of AgNP stems from its antibacterial
activities and indeed several mechanisms of action have been proposed for its cytotoxic effect. In addition to this, recent investigations have now shifted to investigate the anticancer properties of AgNP with interesting results. AgNP have been shown to interact with the DNA inducing DNA damage. AgNP can also induce ROS which further causes DNA single and double strand breaks in addition to DNA adducts due to oxidation of certain nucleotides like guanine to 8-oxo-2-deoxyguanosine which can base pair with deoxyadenosine resulting in mutation (Liu et al., 2020, Tang et al., 2019, Salehi et al., 2018) (Figure 1-4). This in addition to permeabilisation of mitochondria membrane can lead to activation of caspase dependent cell death. However, the overall effect of both anticancer and antibacterial applications of AgNP possesses increased toxicity risk due to increased and repeated human exposure to the free silver ion (Ag⁺) released into the local environment by the nanoparticle. Ag⁺ released from AgNP has been documented to cause several side effects such as skin irritation and discolouration, hepatotoxicity, kidney damage, DNA damage and epithelia cell damage (León-Silva et al., 2016).
AgNPs have a dose and size dependent effect on cellular cytotoxicity which influence the dynamic changes within the cell. AgNP can induce apoptosis via the caspase-dependent mitochondrial cell death pathway facilitating cellular dynamics that can damage the cell barrier, inactivate ATPase activity to cause inactivation of Ca\(^{2+}\) ATPase and Na\(^+/\)K\(^+\) ATPase. This in addition to single and double strand breaks that is caused by AgNP-induced DNA damage can excessively generate and accumulate ROS causing permeabilisation of mitochondrial membrane and release of cytochrome C and pro-apoptotic protein into the cytoplasm followed by activation of the caspase cascade, and finally apoptosis.
So, this thesis sought to mitigate the negative effects of AgNP by altering the delivery vehicle of the AgNP to that of a liposome-based delivery. The rationale behind this research is to improve the cytotoxicity of AgNP and suppress the AgNP-associated inflammation such that lower concentration of AgNP will be applied commercially lessening the dangers of human exposure. Different drugs have been encapsulated in different DDS such as liposomes and carbon nanotubes to increase drug payload delivery intracellular. This type of delivery has a myriad of benefits in cancer research, one of which is the ability to modify the delivery vesicle to a targeted delivery in order to deliver a high concentration of the drug to only tumour cells in the tumour microenvironment. The use of DDS in drug delivery may result in lowering the concentration of nanoparticles or drugs required to achieve a greater pharmacologic response. This could limit the development of resistant strains of bacteria and cancer cells while at the same time reduce the amount of nanoparticle that leaches into the ecosystem.

This study aims as previously stated to encapsulate AgNP in a natural biosurfactant such as dipalmitoyl phosphatidylcholine (DPPC), which is documented to have low immunogenicity and also suppress AgNP-induced inflammation (Murphy et al., 2015b), as a means of delivering AgNP to cancer cells for improved cytotoxic effect. Additionally, liposomal encapsulation of conventional drug such as doxorubicin (Doxil®) has been shown to enhance cytotoxicity of these drugs and have been approved by the FDA (Table 1-1). Thus, this thesis aimed at and achieved the following in attempt to improve upon AgNP cytotoxicity with minimal provocation of inflammation,

a. Synthesised AgNP in-house, investigated and trial different ways of encapsulating AgNP in a DPPC-based liposome
b. Investigated the delivery efficiency of encapsulated AgNP as compared with free uncoated AgNP in a known immune cell line/cancer cell line by evaluating the cytotoxic effects on cellular viability.

c. Investigated the mechanism of action of encapsulated AgNP compared with that of free AgNP looking at the signalling cascade involved in activating cell death pathways.

d. Investigated the effect of encapsulation on AgNP induced inflammation by investigating the release of pro-inflammatory cytokines like interleukin-1β (IL-1β), IL-6, IL-8 and tumour necrosis-α (TNF-α).
2 Materials and methods

2.1 Cell line

The cell line used throughout this study was THP1 (ATCC®: TIB-202™), an acute monocytic leukaemia cell line obtained from the blood of a one-year old leukaemia patient (Tsuchiya et al., 1980). The cell line was differentiated into macrophages as described in section (XXX) for macrophage studies.

2.1.1 Cell culture and exposure

THP1 (ATCC®: TIB-202™) a suspension line derived from a human peripheral blood monocyte from an acute monocytic leukaemia patient, was used for this study. THP1 cells were cultured in RPMI-1640 media containing 2 mM L-glutamine (Sigma Aldrich, Ireland) supplemented with 10 % FBS and incubated at 37°C, 95 % humidity and 5 % CO₂.

For nanoparticle exposure, cells were seeded into a 96-well plate at a density of 1 x 10⁴ cells in 100 µL of culture media for a 24- and 48-hour time period or in a 24-well plate (VWR, Dublin, Ireland) at a density of 3 x 10⁵ cells/ml in media containing 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for a 24 h to induce maturation of the pre-monocytes into adherent monocytic THP-1 after which the cells were rested in PMA free media for 24 h. The cells were then treated with un-encapsulated (AgNP) and Lipo-AgNP after seeding. For the adherent cells in 24 well plates, the culture media containing PMA was removed from the now adhered monocytic THP1 cells and replaced with fresh RPMI media containing different concentrations of uncoated AgNP, PB-Lipo-AgNP and Ex-Lipo-AgNP. A positive kill control, a 10 % (v/v) dimethyl sulfoxide (DMSO) solution was prepared in serum free media to treat the cells while a negative control of unexposed cells was also incorporated onto the plate for both AgNP and Lipo-AgNP exposed cells. THP1 cells were also exposed to
varying concentrations of AgNO₃ (Ag⁺) solution prepared by dissolving corresponding amount of AgNO₃ in ddH₂O as another positive control to test possible influence of AgNP ionisation into Ag⁺ on its cytotoxic effect. A minimum of three independent experiments were conducted and for each independent experiment, six replicate wells were employed per concentration per plate.

2.2 Other materials and reagents

Appendix A1 lists the materials and reagents used throughout this thesis.

2.3 AgNP synthesis

To synthesize AgNP, 6 ml of 1 mM of AgNO₃ solution was added dropwise into an Erlenmeyer flask containing magnetic stirrer a 350 rpm and ice cold 30 ml of 2 mM of NaBH₄. The stirring was continued until last drop when the stirrer was removed for the solution to turn golden yellow. The obtained AgNP was characterised by UV/Vis in a Spectramax M2 microplate reader while atomic absorption spectrophotometry (AAS) was employed to monitor silver (Ag) concentration using a SpectrAA200 Varian Spectrophotometer (Mulgrave, VC, Australia). The samples were analysed with a silver hollow cathode lamp at an operating current of 7.5 mA. Hydrodynamic size of AgNP and liposomal AgNP (Lipo-AgNP) was carried out with Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK). Nanoparticles were loaded into a pre-rinse-pre-rinsed folded capillary cell up to the marked portion. For zeta potential, an applied voltage of 15 and 50 V was used for Lipo-AgNP and free AgNP respectively.
2.4 X-ray diffraction (XRD) of AgNP

Powdered XRD characterisation of AgNP was conducted with a Philips diffractometer (Phillips Inc., USA) using a monochromatic Cu-K\(\alpha_1\) radiation source (\(\lambda = 1.5406\) Å) operated at 40 keV, 30 mA and a 20 angle pattern. The scanning was carried out in the 20° to 80° region and the crystalline structure was analysed by comparing the obtained result with that of the Joint Committee on Powder Diffraction Standards (JCPDS) library.

2.5 Liposome synthesis, AgNP encapsulation and characterisation

Liposomes were prepared with a combination of DPPC and cholesterol via a modification of a dehydration-rehydration technique originally reported by Mugabe et al. (2006). Liposome was prepared by probe sonication and extrusion methods. DPPC and cholesterol were weighed in a mass ratio such that eventual rehydration of the lipid film obtained will give a 7:3 molar ratio solution respectively. The lipids were dissolved in a fixed amount of chloroform and mixed until the mixture becomes clear. The resulting solution was placed in a vacuum oven set at 52°C for the chloroform to evaporate.

2.5.1 Probe sonication method

The lipid cake formed was then rehydrated in AgNP solution at 60°C. AgNP solution were added to the lipid at 1:300 (w/w) of AgNP:DPPC after which the solution was vortexed briefly for 2 minutes to form multi-lamellar vesicles (MLV). The mixture was probe sonicated at 21% amplitude, 20 sec run and 20 sec pause for 4 cycles to form Small Uni-lamellar Vesicles (SUV). The resultant mixture was then centrifuged at 800 \(x\) g for 10 mins at 4°C to remove any MLVs. The suspension was subjected to DLS and zeta potential analysis for size and stability measurements respectively.
2.5.2 Extrusion method
The lipid film was rehydrated in AgNP solution at 60°C to make the final concentration at 1:300 (w/w) of AgNP:DPPC. The solution was placed in the shaker at 60°C on 140 rpm for another 20 mins after which it was vortexed briefly for 2 mins to form multi-lamellar vesicles (MLV). This was then extruded through a 100 nm “Nanosizer” polycarbonate extruder purchased from T&T Scientific (Knoxville, USA). The suspension was subjected to DLS and zeta potential analysis for size and stability measurements respectively.

2.6 Dynamic light scattering (DLS) analysis and Zeta potential analysis
To measure the hydrodynamic diameter of the nanoparticle in solution, the DLS analysis of both AgNP and Lipo-AgNP was carried out with Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK). The zeta potential measurement of the nanoparticles was also measured with Malvern Zetasizer Nano ZS instrument set at 25°C for all the samples. Nanoparticles were loaded into a pre-rinsed folded capillary cell up to the marked portion (usually filled with 1 ml of sample). An applied voltage of 15 and 50 V was used for Lipo-AgNP and uncoated AgNP respectively and a minimum of three different measurements were made for each sample.

2.7 Temperature-dependent size measurements, stability tests and pH-dependent drug release study
To check the effect of incubation conditions on the nanocapsules, both probe-sonicated (PB-Lipo-AgNP) and Ex-Lipo-AgNP were subjected to temperature dependent size stability tests. A solution of Lipo-AgNP in 10% foetal bovine serum (FBS) supplemented RPMI-1640 was prepared and subjected to DLS size measurements over a temperature range of 20°C to 38°C with 1°C increments of temperature.
For nanoparticle stability determination, variations in nanoparticles mean size and zeta potential of both Ex-Lipo-AgNP and PB-Lipo-AgNP were measured at a specific interval over a period of 6 months at both 4°C (storage temperature) and 24°C (room temperature). 5 ml of PB-Lipo-AgNP and Ex-Lipo-AgNP were incubated at 4°C and 24°C and 1 ml sample was taken at each time point for analyses at specific time interval.

For pH dependent AgNP release from the nanocapsules, 1 ml of the encapsulated AgNP was added into a FLOAT-A-LYZER G2 CE dialysis tube with a 1,000 KDa MW cut off (Spectrum Labs, Breda, Netherlands). The dialysis tube was placed in 6 ml of either an acetate buffer (pH 6.5) or a phosphate buffer saline (PBS) at pH 7.45. The ratio between the inside and outside volumes were maintained as thus to facilitate easy movement of the AgNP as recommended by Shen and Burgess (2013). The tube was then placed on a shaker running a 300 rpm at 37°C. To measure the amount of AgNP released, 200 µl of Lipo-AgNP sample was taken from the dialysis tube at specific time interval for 24 h and the absorbance was measured in the SpectraMax M2 microplate reader at 405 nm. After absorbance measurement, the measured sample was replaced with a fresh buffer to avoid change in volume and sink condition.

### 2.8 Scanning electron micrograph (SEM) and scanning transmission electron micrograph (STEM) analysis

SEM micrographs were obtained for both AgNP and Lipo-AgNPs. Briefly, both PB-Lipo-AgNP and Ex-Lipo-AgNP were microscopically analysed using Hitachi SU-6600 field emission SEM (Hitachi, Maidenhead, UK) with accelerating voltage of 25 kV and 8 mm working distance. At 24 h before analysis was carried out, 5 µl of sample was drop-cast to air dry onto a 5 x 5 mm pure silicon wafer substrate (Ted Pella Inc.,
Redding, California, USA) for SEM or carbon formvar copper grid (Agar Scientific Ltd., Stanstead, UK) for STEM, before micrographs were obtained.

2.9 Estimating encapsulation efficiency of the liposome

To estimate the encapsulation efficiency, both probe-sonicated and extruded Lipo-AgNP were centrifuged at 20,000 x g for 1 h and the supernatant was harvested. The supernatant was then analysed by atomic absorption spectrophotometry to estimate the concentration of silver in the solution. The encapsulation efficiency (E) of the liposome was then calculated using the formula below

\[ E = \frac{\text{Total AgNP added to liposome} - \text{AgNP in supernatant}}{\text{Total AgNP added to liposome}} \times 100 \]

2.10 Cell viability

Cellular viability was evaluated with the Alamar Blue assay (AB). Briefly, a 10 % (v/v) AB solution in serum free media was prepared and kept warm in a water bath at 37°C. The plate containing treated monocytic cells is suspension in 96 well plates was centrifuged in a Heraeus Megafuge 16R (Thermofischer) at 500 x g and 20°C for 5 min and the exposure media removed after which 100 μL of the 10% AB solution was added to each well. The plates were then incubated for 3 h after which the resulting florescence of the converted AB dye was measured at 540 nm and 595 nm excitation and emission wavelengths respectively using SpectraMax® M3 Multi-Mode Microplate Reader and compared to the relative controls. For the adherent cells, the exposure media were removed, and the cells were rinsed with pre-warmed sterile 1 x phosphate buffer saline (PBS) after which 1.5 ml of AB solution was added onto the cells and incubated at 37°C for 2 h. this was followed by absorbance measurement as done for the suspension cells.
2.11 Flow cytometry

Flow cytometry is a revolutionary technique used in measuring multiple features of a single cell based on specific physical characteristic such as the size and granularity or internal complexity as the cell flows or moves amidst population of cells through the measuring device.

For flow cytometry analysis THP1 cells were seeded and cultured in T25 flasks at 2 x $10^5$ cells/mL and were subsequently treated with Lipo-AgNP and AgNP for 24 h. After exposure, cells were harvested into 15 mL tubes and were centrifuged at 300 x g for 5 min at 22°C to form pellets. The pellets were resuspended in 2 mL pre-warmed 1 x phosphate buffer saline (PBS) and centrifuged as above and the process was repeated. Prior to staining the pellet was finally resuspended in 1 mL binding buffer a 0.1 % NaN₃ and 1 % bovine serum albumin (BSA) solution in 1 x PBS.

For live-dead staining the cells were double stained by adding 5 µL of 1 µM calcein-AM stain and 10 µL of 10 µg/mL PI. The cells were then incubated in the dark at RT for 20 min and analysed with a BD Accuri C6 flow cytometer.

For cell cycle analysis, cells were harvested as above and rinsed in warm sterile PBS. The cells were then fixed with ice cold 70 % ethanol. After ethanol fixation, the cells were incubated at 4°C for up to 4 h. Prior to any staining, the cells were washed twice in PBS and centrifuged at 800 x g for 5 min and the cell pellet treated with 50 µL of a 100 µg/mL ribonuclease A to ensure only DNA was stained. The cells were then stained with 400 µL of 50 µg/mL PI and incubated for 10 min before analysing using BD Accuri C6 flow cytometer.

Finally, for Caspase3/7 analysis, as before the THP1 cells treated with AgNP and Lipo-AgNP were harvested and centrifuged at 300 x g for 5 min to remove exposure
solutions. As a positive control, THP1 cells were exposed to 1 µM solution of doxorubicin, which is a known activator of the executioner caspases (Ueno et al., 2006, Yang et al., 2001). The cells were washed in PBS once and then resuspended in binding buffer. The cells were then stained with 500 nM CellEvent caspase 3/7 detection reagent and incubated at 37°C for 30 min. The cells were then stained with 1 µM SYTOX AAdvanced dead cell stain and incubated for 5 min prior to being analysed on a BD Accuri C6 flow cytometer.

2.12 Confocal microscopy

Confocal microscopy uses a laser technology in contrast with normal light used by a widefield microscope where light from mercury or xenon source is shone on the whole sample for viewing. For confocal microscopy, image of a specimen is captured by scanning a focused beam of light from a laser source across the specimen (Paddock, 2000). The light path in the confocal microscope is similar to that of the reflected-light wide-field epifluorescence microscope but the confocal microscope has a pin hole in front of the light source as well as a photodetector (Figure 2-1). This approach has allowed for automated capturing the three-dimensional data for images of cells and subcellular structure in form of Z series even in multiple labelled samples.
Figure 2-1. Light path of laser scanning confocal microscope.

THP1 cells were seeded onto a confocal dish (VWR, Dublin Ireland) at density of 3 x 10^5 cells/ml. The cells were also stimulated with 100 ng/ml of PMA for 24 h and subsequently treated with RPMI media containing 2 µg/ml of either PB-Lipo-AgNP and Ex-Lipo-AgNP for 24 h. Dish containing cells exposed to 0.5 nM doxorubicin were incorporated as positive kill control. After exposure, the media were discarded, and the cells were rinsed with pre-warmed sterile PBS. The cells were stained with 50 µl of 1 µM calcein-AM and 50 µl of 10 µg/ml PI. The cells were then incubated in the dark at RT for 20 min and rinsed with warm PBS afterwards. Prior to imaging, 1 ml of warm PBS was added onto the cells and imaging was carried out with Zeiss LSM.
Confocal Laser Scanning Microscope using a Plan-Neofluor oil immersion lens at 40 x magnification and 1.3 numerical aperture.

2.13 Reactive Oxygen Species (ROS) Assay by spectrophotometric method

ROS generation in the THP1 cells as a result of particle exposure was measured with a 6-carboxy-2′,7′–dichlorofluorescin diacetate (carboxy-DCFDA) assay. In the assay carboxy-DCFDA a non-fluorescent dye is metabolised by esterase into H$_2$DCFDA which is further oxidised by ROS generated in the cell to a green fluorescent DCFDA dye. To quantify ROS generation the THP1 cells were seeded into T75 flask at 5 x 10$^5$ cells/mL in RPMI media containing 2 % FBS and the cells were loaded with 10 μM of carboxy-DCFDA dye and incubated for 30 min under normal culture condition. The loaded cells were then centrifuged at 300 x g for 5 min and the medium containing excess carboxy-DCFDA was removed. The loaded cells were then washed twice in warm sterile PBS and then re-suspended in normal culture media. The cells were then seeded into Corning 96-well solid black plates at 2 x 10$^5$ cells/mL and treated with AgNP or Lipo-AgNP at 0.3 μg/mL.

ROS generation in the THP1 differentiated macrophages (TDMs) consequent of particle exposure was monitored using 6-carboxy-2′,7′–dichlorofluorescin diacetate (carboxy-DCFDA) assay. This assay utilises esterase metabolism of carboxy-DCFDA a non-fluorescent dye, into H$_2$DCFDA which is further oxidised to a green fluorescent DCFDA dye by ROS generated in the cell. To quantify the ROS generation, media on the TDMs seeded onto 24-well plate above were aspirated and replaced with RPMI media containing 2 % FBS. The cells were loaded with 10 μM of carboxy-DCFDA dye and allowed to incubate for 30 min under above culture conditions. After this, the culture media containing carboxy-DCFDA were removed and the cells were rinsed in pre-warmed sterile PBS. The cells were then incubated in media containing 2 μg/ml...
AgNP or Lipo-AgNP for specific time points to be analysed. Before ROS quantification, cells were rinsed once again in PBS and the media was replaced with PBS. ROS generation was then quantified by the fluorescence of the oxidised carboxy-DCFDA, by monitoring the dyes emission at 535 nm by 485 nm excitation on a Spectramax M3 multiplate reader, using multi-well scan at five different points per well or flow cytometry.

2.14 Cell culture and THP1 differentiation into monocytic THP1 and TDMs for inflammation studies

THP1 (ATCC®: TIB-202™) cells were used here were cultured as described in section 2.1.1 The cells were stimulated with 100 ng/ml (162 nM) PMA for either 24 h or 72 h to induce differentiation into matured monocytic THP1 or TDMs respectively. The PMA containing media was then replaced with fresh RPMI media and the cells left to incubate for another 24 h (Chanput et al., 2014).

2.14.1 Cell exposure

After this, the culture media containing PMA was removed from the now adhered monocytic THP1 cells or TDMs and another fresh RPMI media containing 30 µg/mL of polymyxin B was replaced for 1 h to inhibit any LPS that might be present in any reagent used. Polymyxin B is known to inhibit LPS induced inflammatory response and the concentration used here was found to inhibit up to 10 µg/mL LPS contamination (Xiao-Xiao et al., 2017). After incubation with polymyxin B, the cells were then treated with different concentrations of uncoated AgNP and Lipo-AgNP. As a positive control for inflammatory response, cells primed with 10 µg/mL LPS from *E. coli* 0111:B4 were incorporated on the plate while a negative control of unexposed cells were both incorporated onto the plate. The cells were incubated in treatment for specified period and were stimulated with 5 mM ATP for 1 h prior to
collection of the supernatant. In some instances, cells exposed to 20 µM of Ac-YVAD-
CMK, a caspase-1 inhibitor (ICE\textsubscript{inh}), were also incorporated onto the plate. Cells were
exposed to caspase-1 inhibitor to ensure changes in IL-1β secretion is due to AgNP
and not caspase-1. A minimum of three independent experiments were conducted and
for each independent experiment, four replicate wells were employed per
concentration per plate.

2.14.2 Cell viability

Cell viability of nanoparticle exposed TDMs was analysed by Alamar Blue assay
(AB). A pre-warmed solution of AB (10% v/v) in serum free media was prepared and
the exposure media on the TDMs were removed, after which the cells were rinsed with
pre-warmed sterile 1 x phosphate buffer saline (PBS). Then, 1.5 ml of AB solution
was added onto the cells and the plates were incubated at 37°C for 2 h. The florescence
of the converted AB dye was measured at 540 nm excitation and 595 nm emission and
wavelengths in a SpectraMax® M3 Multi-Mode Microplate Reader.

2.15 Cytokine release by sandwiched enzyme linked immunosorbent assay
(ELISA)

Sandwich ELISA is used to measure the quantity of specific antigens between two
layers of antibodies known as the capture antibody, conjugated to the surface of a glass
plate for instance and a detection antibody which is conjugated to a fluorophore for
fluorescence detection. For cytokine release assay, RPMI culture media from THP1
promonocytes, monocytic THP1 and TDMs exposed to AgNP or Lipo-AgNP post
priming with LPS and ATP challenge were collected at different time points after
which IL-1β, IL-6, IL-8 and TNF-α levels in the culture supernatant were carried out
by sandwich ELISA following manufacturer’s instructions. For each of the cytokine
assayed, a standard of pure sample with known concentration (as supplied by the manufacturer) was used (see Appendix 1). Absorbance was measured at 450 nm

2.16 Cell lysate collection

To collect cell lysate for protein expression analysis, culture media was aspirated from the cells after which the cells were rinsed in ice cold 1X PBS while the plate was kept on ice. The cells were lysed with 100 µl of RIPA buffer containing protease inhibitor cocktail (as supplied by MerK, Dublin Ireland) per 3.5 x 10^5 cells and the cells were scrapped off and pipetted up and down gently to break up intact cell membranes. Protease inhibitor was required to prevent degradation of the protein content of the lysate prior to sandwich ELISA, as intracellular proteases will be extracted alongside proteins of interest. The lysate was transferred into a 1.5 ml Eppendorf tube and centrifuged at 13,000 xg for 10 mins at 4°C. The supernatant was transferred into another 1.5 ml Eppendorf tube and kept on ice to be analysed by ELISA as soon as possible or stored at -80°C until analysis.

2.16.1 Cell culture and exposure

THP1 (ATCC®: TIB-202™) cells were used in this study and were cultured in RPMI-1640 media supplemented with 2 mM L-glutamine and 10 % FBS. The cells were incubated at 37°C, 95 % humidity and 5 % CO₂. For nanoparticle exposure, cells were seeded in a 24-well plate (VWR, Dublin, Ireland) at a density of 3 x 10^5 cells/ml of culture media and 1.5 ml of culture media per well. The cells were stimulated with 100 ng/ml (162 nM) PMA for 72 h TDM. The PMA containing media was then replaced with fresh RPMI media and the cells left to incubate for another 24 h (Chanput et al., 2014).
For cell exposure, culture media on cells were aspirated and the cells were rinsed in pre-warmed sterile 1X PBS solution. After this, the cells were incubated in RPMI media containing different concentration of either of AgNP or Lipo-AgNP ranging from 0.3 to 10 µg/ml. A negative control of unexposed cells was incorporated on the plate while a positive kill control of a 10 % (v/v) dimethyl sulfoxide (DMSO) solution and an internal positive control of AgNO₃ were o the plate. AgNO₃ was incorporated to assess the effect of Ag ionisation in AgNP on cell viability.

2.17 Reduced glutathione (GSH) assay

To measure the GSH levels, TDMs prepared as for ROS generation were exposed to both AgNP and Lipo-AgNP under the same conditions as above for 4 h. After exposure, the cells were rinsed in pre-warmed sterile PBS and incubated in RPMI media containing 10 µM of Thioltracker™ violet dye for another 30 mins in the dark under incubation conditions used above. The cells were rinsed again in pre-warmed sterile PBS after which they were covered in warm PBS. GSH level of in the cells were measured using Multi-well scan setting on a Spectramax M3 multiplate reader at 405 nm excitation and 530 emission wavelengths.

2.18 Flow cytometry of TDMs

Flow cytometry analysis was used for both ROS generation and nanoparticle uptake. For ROS generation, TDMs already treated with carboxy-DCFDA were exposed to nanoparticles as above, rinsed in PBS and then gently scrapped off. The scrapped cells were resuspended in PBS containing 1% BSA and this method was subsequently used to detach the cells before analysis.

For nanoparticle uptake, RPMI media from TDMs cultured as described above were aspirated and cells rinsed in PBS. The cells were then incubated in media containing
2 µg/ml and the exposure media was removed at specific time points after which the cells were rinsed in PBS and detached as described above before flow cytometry analysis.

For live-dead staining, the TDMs were double stained by adding 5 µL of 1 µM calcein-AM stain and 10 µl of 10 µg/ml PI per 1 ml of RPMI media. The cells were then incubated in the dark at RT for 20 min after which they were detached by scrapping and processed as above for analysis by flow cytometry.

For γH2AX activation analysis, unexposed TDMs and TDMs exposed to 2 µg/ml AgNP and Lipo-AgNP for 24 h after which the culture media on the cell was discarded. The cells were detached by gently scrapping off from the plate as described above and were washed in pre-warmed sterile PBS followed by centrifugation at 300 x g for 5 mins to pellet the cells while the supernatant was discarded. The pellets were washed as before and then fixed in ice cold 70% (v/v) ethanol for alcohol fixation at -20°C for 2 h. The cell suspension was centrifuged at 500 x g for 5 mins to discard the ethanol, the cells washed as previously three times in flow buffer made up of PBS-BSA containing 0.1% sodium azide (NaN₃) using 800 x g centrifugation speed. The cells were permeabilised by resuspension in 0.25% PBS-Triton-X100 (PBS-T) for 5 min at RT. PBS-T was completely removed by centrifugation at 400 x g for 5 min as above. The cells were then blocked by resuspension in 2% (w/v) PBS-BSA and incubation at RT for 30 min.

To immunostain the cells with anti- H2AX antibody, the blocking solution was removed by centrifugation as described above. The cells were resuspended in flow buffer containing the antibody at 1:100 and incubated at 37°C for 30 mins. The cells were ashed to remove excess unbound antibodies as above using PBS. The cells were
resuspended in fresh flow buffer solution analysed by flow cytometry in BD Accuri C6 flow cytometer.

Finally, for Caspase3/7 analysis, as before the TDMs were exposed to 2 µg/ml of either of AgNP or Lipo-AgNP for 24 h. The cells were harvested as above and then resuspended in flow buffer of 1X PBS containing 0.1% sodium azide (NaN₃) and 1% BSA. The cells were then stained with 500 nM CellEvent caspase 3/7 detection reagent and incubated at 37°C for 30 min. After this, the cells were stained with 1 µM SYTOX Advanced dead cell stain and incubated for 5 min prior to being analysed by flow cytometry. All analyses were performed on a BD Accuri C6 flow cytometer.

2.19 Cell lysate collection and Sandwiched Enzyme Linked Immunosorbent Assay (ELISA)

To collect cell lysate for protein expression analysis, culture media was aspirated from the cells after which the cells were rinsed in ice cold 1X PBS while the plate was kept on ice. The cells were lysed with 100 µl of RIPA buffer containing protease inhibitor cocktail per 3.5 x 10⁵ cells and the cells were scrapped off and pipetted up and down gently to break up intact cell membranes. The lysate was transferred into a 1.5 ml Eppendorf tube and centrifuged at 13,000 xg for 10 mins at 4°C. The supernatant was transferred into another 1.5 ml Eppendorf tube and kept on ice to be analysed by ELISA. Sandwich ELISA of the collected lysate for Bax and Bcl-2 protein expression from TDMs exposed to AgNP or Lipo-AgNP was carried out following the manufacturer’s instructions. Absorbance was measured at 450 nm.

2.20 Statistical analysis

Statistical analysis of acquired data was carried out with GraphPad Prism version 7. Data was analysed by Two-way analysis of variance (ANOVA) with Sidak or Turkeys
multiple comparisons test to detect significance in effects between exposure groups. Statistically significant differences in tests were indicated for $p$ value $< 0.05$. 
Chapter 3

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Silver nanoparticles (AgNP) are a widely used nanoparticle for its antibacterial activities and many of the already commercialized products contain AgNP in high concentrations as the active ingredient. For example, AgNP is widely used as antibacterial coating on medical garments and surgical equipment and even on food materials to prolong shelf life by preventing food degradation consequent upon bacterial metabolism and growth (Polivkova et al., 2017, Carbone et al., 2016). In addition, AgNP are currently being investigated by different studies as a chemotherapeutic in cancer treatment (Asharani et al., 2012, Juarez-Moreno et al., 2016, Foldbjerg et al., 2011, Foldbjerg et al., 2012). Unfortunately, with the rise in the biomedical applications of AgNP, development of adverse conditions due to repeated human exposure to AgNP is imminent either from direct contact with products containing AgNP or AgNP that has leached into the ecosystem. AgNP has been reported to cause several adverse effects such as skin irritation and discolouration, hepatotoxicity, kidney damage, DNA damage and epithelia cell damage (León-Silva et al., 2016).

Adverse reactions of conventional drugs are not uncommon and improvement on the delivery mechanisms has been a major way to limit these setbacks. For AgNPs however, there has been little or no research into how to improve upon the delivery mechanism to enhance their antibacterial or anticancer activities. The applications of liposomes in drug delivery systems (DDS) have been studied for more than two decades and there have been significant improvements in the formulations and
methods by which liposome are prepared. For instance, phosphatidylcholine (PC) based lipids are highly used in liposome preparation likely due to the fact that PC makes up about 80% of the surfactants found on epithelial lining of human airways and lungs. Interestingly, the majority of the PC in the human airways is present in the form of dipalmitoyl phosphatidylcholine (DPPC), and this makes up about 60% of the natural surfactants found in the human airways and lungs (Agassandian and Mallampalli, 2013). Consequently, DPPC is highly unlikely to elicit immune response when incorporated in a liposomal formulation compared to the other derivatives.

Liposomes are designed to mimic the lipid bilayer of the cell membrane and while the natural bilayer of the cell membrane is made up different phospholipids, they also contain cholesterol molecules that help restrict the movement of the fluid phospholipid molecules. In the same manner, it has been shown that cholesterol, when incorporated in liposomal formulations at the right concentration can produce such rigidity to protect the liposomal content (Briuglia et al., 2015). In this study, AgNP synthesised by chemical reduction of silver nitrate (AgNO₃) using sodium borohydride (NaBH₄) was encapsulated in a DPPC/cholesterol liposome to both stabilize and improve the uptake of the AgNP in vitro for enhanced cytotoxicity. Two simple encapsulation methods were trialled AgNP, followed by nanoparticle characterization and evaluation of cytotoxicity on a THP1 cell line, a monocytic cell line which acts as first line of defence against nanoparticle during exposure (Wu et al., 2017).
3.1 Results

3.2 DLS characterisation

Results of the DLS characterization of AgNP is summarized in Table 3-1 for dispersions in water (ddH₂O) and RPMI-1640 culture media. DLS analysis of AgNP shows an increase in mean particle size (MPS) of AgNP when dispersed in ddH₂O to RPMI-1640 media from 21.14 nm to 79.15 nm with polydispersity index (PDI) 0.230 to 0.566 respectively. The zeta analysis for AgNP in ddH₂O was -26.5 mV which dropped to -7.90 mV in RPMI-1640 media. There was also change in AgNP colour from golden yellow in ddH₂O to dark grey when dispersed in RPMI-1640 media which is likely due to agglomeration of the nanoparticle.

Table 3-1. Size and Zeta potential of uncoated AgNP, PB-Lipo-AgNP and Ex-Lipo-AgNP in ddH₂O and RPMI-1640

<table>
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<th></th>
<th>ddH₂O</th>
<th>media</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Peak 1 (%)</td>
<td>Peak 2 (%)</td>
</tr>
<tr>
<td>Uncoated AgNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLS Intensity PSD (nm)</td>
<td>21.14±9.48</td>
<td>-</td>
</tr>
<tr>
<td>Zeta (mV)</td>
<td>-26.50</td>
<td>-</td>
</tr>
<tr>
<td>PDI</td>
<td>0.230</td>
<td>-</td>
</tr>
<tr>
<td>PB-Lipo-AgNP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLS Intensity PSD (nm)</td>
<td>143.7±64.18(98.7)</td>
<td>5005±605.6 (1.3)</td>
</tr>
<tr>
<td>Zeta (mV)</td>
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<td>PDI</td>
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<td>Ex-Lipo-AgNP</td>
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<td>Zeta (mV)</td>
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3.3 SEM/STEM and spectra analysis of AgNP

SEM analysis of the AgNP showed a spherical nanoparticle with average size of 14.3 ± 1.9 nm (Figure 3-1A). The UV-Vis spectra of the different AgNP concentration ranging from 0.625 to 10 µg/ml are depicted in Figure 3-1B, showing a characteristic peak absorption (λmax) corresponding to the surface plasmon resonance (SPR) of 20 nm AgNP at around 400 nm, which was the approximate size obtained by DLS. The peak flattening corresponds to decrease in concentration of AgNP, explained by the reduction in the amount of AgNP particles that absorbs UV light at the wavelengths indicated.
Figure 3-1. SEM/STEM and UV-Vis Spectra analysis of AgNP

(A) SEM and of AgNP with STEM image inset (B) UV spectra analysis of 0.625 to 10 µg/mL of AgNP measured at 22.6°C.
3.4 Liposome characterisation

PB-Lipo-AgNP size increased from 143.7 when in ddH$_2$O to 268.7 nm after dispersion in RPMI-1640 media (Table 3-1). A second peak of larger sized particles was observed in both ddH2O (1.3%) and RPMI media (19.6%) likely due to agglomeration. The PDI of PB-Lipo-AgNP also increased from 0.305 to 0.437 after resuspension in RPMI-1640 media but there was a reduction in zeta potential from -25.9 mV in ddH$_2$O to -0.96 after dispersion in RPMI-1640 media.

For Ex-Lipo-AgNP, there was a small decrease in size from 140.1 nm in ddH$_2$O to 138.9 nm (half that of PB-Lipo-AgNP) when dispersed in RPMI-1640 media. Unlike the PB-Lipo-AgNP, extrusion produced Lipo-AgNP that was 100% uniform in size in ddH$_2$O, however, a second peak was found at 3.9 µm for 14% of the particles in RPMI media (Table 3-1). In contrast, Ex-Lipo-AgNP had a PDI of 0.105 in ddH$_2$O but this increased to 0.421 in RPMI-1640 media. There was also a reduction in zeta potential of Ex-Lipo-AgNP from -31.9 mV in ddH$_2$O, higher than that of PB-Lipo-AgNP to -0.61 mV in RPMI-1640.

An overlay of DLS size values of the uncoated AgNP in ddH$_2$O was carried out with the size values of the PB-Lipo-AgNP obtained with the same AgNP solution both in ddH$_2$O and in RPMI media (Figure 3-2C). Overlap in AgNP size value with that of the PB-Lipo-AgNP dispersed in ddH$_2$O was observed, indicating some of AgNP had not been encapsulated within the PB-Lipo-AgNP. In addition, a shift in the major peak of the PB-Lipo-AgNP was observed for a 120 nm increase in size from dispersion in ddH$_2$O to RPMI, accounting for 20% of the total nanoparticle. Ex-Lipo-AgNP exhibited no overlap with AgNP in both dispersion media, indicating both nanoparticles have distinct populations (Figure 3-2D). In addition, there was only a single peak observed for Ex-Lipo-AgNP dispersed in ddH$_2$O indicative of uniform
nanoparticle although there was a slight shift in the major peak to the left as the size reduced by 1.2 nm while a second peak was also visible, accounting for 14% of the total nanoparticle likely due to agglomeration in RPMI.

Figure 3-2. SEM/STEM of PB-Lipo-AgNP and Ex-Lipo-AgNP:
(A) SEM with STEM (inset) of PB-Lipo-AgNP, and overlay of AgNP size value with PB-Lipo-AgNP (B) SEM with STEM (inset) Ex-Lipo-AgNP and overlay of AgNP size value with Ex-Lipo-AgNP
3.5 UV-Vis spectra analysis of encapsulated AgNP and encapsulation efficiency

Different concentrations of PB-Lipo-AgNP and extruded AgNP, were analysed by UV-Vis spectra to investigate whether the AgNP has been successfully encapsulated (Figure 3A and B). PB-Lipo-AgNP showed a similar spectra characteristic with AgNP especially at 10 µg/ml but there was a red shift in the AgNP peak at around 410 nm, observable for both 5 µg/ml and 10 µg/ml. There was considerable peak flattening at concentration ≤ 5 µg/ml (Figure 3-3A). It was observed that PB-Lipo-AgNP was cloudy with lipids and retained the golden yellow colour of AgNP showing presence of free AgNP (Figure 3-2A inset). On the contrary for Ex-Lipo-AgNP, the peak absorbance was barely observed even at 10 µg/ml and there was also a red shift in the peak at around 410 nm (Figure 3-2B). Ex-Lipo-AgNP solution was clear and did not retain the golden yellow colour of AgNP (Figure 2B inset), likely because of the refraction due to the lipid layer of the liposome. PB-Lipo-AgNP also had higher absorbance compared to Ex-Lipo-AgNP (at 10 µg/ml) which has similar baseline with uncoated AgNP (Figure 3-3C), indicating no agglomeration of Ex-Lipo-AgNP. The EE was determined to be 67.8% and 86.5% for the PB-Lipo-AgNP and Ex-Lipo-AgNP respectively, which may explain the similarities between the UV-Vis spectra of free AgNP and PB-Lipo-AgNP since less AgNP was encapsulated.
Figure 3-3. UV-Vis Spectra of PB-Lipo-AgNP and Ex-Lipo-AgNP
UV-Vis spectral analysis of (A) PB-Lipo-AgNP and (B) Ex-Lipo-AgNP at different concentrations between 0.625 µg/ml to 10 µg/ml (C) combined UV-Vis spectra of 10 µg/ml AgNP, Ex-Lipo-AgNP and PB-Lipo-AgNP.
3.6 SEM/STEM analyses of Lipo-AgNP

PB-Lipo-AgNP and Ex-Lipo-AgNP were analysed microscopically by SEM and STEM (Figure 2A and B). As shown, PB-Lipo-AgNP formed agglomerates unlike Ex-Lipo-AgNP. SEM analysis of Ex-Lipo-AgNP showed non-agglomerating spherical liposomes with a well-defined structure. STEM of the PB-Lipo-AgNP (Figure 2A inset) showed AgNP found coated on the liposome with very few nanoparticles encapsulated within. The AgNP in Ex-Lipo-AgNP shown in the STEM (Figure 2B inset) were all encapsulated within the liposome (grey sphere). This alludes to the EE and spectra characteristics of both PB-Lipo-AgNP and Ex-Lipo-AgNP. Size estimation from SEM indicated Ex-Lipo-AgNP was 162.73 ± 29.23 nm while the PB-Lipo-AgNP was 204.22 ± 45.39 nm representing the average of 20 particles counted and similar to the value obtained by DLS.

3.7 Temperature-dependent size change, stability analyses and load release profile of Lipo-AgNP

The practicability of the Lipo-AgNP to retain their contents in *in vitro* experiments was tested under incubation conditions. Sizes of both PB-Lipo-AgNP and Ex-Lipo-AgNP with respect to temperature changes was monitored using DLS in RPMI-1640 media containing 10% FBS over 6 h at 20 mins interval for a degree rise in temperature. The initial size of PB-Lipo-AgNP doubled that of Ex-Lipo-AgNP confirming the values in Table 3-1. PB-Lipo-AgNP size reduced from 334 nm at 20°C to 150.2 nm, a 55% reduction in size at 37°C. For Ex-Lipo-AgNP, a reduction from 174.7 nm at 20°C to 113.1 nm at 37°C, a 35.3% reduction in size was observed (Figure 3-4B). This reduction in size could be as result of loss of liposomal content due to increase in temperature.

Stability analyses of the liposomes over a 6-month period is shown in Table 3-2. After
6 months of incubation, the MPS and zeta potential of PB-Lipo-AgNP increased by 10.3 nm and 5.1 mV respectively at 4°C. Compared to 4°C, PB-Lipo-AgNP at 24°C exhibited a higher reduction in MPS and zeta potential of 19 nm and 4.3 mV for the 6 months in addition to the sedimentation of the lipids that was observed. On the contrary, Ex-Lipo-AgNP showed slight increase in size as well as zeta potential over the 6-month period. At 4°C, an overall 3.2 nm and 2.0 mV MPS and zeta potential was observed, which was comparable to that observed at 24°C (5.9 nm and 2.5 mV MPS and zeta potential respectively), and lower to that of PB-Lipo-AgNP for the same time points.
Figure 3-4. Stability kinetics of probe-sonicated and extruded Lipo-AgNP
Temperature dependent changes in the sizes of (A) PB-Lipo-AgNP and (B) Ex-Lipo-AgNP dispersed in RPMI-1640 culture medium were analyzed by DLS. Values are mean ± SD from average of three independent measurements.
Table 3-2. Stability of PB-Lipo-AgNP and Ex-Lipo-AgNP over a 6-month period

<table>
<thead>
<tr>
<th>Temp</th>
<th>Initial size (nm)</th>
<th>Initial Zeta (mV)</th>
<th>Month 1</th>
<th>Month 3</th>
<th>Month 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB-Lipo-Figure 4°C</td>
<td>143.7±64.18</td>
<td>-25.9</td>
<td>149.4±9.7</td>
<td>151±13.3</td>
<td>154±20.3</td>
</tr>
<tr>
<td>24°C</td>
<td>153.2±9.61</td>
<td>-24.1</td>
<td>156.26±8.9</td>
<td>161.34±14.5</td>
<td>-19.6</td>
</tr>
<tr>
<td>Ex-Lipo-4°C</td>
<td>140.1±47.49</td>
<td>-31.9</td>
<td>142.2±3.4</td>
<td>144.4±2.5</td>
<td>143.33±1.5</td>
</tr>
<tr>
<td>24°C</td>
<td>141.3±1.72</td>
<td>-30.9</td>
<td>145±1.98</td>
<td>146±2.4</td>
<td>-29.4</td>
</tr>
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The load release profile of both Lipo-AgNPs was carried out to evaluate AgNP release from the nanocapsule using dialysis. Due to the large volume of fluid outside the dialysis tube and the effect this will have on the absorbance of minute quantity of released nanoparticles from the dialysis tube, the absorbance of the sample inside of the dialysis tube was measure instead, as drop in absorbance will corresponds to the amount of AgNP released into the buffer. As shown in Figure 3-5A, PB-Lipo-AgNP appeared to have initial burst release of AgNP as more than 25% of the encapsulated AgNP was released within the first 2 h at pH 6.5. Afterwards, a release of 29% to 30% at 4 and 6 h respectively was observed. Unlike PB-Lipo-AgNP, the extruded AgNP showed a steady release from 2 h up till 6 h, releasing only 15% of the encapsulated AgNP at 6 h, a significantly lower release to that of PB-Lipo-AgNP. Both nanocapsules exhibited similar release at 24 h with PB-Lipo-AgNP releasing 80% of encapsulated AgNP while Ex-Lipo-AgNP released 74%. At physiological pH of 7.45, PB-Lipo-AgNP exhibited lower release rate of AgNP from 2 h to 6 h releasing 0.8% to 12.5% respectively. In the same time point, Ex-Lipo-AgNP only released 0.7% to 3.5% respectively, a significantly lower release than that of PB-Lipo-AgNP. At 24 h, Ex-Lipo-AgNP released 70%, a significantly lower release compared with PB-Lipo-AgNP exhibiting 79% AgNP release (Figure 3-5B).
Figure 3-5. pH dependent drug release profile of PB-Lipo-AgNP and extruded Lipo-AgNP
Encapsulated AgNP in (A) acetate buffer at pH 6.5 or (B) PBS at pH 7.45 and at specific time interval, 200 µL of the sample was taken out for absorbance measurement. Data is presented as mean ± SD of 3 independent experiments * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. 
3.8 Cell viability

To evaluate if the stability of Ex-Lipo-AgNP translates to enhanced cytotoxicity, THP1 cells were first stimulated with 100 ng/ml PMA to induce adherence of the cell line prior to exposure to facilitate easy removal of uninternalized liposome and prevent cell loss during wash steps. After 24 h of exposure to the nanoparticles, viability of the PMA-stimulated THP1 cells was evaluated by their ability to convert the non-fluorescent resazurin in AB dye into a fluorescent resorufin. As shown in Figure 3-6A, Ex-Lipo-AgNP induced significant reduction in cell viability at concentration ≥ 1.25 µg/ml while uncoated AgNP and PB-Lipo-AgNP induced significant reduction in the THP1 cell viability at 5 µg/ml. It was observed that Ex-Lipo-AgNP at concentrations of 1.25 and 2.5 µg/ml were significantly more cytotoxic on THP1 cell than the PB-Lipo-AgNP at the same concentration.

A flow cytometry cell viability study was carried out to confirm AB assay findings. THP1 monocytes exposed to AgNP, PB-Lipo-AgNP and Ex-Lipo-AgNP were stained with calcein-AM and PI. Calcein-AM is a non-fluorescent stain hydrolysed by esterase activity of viable cell into a fluorescent calcein derivative that is maintained within cell with intact cell membrane (Uggeri et al., 2000), while PI only permeates compromised membrane of dead cells. As expected, Ex-Lipo-AgNP induced significantly more cell death compared to free AgNP and PB-Lipo-AgNP (\(p < 0.001\)). A significantly higher proportion of early apoptotic cells positive for both calcein and PI (9.7%) and late apoptotic cells that are only positive for PI (26.4%) was observed in Ex-Lipo-AgNP exposed cells compared to unexposed control cells, free AgNP and PB-Lipo-AgNP exposed groups (Figure 3-6B). In addition to this, PB-Lipo-AgNP exposure resulted in higher proportion of cells identified as cellular debris (22.6%) compared to Ex-Lipo-AgNP (1.1%) which was similar to that in untreated controls.
and free AgNP exposed cells (0.2%) ($p < 0.001$). This cell population are likely due to PB-Lipo-AgNP identified as cellular debris due to the larger and ununiform sizes.

To further confirm the effect of the Lipo-AgNPs on cell viability, confocal microscopy was used to analyse calcein-AM and PI stained PMA-stimulated THP1 cells exposed to nanocapsules containing equivalent amount of 2 µg/ml AgNP for 24 h. THP1 cells that were exposed to either of PB-Lipo-AgNP or Ex-Lipo-AgNP appeared to have spotted calcein fluorescence (Figure 3-7A and B). This was unlike the control-untreated THP1 cells which appeared to have uniform calcein stain throughout the cytoplasm. In addition, only Ex-Lipo-AgNP induced significantly higher cytotoxicity on THP1 cells compared with control-untreated or PB-Lipo-AgNP exposed cells ($p < 0.01$) (Figure 3-7A and B). Similarly, only Ex-Lipo-AgNP resulted in significantly higher PI fluorescence when compared to both PB-Lipo-AgNP exposed and control-untreated cells ($p < 0.001$). Thus, verifying the result of the AB and flow cytometry assays.
Figure 3-6. Cell viability of PMA-stimulated THP1 cells post-exposure to AgNP nanocapsules

(A) AB assay determining viability of THP1 cells exposed to 0.3 to 5 µg/mL AgNP, PB-Lipo-AgNP and Ex-Lipo-AgNP for 24 h (B) THP1 monocytes cell viability by flow cytometry after exposure to 2 µg/ml of AgNP, PB-Lipo-AgNP and extruded Lipo-AgNP. Calcein was assessed on FL-1 channel while PI was assessed in the FL-3 channel. Data is presented as mean ± SD of the 3 independent experiments and similar values were obtained. ***p < 0.001 and ****p < 0.0001
**Figure 3-7. Cell viability by confocal microscopy**

(A) Confocal microscopy assessment of PMA-stimulated THP1 cell viability after exposure to 2 µg/ml of AgNP, PB-Lipo-AgNP and extruded Lipo-AgNP. Calcein fluorescence is shown in green and PI fluorescence in red. (B) Fluorescence intensities quantified by ImageJ software from 50 different cells. Data is presented as an abstract value and as mean ± SD of 3 independent experiments. *$p \leq 0.05$ and **$p < 0.01$. 

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[Image of confocal microscopy results]

[Graph showing fluorescence intensities]
3.9 Discussion

AgNP can be synthesized from AgNO$_3$ by different methods such as using reducing agents like citrate or NaBH$_4$ with further stabilization of the nanoparticle with compounds such as polyvinyl alcohol (PVA) (Becaro et al., 2015, Cheon et al., 2015, Dong et al., 2009). A citrate-based reduction is most commonly used in the synthesis of AgNP because of its reducing and stabilizing functionality. However, reduction of AgNO$_3$ with citrate results in formation of AgNP in complex with the citrate ions which prevents the release of elemental silver (Djokic, 2008), limiting its effects. It is reported here that the encapsulation of AgNP in a DPPC based liposome through different methods enhances its associated cytotoxicity. The AgNP synthesis employed here was designed to yield elemental AgNP through reduction of AgNO$_3$ by NaBH$_4$ as in the equation below;

$$\text{AgNO}_3(\text{aq}) + \text{NaBH}_4(\text{aq}) \rightarrow \text{Ag}^0(\text{s}) + \frac{1}{2} \text{B}_2\text{H}_6(\text{g}) + \frac{1}{2} \text{H}_2(\text{g}) + \text{NaNO}_3(\text{aq})$$

One of the aims of this study was to encapsulate AgNP in a DPPC liposome, as DPPC is a natural biosurfactant in human airways. Thus, it is hoped that such a system will result in a very low capability of inducing adverse immune responses. SEM images of PB-Lipo-AgNP indicated a high agglomeration while that of Ex-Lipo-AgNP indicated a uniform spherical nanoparticle. In addition, DLS analyses indicated higher average size for PB-Lipo-AgNP compared to Ex-Lipo-AgNP both in ddH$_2$O and RPMI media. It is believed that PB-Lipo-AgNP increased size could have significant impact on cellular response. It is known that larger nanoparticles have reduced bioavailability as they are quickly eradicated by the reticulo-endothelial system (Maruyama, 2011), making PB-Lipo-AgNP less practical for in vitro applications as a drug delivery system. PB-Lipo-AgNP exhibited a lower zeta potential -25.9 mV while that of Ex-Lipo-AgNP was -31.9 mV. Nanoparticles with zeta potential value between -30 and
+30 mV are considered less stable owing to the increased agglomeration potential due to reduced repulsion between the particles (Shieh et al., 2012), indicating the Ex-Lipo-AgNP is more stable. In addition to this, the PDI of PB-Lipo-AgNP was found to be higher than that of Ex-Lipo-AgNP in ddH₂O, indicating that Ex-Lipo-AgNP are of more uniform size compared to PB-Lipo-AgNP.

The UV-Vis spectra analysis of free AgNP conformed with reported SPR characteristic of a 20 nm AgNP which is at 400 nm (Zuber et al., 2016), in a way confirming the DLS size of 21 nm. UV-Vis spectra analysis of both PB-Lipo-AgNP and Ex-Lipo-AgNP also allude to the success of the encapsulation process. PB-Lipo-AgNP and AgNP had a similar spectra profile with same λmax although PB-Lipo-AgNP spectra exhibited a broadened peak with a raised baseline and a red shift in the λmax, which are indicative of agglomeration/size increase. The λmax also indicates free AgNP that are not successfully encapsulated absorbing UV emission to produce the observed spectrum. In support of this, an overlap in the DLS size value of AgNP with the ddH₂O dispersed PB-Lipo-AgNP observed indicates that the PB-Lipo-AgNP particles in the overlap region is more likely to be uncoated AgNP. Contrastingly, Ex-Lipo-AgNP spectra depicted a flat peak with same baseline as free AgNP which hints at non-agglomeration of the nanoparticle. The spectra observed at 10 µg/ml was similar to that of 1.25 µg/ml of free AgNP indicating less free AgNP that are able to absorb at the UV-Vis wavelength. This observation is also supported by non-overlap of the AgNP and Ex-Lipo-AgNP DLS size values.

Interaction between nanoparticles and culture media proteins is not uncommon based on their surface reactivity. This interaction was monitored through the size and zeta potential of the liposomes in RPMI-1640 medium. There was increase in the size of PB-Lipo-AgNP and drastic reduction in its zeta potential. The dramatic increase in
PB-Lipo-AgNP size in RPMI-1640 could be due to the AgNP on the surface interacting with the proteins in the culture medium as also observed for free AgNP. This is in agreement with the findings of Sabuncu et al. (2012) who also reported an increase in gold nanoparticle size and decrease in the zeta potential when dispersed in foetal calf serum (FCS) supplemented DMEM culture. This is supported by the DLS overlay of PB-Lipo-AgNP in ddH$_2$O and RPMI which indicates increase in size of the nanoparticle from dispersion in ddH$_2$O to RPMI medium. On the contrary, there was a considerable drop in the zeta potential of Ex-Lipo-AgNP, with only a small increase in the percentage of nanoparticles with increased size (14%). This could mean that Ex-Lipo-AgNP do not readily react with proteins in the culture medium, resulting in no net increase in the size after dispersion in FBS containing RPMI-1640 medium. Interestingly, the charges on the protein amino acids may have a masking effect on Ex-Lipo-AgNP zeta potential. The spectra characteristic of Ex-Lipo-AgNP was less similar to that of AgNP, although with a red shift in $\lambda_{\text{max}}$ at 410 nm. Taken together with the similar baseline as free AgNP and the low absorbance at $\lambda_{\text{max}}$ which is about 50% less than that of free AgNP and PB-Lipo-AgNP, the shift is likely due to the increase in size contributed by the liposome. This also shows that the AgNP is bound to the liposome assuming a larger size than prior to encapsulation such that less AgNP particles are available to interact with proteins in the RPMI media and absorb UV emission. In a study investigating the use of AgNP as biosensor, a red shift in the spectra of a 19 nm AgNP was reported to be consequent upon the binding of the nanoparticle to protein ligands present on the biosensor platform (Liao et al., 2009), explaining why there was no considerable change in the Lipo-AgNP size in the media. In temperature dependent study, it was noted that the PB-Lipo-AgNP size decreased by more than half at 37$^\circ$C whereas Ex-Lipo-AgNP only decreased in size by about a
quarter of the original size. The reason for reduction in their sizes with increased temperature is not known, but this could be as a result of the increased fluidity of the lipid bilayer at temperature close to the transition temperature. Increased fluidity could result in the movement of the liposomal water content out of the liposome into the more concentrated culture medium by osmosis. A previous report indicated liposome often lose their aqueous content when dispersed in medium of high osmolarity (Monteiro et al., 2014), such that water moves from region of lower concentration to region of higher concentration through the lipid bilayer. As such, Ex-Lipo-AgNP appeared to be more stable with respect to its ability to retain its content at 37°C. The stability study over a 6-month period also indicated Ex-Lipo-AgNP to be more stable with minimal overall increase in size and zeta potential at both 4°C and 24°C compared to PB-Lipo-AgNP which was also found to sediment unlike the Ex-Lipo-AgNP that remained clear.

Encapsulation of AgNP in liposome here was carried out with the intent of improving its cytotoxicity as a chemotherapeutic agent. Hence, it became pertinent to carry out drug release studies. Considering the possible route of administration and target site for the encapsulated AgNP, pH of 7.45 which is the physiologic pH and most culture media (relevant for in vitro studies) and pH 6.5 which is known to be the pH of the tumour microenvironment and inflamed tissue (Ueno et al., 2008, Huber et al., 2017, Som et al., 2016), were considered. One of the major problems associated with drug delivery systems is the initial burst release which is associated with an initial hypertoxicity and suboptimal concentration of the drug at the time it reaches the target. A good drug delivery system is expected to protect the drug against the harsh physiological environment of immune cells, minimize the burst release and maintain a steady release of the drug for optimal concentration to achieve maximum efficacy
over a period. Findings in this study, showed that PB-Lipo-AgNP possesses initial burst release at pH 6.5 and 7.45. Ex-Lipo-AgNP exhibited and maintained a steady release of AgNP at pH 6.5 with significantly lower release compared to PB-Lipo-AgNP. At 24 h, the two systems have released similar concentration of AgNP. At physiologic pH of 7.45, PB-Lipo-AgNP had already released 12.5% of the encapsulated AgNP compared to 3.5% of Ex-Lipo-AgNP. Initial burst release has been demonstrated for Ag\(^+\) coated with titanium dioxide used as an antibacterial for *Staphylococcus aureus* (Jamuna-Thevi et al., 2011). Although it was found that this rapid release produced an effective antibacterial effect, this effect can be quite adverse in an *in vivo* model.

Initial burst release has been proposed to occur consequent upon rapid dissolution of weakly or poorly encapsulated drugs that might be attached to the surface of the delivery systems (Rivadeneira et al., 2014, Hua et al., 2014, Singh and Lillard, 2009, Tan et al., 2014). This supports the deduction from UV/Vis spectra features of PB-Lipo-AgNP to weakly encapsulate AgNP with some free AgNP attached to the surface of the liposome as also depicted in the STEM image. Contrastingly, the finding here indicated that Ex-Lipo-AgNP can maintain steady AgNP release at both pH 6.5 and 7.45. The advantage is that the absence of initial burst release of Ex-Lipo-AgNP prevents initial hypertoxicity. On the other hand, while Ex-Lipo-AgNP had significantly less drug release at 24 h compared with PB-Lipo-AgNP at pH 7.45, stability of Ex-Lipo-AgNP may facilitate better drug delivery with better net cytotoxicity. In support of the finding for Ex-Lipo-AgNP however, Ruttala and Ko (2015), showed that a liposomal anti-tumour agent with steady load release exhibited enhanced cytotoxicity.
The uncertainty that encapsulation of AgNP translates to enhanced and improved cytotoxicity led to the investigation of the cytotoxicity of PB-Lipo-AgNP and Ex-Lipo-AgNP on THP1, a leukemic cell line in the monocytic lineage. The choice of the cell line for this study is three-folds. Firstly, THP1 is a leukemic (cancer) cell line, allowing investigation of the cytotoxic effect of AgNP encapsulation on a cancer cell line. Secondly, monocytes and similar immune cells act as first line of defence in response to foreign objects including nanoparticles upon human exposure (Mrakovcic et al., 2014, Rueda-Romero et al., 2016, Robbins et al., 2015), making the cell line a perfect model to also study the effect of the nanoparticle on the innate immune system. In addition to this, due to the role of monocytes in diseases such as atherosclerosis and cancer (Lameijer et al., 2013), this cell line is a potential therapeutic targets in treatment of these diseases.

Upon exposure of THP1 monocytes to the different nanoparticles, it was discovered that Ex-Lipo-AgNP induced significantly higher cytotoxicity at lower concentrations compared with PB-Lipo-AgNP and free uncoated AgNP exposed cells. In addition, flow cytometry and confocal microscopy analyses both confirmed Ex-Lipo-AgNP to be more cytotoxic compared to PB-Lipo-AgNP and free uncoated AgNP. There was a significantly higher live cells and less dead cells in the control-untreated, free uncoated AgNP, and PB-Lipo-AgNP exposed cells groups compared to Ex-Lipo-AgNP exposed cells. Another observation was the speckled fluorescence observed in both PB-Lipo-AgNP and Ex-Lipo-AgNP exposed cells but not the control-untreated cells. This is likely due to the loss of membrane integrity upon exposure to the nanoparticles resulting in leakage of calcein from the cytoplasm. Foged et al. (2008) have previously showed that disruption of the cell membrane can result in leakage of calcein.
The enhanced cytotoxicity of Ex-Lipo-AgNP in comparison to AgNP or PB-Lipo-AgNP may be attributed to its superior characteristics and enhanced delivery. This may have been facilitated by the hydrophobic interaction between the lipid bilayer of the cell membrane and that of the liposome encapsulating the AgNP. On the other hand, the slightly enhanced cytotoxicity of the PB-Lipo-AgNP may be because of less encapsulated AgNP and lower endocytosis due to larger size in culture media. This reason may also explain why Ex-Lipo-AgNP enhanced delivery into the cells since its size may have remained unchanged even when reconstituted in culture media. Lastly, flow cytometry detected more cellular debris in PB-Lipo-AgNP exposed THP1 cells than in other exposure groups. These debris were due to the PB-Lipo-AgNP which were larger in size and similar to left over of apoptosed cells. Unfortunately, this identified debris are counted as events in the cytometer, imposing a confounding effect on the number of viable cells that will be analysed. Interestingly, Ex-Lipo-AgNP does not exhibit such anomaly, further alluding to the stability and superior characteristic liposome obtained through the extrusion as compared with that obtained from probe sonication. Taken together, encapsulation of AgNP in DPPC based liposome may help limit the concentration of AgNP used in the various biomedical applications to achieve better cytotoxicity resulting in less human exposure and mitigation of any development of adverse effects.

3.10 Conclusion

Stable AgNP were successfully synthesized at a suitable concentration without the need for stabilizer. Synthesized AgNP were successfully encapsulated in liposome for the first time by both probe sonication and extrusion methods. However, the extrusion method produced a more stable liposome both when dispersed in ddH$_2$O and in culture medium. The spectra analysis confirms probe sonication produced a less successful
encapsulation based on the similarity between PB-Lipo-AgNP and AgNP spectra characteristics. Ex-Lipo-AgNP on the other hand had a different spectra analysis which is believed to be as a result of the shielding effect of the liposome bilayer. In addition, Ex-Lipo-AgNP exhibited a more controlled AgNP release compared with the PB-Lipo-AgNP which showed an initial burst release. Cell viability studies indicated that Ex-Lipo-AgNP exhibited higher cytotoxic effect in comparison to PB-Lipo-AgNP and uncoated AgNP at similar concentrations. This may have been due to the stable characteristic of Ex-Lipo-AgNP facilitating an effective delivery of the nanoparticle into the cell. As such, extrusion method offers a more reliable way for encapsulating AgNP in liposome with repetitive characteristics and enhanced cytotoxicity. This provides with potential of achieving cytotoxicity at lower concentrations compared to those currently in application limiting possible exposures.
Chapter 4
Silver nanoparticles (AgNP) are one of the most crucial metals in nanomedicine/nanotechnology. They have been investigated in the treatment of bacterial diseases and are found to possess a striking antimicrobial activity. AgNP are known to cause oxidative lesion that destroys bacterial cell wall, facilitating its entry into the cell to bind to sulphydryl groups of key proteins and bacterial DNA to disrupt crucial metabolic processes and halt cell proliferation (Feng et al., 2000, Wigginton et al., 2010, Grigor'eva et al., 2013). This has also led to the worldwide commercialisation of AgNP in an array of formats with up to 24 % of nanotechnology driven everyday consumer products worldwide containing AgNP (Vance et al., 2015).

Some of the AgNP based consumable products include but are not limited to food packaging, antibacterial creams, coatings in antimicrobial textiles and domestic cleaning products. AgNP are widely used in medicine for dressing wounds, in making orthodontic materials, coating of bone prosthesis, coating of stents or catheters, cleaning surgical equipment and in certain contraceptive devices (Blaske et al., 2013, Correa et al., 2015, Knetsch and Koole, 2011, Samuel and Guggenbichler, 2004). In recent years, research involving AgNP has moved beyond investigating its antibacterial properties. The possibility of AgNP as an anticancer drug has been the subject of research due to the emergence of drug resistant cancer cells and the discovery that AgNP possess an inherent cytotoxic effect on cancer cells (Zhang et al., 2016).

With increasing applications of AgNP in consumable products and in medicine there is an increased risk of exposure to the toxic side effects of AgNP that may have leached
into the environment. (Benn and Westerhoff, 2008) showed that a sock coated with about 1.36 mg of AgNP can release 48 % of its coating into water by simply shaking the sock in water or as much as 100 % in four consecutive washes. AgNP can also be released in the same manner from shampoos, toothpaste, detergents and medical apparels (Benn et al., 2010). It has been reported that AgNP route of entry could be through oral, pulmonary or dermal routes. Irrespective of the entry route, AgNP induced cell death through the generation of ROS, increased DNA damage and impairment of mitochondrial functions (Kang et al., 2012). As such, continuous use of AgNP at these high concentrations comes with a potential danger in the near future. For instance, inefficient delivery of AgNP to target cells could result in systemic toxicity as it has been previously reported that prolonged bio-distribution of AgNP could result in systemic transport to different tissues especially to the spleen and liver (Xue et al., 2012). As such, devising ways by which low concentrations of AgNP can be efficiently delivered to target cells thus becomes pertinent. Generally, the unspecific systemic activities of conventional anticancer drugs are the major limiting factor in their application as chemotherapeutics. To subvert this negative side effect, encapsulation of drugs in lipid bilayer has been investigated for improved delivery with promising results (Sercombe et al., 2015). For example, doxorubicin is a potent anticancer drug that inhibits topoisomerase II preventing accessibility of DNA polymerase to the DNA. Regardless of its potency, there is limitation to the use of doxorubicin in cancer treatment due to the negative side effects following its administration such as alopecia, hepatotoxicity, cardiotoxicity and nephrotoxicity (Tacar et al., 2013). However, liposomal encapsulation of doxorubicin has been shown to improve its delivery in addition to alleviating the negative side effects of the drug,
reducing the required concentration to achieve cell death (Brown and Khan, 2012, Camacho et al., 2016, Souto et al., 2016).

Liposomes like many other nanoparticles have unique characteristics that make them efficient drug delivery vehicles. They are suitable for transporting both hydrophobic and hydrophilic molecules, the lipid layer of liposomes can entrap hydrophobic drugs due to hydrophobic interactions between the fatty acid chains and the drug while the aqueous core of the liposome can also hold hydrophilic drugs (Bozzuto and Molinari, 2015). In general, liposomes are often prepared from phospholipids which then form the outer layer of the nanostructure that can be similar to the lipid bilayer of the cell membrane. The lipid layer of liposomes aids the passive transport of the liposome and its associated content into the cell. This prevents the requirement of membrane channels or pumps normally controlling the entry of charged or polar molecules such as peptides, metals and synthetic compounds that can be encapsulated in the lipid vesicle. These attributes allow for dose intensification of compounds which are encapsulated within the liposome while limiting any negative side effects associated with excess free drug present in a non-liposome delivery method (Silverman et al., 2013). Liposomes have controllable sizes and their surface properties are easily modified in the synthesis process and can be tailored to any functional delivery requirements. These properties coupled with an enhanced permeability and retention (EPR) effect in vivo, increases their ability to accumulate in a tumour microenvironment, which is typically composed of leaky vessels and abnormal cell junctions (Nehoff et al., 2014, Xing et al., 2016).

In this study, AgNP were encapsulated in a liposome made of natural bio-surfactant, DPPC to improve delivery due to increased permeabilisation into the target cells. It is hoped that such a system will not only reduce the non-specific cytotoxic effect
observed during AgNP treatment, but it may also reduce the concentration of AgNP required to elicit the expected cytotoxic response. Subsequently, this may help reduce the development of resistant strains to AgNP based drug as well as eliminate the release of AgNP in large quantities into the ecosystem.
4.1 Results

4.1.1 Particle characterisation

It was demonstrated in Chapter 3 that extruded Lipo-AgNP is more stable than the probe sonicated Lipo-AgNP with more superior characteristics. Hence, the extruded Lipo-AgNP was used in subsequent studies. A summary of the synthesised nanoparticles characterisation using DLS is shown in Table 4-1 for different dispersion environments. DLS analysis of AgNP showed a dramatic increase in mean particle size when dispersed in RPMI-1640 media when compared to dispersion in ddH₂O from 21.24 nm to 79.15 nm respectively. This increase in size was accompanied by a change in colour of the AgNP when dispersed in RPMI-1640 from the usual golden yellow colour to dark grey colour. In contrast, there was no significant change in the mean particle size of Lipo-AgNP when dispersed in RPMI-1640 compared to ddH₂O and no colour change when dispersed in RPMI-1640 media. There was an observable increase in polydispersity index (PDI) of the AgNP (PDI = 0.23 to 0.566) and the Lipo-AgNP (PDI = 0.105 to 0.421) when dispersed in water compared to dispersion in RPMI-1640 media. However, no sedimentation was observed for the AgNP when dispersed in RPMI-1640 likely due to the concentration used. There was a measurable drop in zeta potential values for AgNP (zeta = -26.5 mV to -7.9 mV) and Lipo-AgNP (zeta = -31.9 mV to -0.61 mV) when dispersed in ddH₂O to RPMI-1640 media, indicating a less stable nanoparticle in RPMI-1640 media.

As shown in Figure 1B, EDX analysis confirmed the presence of elemental AgNP in the sample as the strong peak at 3 keV. The other peaks observed correspond to other elements due to sample preparation, the substrate (Si wafer) and carbon tab for conductive support. Result from XRD characterisation of AgNP is shown in Figure 1C. The XRD pattern shows four main peaks for AgNP at 2θ values of 38.30°, 44.55°, 77.41°, and 130.86°.
64.60° and 77.55° which corresponds to (111), (200), (220) and (311) planes respectively for the face centred cubic (fcc) structure of metallic silver. This pattern is in agreement with that of the JCPDS file no. 04-0783 confirming the crystalline structure of the synthesised AgNP (Luna et al., 2016). The average size of synthesised AgNP was estimated by the Debye–Scherrer’s equation (Equation 3)

\[ D = \frac{0.9\lambda}{\beta \cos \theta} \] ----> Equation 3

Where D is the size of AgNP crystal in nm, \( \lambda \) is the wavelength of the diffractometer, \( \beta \) is the full width at half maximum intensity and \( \theta \) is the Bragg angle. Based on the equation, the size of AgNP crystal was estimated to be 18.7 nm which was similar to that of the average size as determined by DLS measurement of 21.24 nm.

**Table 4-1. Size and Zeta potential of AgNP, lyophilised and extruded Lipo-AgNP nanoparticles in ddH₂O and RPMI-1640 media**

<table>
<thead>
<tr>
<th></th>
<th>In ddH₂O</th>
<th>In media</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intensity PSD</td>
<td>Intensity PSD</td>
</tr>
<tr>
<td><strong>AgNP</strong></td>
<td>DLS (nm)</td>
<td>21.14±9.48</td>
</tr>
<tr>
<td></td>
<td>Zeta (mV)</td>
<td>-26.50</td>
</tr>
<tr>
<td></td>
<td>PDI</td>
<td>0.230</td>
</tr>
<tr>
<td><strong>Extruded</strong></td>
<td>DLS (nm)</td>
<td>140.1±47.49</td>
</tr>
<tr>
<td><strong>Lipo-AgNP</strong></td>
<td>Zeta (mV)</td>
<td>-31.9</td>
</tr>
<tr>
<td></td>
<td>PDI</td>
<td>0.105</td>
</tr>
</tbody>
</table>

SEM and STEM were used to evaluate the morphology of the nanoparticles and to estimate the size distribution values of the nanoparticles in dry state. The STEM analysis of AgNP showed all particles produced were spherical with an average size of 14.3 ± 1.9 nm which is similar to that estimated by the XRD analysis (Figure 4-1A).
SEM analysis of Lipo-AgNP showed spherical and uniformly extruded vesicles while the contrasting image of the STEM showed encapsulation of the AgNP in the liposome with an average size of 82.73 ± 29.23 nm (Figure 4-2A and B) which displays size values less than the DLS values which considering the hydrodynamic radius of the nanoparticles was expected (Table 4-1). To confirm the encapsulation, DLS values of both AgNP prior to and after encapsulation were overlaid to determine if any unencapsulated AgNP were present in the test sample (Figure 4-2C). As expected, there was no overlap in the size values showing successful encapsulation of AgNP.
Figure 4-1. AgNP characterisation by STEM, EDX and XRD

Characterisation of nanoparticles showing (A) STEM (B) EDX analysis of AgNP on a Si wafer (C) XRD pattern of AgNP synthesised by chemical reduction method.
Figure 4-2. Lipo-AgNP characterisation by SEM/STEM and DLS

Characterisation of nanoparticles showing (A) STEM (B) SEM and (C) Superposition of AgNP DLS analysis before and after encapsulation in extruded liposome.
4.1.2 Encapsulation efficiency
Encapsulation efficiency of Lipo-AgNP was estimated based on Equation 2. A 10 µg/ml AgNP solution (representing total AgNP added) was used to rehydrate the lipid film and the liposome was prepared as described above. The encapsulation efficiency of the different Lipo-AgNP preparations (Lipo-AgNP-L1 to L3) are shown Table 4-2 was between 85 and 89 % of the total AgNP added prior to encapsulation.

Table 4-2. Encapsulation efficiency of Lipo-AgNP

<table>
<thead>
<tr>
<th></th>
<th>Lipo-AgNP-L1</th>
<th>Lipo-AgNP-L2</th>
<th>Lipo-AgNP-L3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total AgNP (µg/ml)</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>AgNP in Supernatant (µg/ml)</td>
<td>1.13</td>
<td>1.50</td>
<td>1.43</td>
</tr>
<tr>
<td>Encapsulation efficiency</td>
<td>88.7 %</td>
<td>85.0 %</td>
<td>85.7 %</td>
</tr>
</tbody>
</table>

4.1.3 Cellular viability study
The cell viability of THP1 cell line was evaluated by AB assay. The assay evaluates the ability of metabolically active cells to reduce resazurin, a non-fluorescent blue dye to resorufin, a pink fluorescent product. Reduction of resazurin is done by both mitochondrial and cytoplasmic reducing agents such as NADPH, NADH, FMNH and FADH as such the assay gives a broad indication of cellular viability (Rampersad, 2012). THP1 cells were exposed to varying concentrations of AgNP and Lipo-AgNP (0.3 – 5 µg/mL) for 24 and 48 h. To evaluate possible ionisation of AgNP into Ag⁺, THP1 cells were exposed to same concentration of Ag⁺ for 24 h. At 24 h, there was significant reduction in THP1 cell viability treated with 2.5 µg/mL of Lipo-AgNP and cells treated with 5 µg/mL AgNP and Lipo-AgNP when compared with the unexposed control cells (Figure 4-3A). Interestingly, the cytotoxic effect of Ag⁺ was 3-fold that
of AgNP, showing a highly significant reduction in THP1 cell viability at concentrations 0.625 to 2.5 µg/ml and 2-fold reduction at 5 µg/ml after 24 h. Ag⁺ induced significant reduction in THP1 cell viability when compared with AgNP at concentrations ≥ 0.625 µg/mL. The IC₅₀ of AgNP, Lipo-AgNP and Ag⁺ at 24 h was estimated to be 4.991 µg/mL, 3.045 µg/mL and 0.3226 µg/ml respectively. At 48 h, there was significant reduction in cell viability for all exposure groups and concentration when compared to control. Interestingly, it induced a significant reduction in THP1 cell viability by 2.5 µg/mL of Lipo-AgNP compared to the AgNP at the same concentration (Figure 4-3B). Cell viability for Ag⁺ was not carried out at 48 h as ≤ 20 % cell viability was already observed at 24 h. To ensure the cytotoxic effect of Lipo-AgNP was due to the encapsulated AgNP and not the liposome, THP1 cells were exposed to empty liposome at same concentration present in 1.25 and 2.5 µg/ml or Lipo-AgNP. THP1 cell viability after 24 h exposure showed no significant difference when compared with unexposed THP1 cells (Figure 4-4).

To verify the AB findings, the levels of THP1 cell viability after exposure were monitored by flow cytometry. Flow cytometry is a single cell analysis method and as a result it gives a better indication of the exact viability levels in the exposures when compared to the AB assay. THP1 cell death was evaluated by initially staining the cells with calcein-AM and PI 24 h after being exposed to 0.625 µg/mL of AgNP and Lipo-AgNP. Compared to an unexposed control as with the previous viability measurements, 10 % DMSO medium solution was used as positive control.

From the results obtained, there was no significant difference in the percentage of live cells between the control (98.35 %) and AgNP treated cells (98.55 %) after 24 h. A highly significant difference in the percentage of live cells treated with Lipo-AgNP (66.75 %) was discovered in comparison with AgNP (Figure 4-5). In both control-
unexposed and AgNP-treated cells, only 0.5 % cell death was observed after 24 h. Contrarily, Lipo-AgNP treatment induced cell death in 32.5 % of THP1 cells, a highly significant increase in cell death compared with both control-unexposed and AgNP-treated THP1 cells after 24 h. This result suggests Lipo-AgNP is capable of inducing cell death at lower dose than that established by AB assay.

Figure 4-3. Cell viability of THP-1 cells post exposure to AgNP and Lipo-AgNP (a) AgNP, Lipo-AgNP and Ag⁺ exposed cells at 24 h and (b) AgNP and Lipo-AgNP at 48 h as determined by the Alamar Blue assay. Data is expressed as a percentage of three independent experiments ± SEM of the 4 individual experiments and relative to a 2D culture control. Statistically significant differences between the exposed viability
responses and that of the control cultures are denoted by * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

Figure 4-4. Cell viability of empty liposome exposed cells at 24 h as determined by the Alamar Blue assay. Data is expressed as a percentage of three independent experiments ± SEM of the 4 individual experiments and relative to a 2D culture control.

Figure 4-5. Lipo-AgNP induced THP1 cell death.

Control THP1 cells, and cells treated with 0.625 μg/mL of AgNP and Lipo-AgNP and 10 % DMSO were incubated for 24 h and were washed in PBS before staining with
10 μg/mL PI and 10 nM calcein-AM. Data represent mean percentage ± SEM (n = 3). **** p < 0.0001.

4.1.4 Cell cycle analysis

THP1 cells were exposed to 0.625 μg/mL of AgNP and Lipo-AgNP while unexposed cells were used as control. Cell cycle analysis after 24 h of exposure showed that exposure of THP1 cells to both AgNP and Lipo-AgNP did not affect the cell cycle progression from G1 to S phase and there was no significant difference between the exposed cells to control-unexposed cells. Exposure of THP1 cells to Lipo-AgNP resulted in 21.1 % cells in the G2/M phase of the cell cycle. As expected, AgNP treatment did not show any effect on cells in the G2/M phase having 32.65 % cells comparable to the 30.85 % unexposed-control cells in G2/M phase. Interestingly, it was found that Lipo-AgNP treatment resulted in 8.4 % cells in the SubG1 phase compared with 0.65 % and 0.8 % cells for both unexposed-control and AgNP treated cells respectively (Figure 4-6). This indicated that Lipo-AgNP at low doses caused DNA fragmentation which was evident by the characteristic of Sub-G1 peak. The percentage of cells in the G0/G1 and S phases remained the same in all groups, suggesting no apparent effect of treatment on cells in all exposure groups.
Figure 4-6. Lipo-AgNP treatment induced DNA fragmentation

THP1 cell lines were treated with 0.625 μg/mL of Lipo-AgNP and AgNP while unexposed cells and 10 % DMSO treated cells were used as controls. Data represent mean percentage ± SEM (n = 3), *p < 0.05
4.1.5 ROS induction

DNA fragmentation is one of the consequences of ROS generation prior to apoptosis. To determine if the low dose Lipo-AgNP was inducing generation of ROS at sufficient levels to induce DNA damage, ROS levels were monitored in THP1 cells exposed to 0.3 µg/mL Lipo-AgNP at varying time points indicated in Figure 4-7 alongside an unexposed-control and uncapped AgNP in order to plot ROS generation as a function of time. From 30 min to 5 h, there was an average of 1.5-fold induction of ROS in cells exposed to 0.3 µg/mL of AgNP compared to control cell ROS. Cells exposed to the same concentration of Lipo-AgNP showed mild suppression of ROS induction up to 4 h, which significantly increased to approximately 3 folds of control ROS at 24 h. This indicates that ROS generation was not responsible for the observed cell death in THP1 cells exposed to 0.3 µg/mL of Lipo-AgNP.

![Graph showing ROS induction over time for AgNP and Lipo-AgNP](image)

**Figure 4-7.** ROS generation post exposure to AgNP and Lipo-AgNP

THP1 cell lines were loaded with 10 µM carboxy-DCFDA dye for 20 min: After which they were exposed to 0.3 µg/mL AgNP and Lipo-AgNP for up to 4 h. Control value was set as baseline for all time point and represented as mean ± SEM (n = 3).
4.1.6 Caspase activation

ROS and DNA damage are known to be indicators of apoptosis and to verify this was the mechanism of action utilised by Lipo-AgNP, the CellEvent Caspase-3/7 Green Detection Reagent and SYTOX AADvanced Dead Cell Stain were employed. THP1 cells exposed to 0.625 μg/mL of AgNP and Lipo-AgNP were stained after 1 h, 4 and 24 h of exposure. The result showed basal caspase activities at 1 hr and 4 h in all exposure groups with an average of 2 % of the total cell population exhibiting caspase activities. After 24 h of exposure, the level of caspase activation remained the same for control-unexposed cells and AgNP-treated cells. Conversely, 33 % of total THP1 cells exposed to Lipo-AgNP were positive for caspase activation at 24 h (Figure 4-8). This result suggested the cell death observed in Lipo-AgNP exposed cells was likely a consequence of caspase-3/7 activation.

Figure 4-8. Lipo-AgNP activates caspase-dependent apoptosis in THP1 cells after 24 h

THP1 cells that were unexposed, treated with 0.625 μg/mL of AgNP, 0.625 μg/mL of Lipo-AgNP and 1 μM Doxorubicin were stained with 500 nM CellEvent™ for 30 min
and co-stained with 1 µM Sytox AADvanced during the last 5 min. Data is represented as mean ± SEM (n = 3), **** p < 0.0001.

4.2 Discussion

Currently AgNP are the main active ingredient in multiple nano-enabled commercial products worldwide, primarily due to their antimicrobial activities. Despite this, several studies have investigated and identified the cytotoxicity related hazards associated with AgNP (Connolly et al., 2015, Nowrouzi et al., 2010, Zhang et al., 2014). Interestingly, the increasing investigation of AgNP cytotoxicity has now moved beyond antibacterial applications and recent studies are investigating the potential of AgNP as anticancer drugs. This shift is partially due to the evolution of multidrug resistant cancer cells similar to that of antibiotic resistant bacteria. Foldbjerg et al. (2012) noted that AgNP at an EC$_{20}$ of 12.1 µg/mL showed potential anticancer properties such as induction of ROS, modulation of cell division and growth regulatory gene expressions such as cyclin B1 and histone H1B and the arrest of cells in the G2/M phase. Lin et al. (2014) showed that 10 µg/mL of AgNP produced enhanced cytotoxicity in HeLa and B16 mouse melanoma cell lines that have undergone chemotherapeutic or genetically induced autophagy inhibition by preventing formation of anti-apoptotic autophagosomes normally induced by AgNP in these cell lines. Asharani et al. (2012) showed that 400 µg/mL of AgNP induces activation of γH2AX foci, suppresses expression of key cell cycle proteins such as p21, p53 and cyclin B, and induces expression of DNA damage response genes RPA1 and FEN1. Several other studies evaluating the antibacterial properties and mechanism of action of AgNP have shown the minimum inhibitory concentration of AgNP from 10 µg/mL to as high as 180 µg/mL for different bacteria strains (Bao et al., 2015, Guzman et al., 2012, Amato et al., 2011, Maiti et al., 2014, Hsueh et al., 2015).
Nonetheless, all of these studies have investigated AgNP activities at high concentrations which are likely to be detrimental to human health, thus limiting the potential therapeutic applications. Hence, this necessitates development of delivery systems capable of delivering AgNP intracellularly at a lower concentration to achieve the desired pharmacological response.

In this study, an in-house synthesised AgNP was encapsulated to improve the \textit{in vitro} delivery of the AgNP to enhance their \textit{in vitro} effects and simultaneously reduce the dose required. Nanoparticles are found in bodily tissues and fluid after entering the body through different routes such as oral, intravenous, transdermal or inhalation (Yildirim et al., 2011). The use of THP1 in the study was to mimic such presence in the blood system as a result of relocation in a diseased condition and as an enhanced delivery mechanism for cancer. There are multiple reports on the effect of nanoparticle size on drug delivery and cellular internalisation. Nanoparticles of larger sizes (< 500 nm) are, in addition to hepatic uptake, more prone to clearance by the reticuloendothelial system resulting in reduced bioavailability and pharmacological potential (Alexis et al., 2008, Omar Zaki et al., 2015). Both the DLS and SEM/STEM characterisation showed the sizes of the AgNP and Lipo-AgNP to be below 30 nm and 200 nm respectively. In general, DLS values were slightly higher than that of the SEM values and this was as expected as the DLS is an intensity measurement that tracks the hydrodynamic radius of the solvated nanoparticle while SEM/STEM imaging is a physical snapshot of the dry particles with no influence from surrounding solvent. As such, the hydrodynamic radius which includes the aqueous layer tends to be slightly larger than dry particle radius. The XRD pattern analysis using the Debye-Scherrer equation estimated a size of 18.7 nm and peak pattern that is characteristic of an fcc crystalline structure. This finding is in agreement to those of other studies further
confirming the sample as metallic silver (Anandalakshmi et al., 2016, Bindhu and Umadevi, 2015). However, there are two peaks around 26° and 30.7° that are not characteristic of silver. These peaks may be generated by the NaBH₄ reducing agent used in chemical reduction of AgNO₃ and it is similar to those reported in other studies for NaBH₄ (Su et al., 2012, Agnihotri et al., 2014).

Several studies have noted particle size changes of AgNP in cell culture environments; as such the size distributions of AgNP and Lipo-AgNP in culture media were monitored and compared to those in water. AgNP size increase from 21.14 nm to 79.15 nm was observed in media compared to when dispersed in water. This size increase was presumed to be an indication of nanoparticle agglomeration in the solution. In contrast, this was not the same for Lipo-AgNP where there was a slight reduction in recorded sizes (140.1 nm in water and 138.9 nm in media). Previous studies have reported an increase in AgNP size in culture media and this increase in size has been proposed to be due to association of AgNP with proteins (Hansen and Thunemann, 2015, Shannahan et al., 2013, Murphy et al., 2015a, Mukherjee et al., 2012). The interaction of AgNP with protein in culture media was most likely prevented by Lipo-AgNP due to the presence of the protective lipid layer, preventing the encapsulated AgNP from directly interacting with biomolecules in the milieu of the culture media. Additionally, there was an increase in PDI values for AgNP in both water and RPMI-1640, the difference in these PDI values for AgNP was higher than that of Lipo-AgNP. PDI value increases indicate an increased non-uniformity of the nanoparticles when they were dispersed into the culture media. These findings suggest encapsulation and formation of the Lipo-AgNP suppresses the changes in morphology of AgNP in culture media and ultimately improves their stability in comparison to standard AgNP.
After dispersion, cellular viability levels of THP1 cells exposed to varying concentration of AgNP and Lipo-AgNP (0.3-5 µg/mL) for 24 h and 48 h were monitored with the AB assay. The results showed a dose-dependent reduction in cell viability with a greater level of cytotoxicity observed for the Lipo-AgNP treated cells. A significant THP1 cell viability reduction was observed at 2.5 µg/mL Lipo-AgNP treatment ($p < 0.05$) and interestingly not for AgNP of the same concentration after 24 h. In contrast, there was a significant reduction in cell viability of THP1 cells treated with 2.5 µg/mL Lipo-AgNP compared to AgNP of the same concentration at 48 h ($p < 0.0001$), suggesting Lipo-AgNP were more cytotoxic than AgNP. AgNP only induced significant reduction in cell viability at 5 µg/mL and ≥ 2.5 µg/mL at 24 and 48 h respectively when compared to control. This finding is in agreement with those of other studies that investigated dose-dependent cytotoxicity of AgNP on different human cell lines. (Jiang et al., 2013) reported significant reduction of Chinese Hamster Ovary (CHO-k1) cell viability at concentrations ≥ 5 µg/mL at 24 h using MTT assay. (Juarez-Moreno et al., 2016) in their study reported similar AgNP IC$_{50}$ to that of this study in addition to significant reduction in cell viability at AgNP concentration ≥ 1.25 µg/mL for eight different human cancer cell lines where viability was monitored with the MTT assay. Variations in the cytotoxicity of AgNP in these cell lines could be attributed to the difference in sensitivity of the cell lines to the nanoparticle but also differences in assays employed to monitor viability. Interestingly, (Murphy et al., 2016) who used THP1 cells reported dose dependent cytotoxicity of AgNP (50-70 nm size) with significant difference from ≥ 1.9 µg/mL. This contrasted with the finding here and could be due to polyvinyl pyrrolidine (PVP)-coated AgNP used in the study, which may have its own inherent toxicity. Of course, it may be because of a difference
in particle sizes, which have been previously shown to mediate cytotoxicity irrespective of capping agent used (Gliga et al., 2014).

AgNP ionisation into Ag\(^{+}\) has been widely proposed as its mechanism of action in inducing cytotoxicity (Hsueh et al., 2015, Lansdown, 2010). As such, the cytotoxicity of Ag\(^{+}\) on THP1 cells was investigated and findings indicated that Ag\(^{+}\) at concentrations between 0.625 and 2.5 µg/ml reduced THP1 cell viability by more than 3-folds while 2-fold reduction in cell viability was observed for THP1 exposed to 5 µg/ml Ag\(^{+}\) when compared with AgNP at the same concentrations. This finding is in agreement with that of (Foldbjerg et al., 2009) who used flow cytometric analysis to determine viability of THP1 cells after exposure to AgNP and Ag\(^{+}\). Their findings showed at least 2-fold reduction in THP1 cell viability after exposure to ≥ 0.625 µg/ml Ag\(^{+}\). There was a similar concentration-dependent cytotoxicity profile for both AgNP and Ag\(^{+}\) although Ag\(^{+}\) had higher toxicity. Taken together, ionisation of AgNP into Ag\(^{+}\) is likely responsible for the cytotoxic effect of AgNP. The lower cytotoxic effect of AgNP may be as a result of its slow ionisation rate as it has been reported that AgNP ionisation rate correlates with its concentration (Maurer-Jones et al., 2013). This is also supported by the reduction in Ag\(^{+}\) cytotoxic effect to 2-fold relative to that of AgNP at 5 µg/ml. This may be consequent upon increased ionisation of AgNP with increased concentration. In addition, the steady reduction in THP1 cell viability after 48 h of exposure to 0.625 to 2.5 µg/ml of AgNP also supports this notion.

To verify the results of the AB assay, flow cytometry, which is a more sensitive technique, was employed to evaluate cell viability after exposure to THP1 cells with AgNP and Lipo-AgNP. Calcein-AM and PI staining of THP1 cells after 24 h of treatment with 0.625 µg/mL Lipo-AgNP showed induction of significant amount of death in the cells \((p < 0.0001)\). Conversely, AgNP treatment showed a comparable
outcome to control-unexposed cells with little or no cell death observed, verifying the AB assay results that the Lipo-AgNP exhibited a higher level of cytotoxicity that the AgNP alone.

Several studies have shown that AgNP can initiate cell cycle arrest at different phases of the cell cycle including the G0/G1, S and G2/M phases (Asharani et al., 2012, De Matteis et al., 2015, Eom and Choi, 2010). As such the effect of a low dose Lipo-AgNP on THP1 cell cycle was monitored. the finding here showed normal progression of THP1 cells from the G1 to the S-phase for all exposure and control groups. On the contrary, Lipo-AgNP exposure resulted in significant reduction in G2/M cell population compared with the control and AgNP treated cells ($p < 0.05$). In the S-phase, cells that have successfully passed through the G1/S checkpoint replicate their DNA in preparation for progression into the G2/M phase where they divide during mitosis (Takeda and Dutta, 2005). DNA damage in the S phase results in activation of ataxia-telangectasia mutated (ATM) and ataxia-telangectasia related (ATR) kinases which in turn activate CHK2, γH2AX and the BRCA genes to slow down replication through abrogation of origin firing and signal DNA damage for repair. Increased DNA damage however results in induction of apoptosis by ATM, ATR and CHK2 activation of p53 (Norbury and Zhivotovsky, 2004, Willis and Rhind, 2009). A population in the Sub-G1 upon Lipo-AgNP exposure was noted and such populations are known to be cells with fragmented DNA that are undergoing or have undergone apoptosis. A genomic DNA fragmenting effect of AgNP has indeed been previously demonstrated by Awasthi et al. (2013) and may be occurring in this study after exposure to Lipo-AgNP. The proportion of cells observed in the sub-G1 recorded as a result of Lipo-AgNP exposure matches the reduction in the G2/M phase. This may indicate the cells
were forced out of the cell cycle due to the increased DNA damage because of Lipo-AgNP exposure and died via apoptotic mechanisms.

The cell induces apoptosis for many reasons, one of which is to maintain genome integrity by killing cells with high amount of DNA damage. ROS induction is often used to activate the signalling cascade that result in apoptotic induction in such cell. ROS are known to disrupt the mitochondrial membrane which results in the release of cytochrome c. Release of cytochrome c activates the proapoptotic Bcl2 proteins Bax and Bak which in turn activate executioner caspases 3 and 7 (Quast et al., 2013). There are reports in literature indicating that AgNP cause apoptosis by inducing increased generation of ROS. Indeed Kang et al. (2012) and Foldbjerg et al. (2011) reported a dose and time-dependent ROS induced apoptosis in dendritic cell line (DC2.4) and A549 lung cell lines respectively as a result of AgNP exposure. Awasthi et al. (2013) also reported a dose-dependent ROS generation and induction of apoptosis in CHO cell lines. To monitor if this was occurring here as a result of the Lipo-AgNP exposure, ROS generation was monitored in THP1 cell lines. The cells were treated with a low concentration of AgNP and Lipo-AgNP (0.3 µg/mL) for up to 4 h to evaluate the onset of ROS generation. It was noted that AgNP exposure did induce ROS, but non-significant levels of ROS with respect to the controls, most likely because the concentrations considered were not cytotoxic to the cells. In contrast to above studies, this study showed that Lipo-AgNP did not induce ROS generation to cause THP1 cell death. In fact, there was an observable suppression of ROS induction. The variation in these studies can likely be due to several factors which include but not limited to difference in cell lines, size and surface characteristics of the AgNP used.

Literature indicates cell death as a result of AgNP exposure is typically due to ROS generation with subsequent DNA damage to the cells followed by apoptosis. But
findings of this study suggested ROS was not involved in the induction of observed cell death when studying the Lipo-AgNP. One of the downstream factors activated by p53 signalling after sensing DNA damage is caspase 3. Different studies have shown that activation of p53 in response to DNA damage results in p53 transcriptional upregulation of Puma and Noxa, inhibitors of Bcl-2, Bcl-xl and Mcl-1. Inhibition of this Bcl proteins result in Bax and Bak activation which ultimately result in cytochrome c release and activation of the executioner caspases (Ashkenazi, 2008).

To explore the possibility of ROS independent induction of apoptosis, activation of caspases 3/7 in THP1 cells treated with same low dose AgNP and Lipo-AgNP at 1 hr, 4 h and 24 h to monitor onset of activation was investigated. For all cell groups, there was no significant activation of caspases 3/7 at 1 h and 4 h. On the other hand, there was significant induction of caspases 3/7 in THP1 cells treated with 0.625 μg/mL of Lipo-AgNP after 24 h ($p < 0.0001$).

Previous studies have linked AgNP mechanism of action to increased generation of ROS, which causes depolarisation of mitochondrial membrane potential and subsequent rupture of the membrane for release of cytochrome c (Kang et al., 2012, Foldbjerg et al., 2011, Jiang et al., 2013). This has been proposed to be achieved by the intracellular oxidation of AgNP to Ag$^+$ by the acidic environment in the lysosome (De Matteis et al., 2015). Endocytosis of AgNP is believed to result in the compartmentalisation of the nanoparticle in an endosome, which is metabolised in the endolysosomal pathway thus leading to its oxidation and generation of ROS. The finding here have interestingly opened an alternative pathway to ROS-dependent apoptosis induction by AgNP. In this study, it was shown that encapsulation of AgNP in liposomes was able to achieve greater cytotoxicity at low dose compared to what was achievable at high doses, in a ROS-independent way. This may be attributed to
the improved delivery of AgNP into the cells via the liposome since the lipid layer can easily traverse the lipid bilayer of the cell membrane due to the hydrophobic interactions when compared to un-encapsulated AgNP. Lipo-AgNP may also suppress the ionisation of AgNP as part of its mechanism to prevent ionisation-dependent generation of ROS. Conventional drugs encapsulated in liposome are known to have an increased bioavailability, enhanced permeability and retention (EPR) effect (Maeda, 2012). These characteristics enhance the pharmacokinetics of Lipo-AgNP in the cells resulting in efficient delivery of AgNP into the cell. Lysosomal breakdown of the lipid layer will result in overwhelming delivery of AgNP into the cytoplasm and subsequently in the nucleus allowing interaction between AgNP and the DNA causing DNA damage and halted replication (Li et al., 2013a, Pramanik et al., 2016). The DNA damage could be responsible for the activation of executioner caspases consequent upon activation of p53. Taken together, this suggests a “Trojan Horse” effect mechanism for Lipo-AgNP due to the sudden leakage of AgNP into the cytoplasm and subsequently the nucleus after degradation of the liposome. This stealth mechanism nonetheless, is in contrast with the type previously described for AgNP in other studies where AgNP is phagocytosed, ionised and released to enhance generation of ROS which in turns stimulate inflammatory responses that can mediate cell death (Park et al., 2010a).

The cytotoxicity induced by Lipo-AgNP independent of ROS generation offers some advantages if properly harnessed. ROS is known to induce cellular senescence in neighbouring cells and this is accompanied by up-regulation of stress and inflammatory genes through secretion of pro-inflammatory molecules like nuclear factor kappa B (NF-κB) (Correia-Melo et al., 2014). In addition, ROS mediated necrosis of cells could result in release of cytoplasmic wastes that can cause
exaggerated and unscheduled inflammatory responses, which may promote tumour development (Vakkila and Lotze, 2004). As such, these secondary effects can be prevented by coating AgNP surface in a protective layer as demonstrated in this study.

4.3 Conclusion

In this chapter, the encapsulation of AgNP in a liposome has been demonstrated to enhance AgNP cytotoxicity at low concentrations through increased DNA damage with suppression of ROS. This contrast with several other studies that have shown that AgNP cytotoxic effect can only be achieved through generation of ROS. It is postulated that the encapsulation of AgNP in liposomes could eliminate the negative side effects of ROS making it possible to achieve a greater level of cytotoxicity that would otherwise only be possible at high concentrations. As such, Lipo-AgNP could reduce the concentration of AgNP required thereby increasing any potential biological activity with reduced associated side effects typically caused from high dose exposures.
Chapter 5
“Liposomal encapsulation of silver nanoparticles (AgNP) improved nanoparticle uptake and induced redox imbalance to activate caspase-dependent apoptosis.” Yusuf et al. 2018

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While macrophages are an essential component and are key players in the innate and adaptive immune system, they also play a central role in maintenance of tissue homeostasis and developmental processes in defining physiological and pathological conditions. Due to this, macrophages have been widely studied as therapeutic target for diseases like rheumatoid arthritis, atherosclerosis and cancer as the role of this immune cell is known to be tilted to favour disease development under specific conditions, particularly due to their induced inflammation (Ponzoni et al., 2018). The role of macrophages in cancer for instance involves the release of pro-inflammatory cytokines such as interleukin 1β (IL-1β) and tumour necrosis-α (TNF-α) by tumour associated macrophages (TAM) within the tumour microenvironment (Fultang et al., 2019). The release of these inflammatory cytokines like IL-1β can drive endothelial cell proliferation and secretion of growth factors such as the vascular endothelial growth actor A (VEGF-A) by the endothelial cells, facilitating angiogenesis (Voronov et al., 2014). In the same manner, production of IL-1β and TNF-α aids recruitment of leukocytes that perpetuate inflammation, which facilitates proliferation of synovial fibroblasts to form pannus that damages the rheumatoid arthritis joint (Kim and Moudgil, 2017). There is evidence that TNF-α can induce activation of extrinsic apoptotic pathway through TNF receptors 1 and 2 (TNFR1/TNFR2). Binding of metalloproteinase cleaved soluble TNF-α or membrane anchored TNF-α to TNFR1 or TNFR2 respectively. This interaction results in internalisation of the receptor-ligand complex where they engage FAD associated death domain (FADD) protein and
Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) protein that subsequently activate caspase 8 and 3 to induce apoptosis (Dreschers et al., 2017).

It appears that the exaggerated inflammatory response of these macrophages may in part be facilitated by the high level of reactive oxygen species that is generated by the immune cell. There is evidence in the literature that macrophages generate a high level of reactive oxygen species (ROS) such super oxides and hydrogen peroxides within a tissue or organ even under minimal perturbation to the normal processes that may characterise a diseased condition (Bae et al., 2009, Kim et al., 2017).

In support of this notion, ROS generation by macrophages have been documented to play key role in driving progression of atherosclerosis through NADH oxidase activity in production of RANTES (regulated on activation, normal T-cell expressed, and secreted), a chemokine that facilitates the homing of immune cells that boost inflammation within the atherosclerotic plaque (Virani et al., 2011, Bae et al., 2009). Macrophages generated ROS also drives progression of cancer through secretion of TNF-α and IL-1β to drive hyperproliferation and arthritis through downregulation of regulatory T cells (Canli et al., 2017, Roberts et al., 2015). The ROS generation has other significant impact as it has been previously demonstrated that ROS can induce activation of caspase 3 and 9 through TNFR activation (Thakor et al., 2017), indicating a link between ROS generation and TNF-α signalling. Interestingly, targeting ROS generated by macrophages has been shown to attenuate the development of atherosclerosis (Vendrov et al., 2007). Likewise in cancer, liposomal bisphosphonates or monoclonal antibodies have been employed to directly induce cytotoxic effect on TAMs to deplete the macrophages, preventing ROS generation or inflammation (Ponzoni et al., 2018).
Silver nanoparticles (AgNP) are widely used as an antibacterial in a variety of applications including wound treatment and food preservation but have also been investigated as anticancer agent in recent years. AgNP has been shown to induce a cytotoxic effect on macrophages even at a very low dose (Haase et al., 2011) and may be effective in treating disease conditions mediated by macrophages as detailed above. Unfortunately, the main mechanism of AgNP action involves induction of ROS which effect myriad of intracellular responses such as DNA damage, increased Bax/Bcl-2 ratio, permeabilization of the mitochondrial membrane, degradation of key cellular protein and subsequent caspase 3/7 dependent apoptosis (Verano-Braga et al., 2014). While the induction of caspase-dependent apoptosis may facilitate macrophage depletion within the atherosclerotic plaque, a tumour microenvironment or the arthritic joint, the induced ROS may result in surge in secretion of inflammatory molecules by the macrophages themselves further favouring the disease condition.

It has been shown in Chapter 4 that encapsulation of AgNP in a dipalmitoyl phosphatidylcholine (DPPC) based liposome suppressed ROS generation in macrophages and that the nanoparticle encapsulation suppressed AgNP-induced ROS generation, enhanced the nanoparticle cytotoxicity in THP-1 monocytes and suppressed AgNP-induced inflammation in THP-1 monocytes and THP1 derived-macrophages (Chapter 6). This Chapter investigated the cytotoxic effect of encapsulated AgNP (Lipo-AgNP) on THP-1 derived macrophages (TDMs) and further probed the mechanistic detail of the pathway utilised by Lipo-AgNP in inducing cell death, highlighting the divergence to the pathway utilised by AgNP.
5.1 Results

5.1.1 Macrophage cell viability

As stated, the cell viability of TDMs upon exposure to AgNP or Lipo-AgNP was evaluated by AB assay. The AB assay is routinely used to estimate cell viability based on the rate at which metabolically active cells reduce resazurin, a non-fluorescent blue dye to resorufin, to a pink fluorescent product. This chemical reduction of resazurin occurs in both the mitochondrion and the cytoplasm, thus it gives a broad indication of cellular viability (Rampersad, 2012). TDMs were exposed to varying concentrations of AgNP, Lipo-AgNP and AgNO\(_3\) (as positive control) ranging from 0.3 µg/ml to 10 µg/ml. As shown in Figure 5-1, the AgNO\(_3\) used as a positive kill control for Ag\(^+\) induced a significantly higher reduction in TDMs viability at all concentrations (p < 0.0001) with a 24 h IC\(_{50}\) of 0.12 µg/ml. In comparison to AgNP induced reduction in cell viability at concentrations ≤ 5.0 µg/ml while Lipo-AgNP induced significant reduction in TDMs viability at concentrations ≤ 2.5 µg/ml (p < 0.001) compared to control unexposed TDMs, making Lipo-AgNP to have a lower IC\(_{50}\) value compared to AgNP (3.98 ± 1.49 µg/ml compared to 5.71 ± 1.09 µg/ml respectively). In addition, Lipo-AgNP induced a significant reduction in TDM viability when compared with AgNP at 2.5 µg/ml. As a negative control, TDMs were also exposed to empty liposome at concentrations equivalent to the liposomes in 2.5 µg/ml and 5 µg/ml Lipo-AgNP. As shown in Figure 5-1B, it was found that the empty liposomes did not affect the cell viability.

A calcein-AM/PI staining of TDMs was also carried out to confirm the result of the AB assay at a cellular level via an alternate technique. To evaluate cell viability here, 2 µg/ml was chosen as a test concentration. This concentration was just below that where a significant difference in both AgNP and Lipo-AgNP toxicity existed. Here
the exposure of TDMs to Lipo-AgNP resulted in a significant reduction in TDM cell viability compared with control unexposed TDMs ($p < 0.05$), which was not observed for AgNP at the same concentration (Figure 5-2). On the contrary, PI entry into the cells was similar in all groups. This was likely because the exposure concentration of the nanoparticles was not toxic enough to cause cell death. To confirm if the liposome was not cytotoxic on the cell, manifesting as the observed cytotoxicity, the TDMs were exposed to an equal amount of empty liposome as contained in 2 µg/ml Lipo-AgNP and it was found that the liposome did not affect the cell viability after 24 h exposure (Figure 5-2).
Figure 5-1. Effect of AgNP, Lipo-AgNP and AgNO₃ on cell viability of TDMs

(A) TDMs were incubated with 0.3 µg/ml to 10 µg/ml of either of AgNP, Lipo-AgNP or AgNO₃ or (B) equivalent concentration of empty liposome as contained in 2.5 µg/ml and 5 µg/ml Lipo-AgNP for 24 h. Cells were incubated in 10% AB solution in 2% FBS-RPMI for another 2 h. IC₅₀ (24 h) was computed for (C) AgNP (D) Lipo-AgNP and (E) AgNO₃. Y-axis represent arbitrary reading from the spectrophotometer for AB assay. Data is represented as mean ± SD of 3 independent experiments. *** $p < 0.001$ and **** $p < 0.0001$. 
**Figure 5-2. Calcein/PI staining of TDMs exposed to AgNP and Lipo-AgNP**

TDMs were exposed to 2 µg/ml of AgNP or Lipo-AgNP or 0.5 µM of Dox or empty liposome containing DPPC and cholesterol as in 2 µg/ml Lipo-AgNP for 24 h before staining with 1 µM calcein-AM and 10 µg/ml PI stain for 20 mins. Conversion of Calcein-AM to fluorescent calcein was measured by detecting the dye MFI in FL-2 channel while PI entry into the cell was detected in the FL-4 of a BD Accuri 6 cytometer. Data is represented as mean ± SD of 3 independent experiments. MFI was calculated from intensity of 50 individual cells per experiment. ** is p < 0.01
5.1.2 Lipo-AgNP suppressed AgNP induced redox imbalance in TDMs

One of the known mechanisms of AgNP cytotoxicity responsible for its antibacterial and anticancer properties is its ability to induce generation of ROS upon entry into the cell. As such, whether the enhanced cytotoxic effect of Lipo-AgNP was due to its ability to enhance ROS generation was investigated. TDMs were exposed to either of 2 µg/ml of AgNP or Lipo-AgNP or 0.5 µg/ml of AgNO3 as positive control after which the ROS generation upon exposure was analysed at different time points using flow cytometry (Figure 5-3). A negative of the empty liposome at the same DPPC concentration as in 2 µg/ml Lipo-AgNP was also incorporated. As expected, AgNP exposure induced significantly higher generation of intracellular ROS at all time points investigated when compared to the control unexposed and Lipo-AgNP exposed TDMs. Although, percentage of cells that were positive for DCFDA fluorescence was comparable in all groups as shown in Figure 5-3A, the MFI readings indicated that AgNP resulted in stronger intensity of ROS dependent DCFDA signals compared to other groups. ROS generation was highest for all groups at 1 h post exposure but this began to decline up until 4 hours. In contrast to AgNP, Lipo-AgNP exposure at all time points resulted in comparable levels of intracellular ROS generation with the unexposed control cells (Figure 5-3B). The empty liposome was also found to induce comparable levels of ROS to the control untreated cells for all time points.

After demonstrating that Lipo-AgNP mediates a time dependent suppression of AgNP induced intracellular ROS, whether this is associated with the GSH levels in the cells and thus the redox balance was investigated. This was done by exposing the TDMs to 1 µg/ml and 2 µg/ml of AgNP or Lipo-AgNP empty liposome at the same DPPC concentration as in 2 µg/ml Lipo-AgNP for 1 h. ROS and reduced glutathione (GSH) levels were then quantified by spectrophotometry. As observed previously, 2 µg/ml
Lipo-AgNP suppressed AgNP-induced ROS generation and in the same manner contrary to what was expected (Figure 5-4A), Lipo-AgNP also significantly reduced the level of GSH in the TDMs compared to both AgNP exposed and control unexposed TDMs (p < 0.05) (Figure 5-4B). As also observed in the confocal microscopy result, the empty liposome induced comparable levels of ROS and GSH as in the control untreated groups (Figure 5-4C and D). This observation is indicative of a redox imbalance due to Lipo-AgNP exposure as a high GSH level is expected due to ROS suppression and that the empty liposome is relatively non-toxic to the cells.
Figure 5-3. ROS generation by TDMs pot-exposure to AgNP and Lipo-AgNP

TDMs were preloaded with 10 μM H2DCFDA for 30 mins before exposure to 2 μM AgNP or Lipo-AgNP or 0.5 μg/ml AgNO3 or empty liposome containing DPPC and cholesterol as in 2 μg/ml Lipo-AgNP for up to 4 h. (A) Plot of percentage of cell population with positive DCFDA fluorescence (B) plot of the MFI of TDMs. Statistical significance was analysed by One-way ANOVA Turkeys multiple comparison tests for each time point. Data represents mean ± SD of 3 independent experiments. * p < 0.05 and ** p < 0.01
Figure 5-4. Redox balance in AgNP and Lipo-AgNP exposed cells

(A) Cells were treated as previously for ROS and the ROS level in cells at 1 h post exposure to 2 µM of AgNP and Lipo-AgNP and 0.5 µg/ml AgNO₃ or (C) empty liposome containing DPPC and cholesterol as in 2 µg/ml Lipo-AgNP was measured spectrophotometrically (B) GSH levels as measured in TDMs by first exposing the cells to 2 µM AgNP or Lipo-AgNP or 0.5 µg/ml AgNO₃ or (D) empty liposome containing DPPC and cholesterol as in 2 µg/ml Lipo-AgNP for 1 h and then to Thioltracker™ for 30 mins before measurement. Statistical significance was analysed by Two-way ANOVA Tukeys test. Data represent mean ± SD of 3 independent experiments. * p < 0.05 and *** p < 0.001.
5.1.3 Caspase 3/7 pathway activation

ROS is one of the major upstream factors in the caspase 3/7 apoptotic pathway, and indeed the main mechanism of AgNP induced apoptosis. The above data demonstrated that Lipo-AgNP induced a higher cytotoxic effect on TDMs compared to AgNP, which is independent of intracellular ROS generation and was accompanied by reduced GSH levels. It has been previously shown that this redox imbalance created by suppressed ROS and GSH levels induced caspase 3-dependent apoptosis (Liu et al., 2014b). Based on this, activation of caspase 3/7 upon exposure of the TDMs to 2 µg/ml AgNP or Lipo-AgNP was probed. It was found that AgNP induced significant activation of caspase 3/7 at 24 h compared to the unexposed (p < 0.0001) which was similar to that induced by Lipo-AgNP (Figure 5-5). However, exposure of the TDMs to AgNP and the observed caspase 3/7 activation did not translate to observable cell death as indicated by fluorescence due to the Sytox AAdvanced stain for dead cells. Contrary to this, Lipo-AgNP exposure resulted to significant number of dead cells after 24 h despite inducing activation of caspase 3/7 at similar level with AgNP (p < 0.001).

Bax and Bcl-2 are respectively pro- and anti-apoptotic factors in the intrinsic cell death pathway that act upstream of caspase 3/7. The protein expression of Bax and Bcl-2 after AgNP and Lipo-AgNP exposure was next investigated. TDMs were exposed to 2 µg/ml of AgNP or Lipo-AgNP for 24 h after which cell lysate was obtained from the cells. Immunoassay of the lysate indicated that none of the nanoparticles influenced Bax protein expression when compared with Bax expression in control unexposed group (Figure 5-6A). On the other hand, Lipo-AgNP was found to significantly inhibit Bcl-2 protein expression (p < 0.001). Bax to Bcl-2 ratio (Bax/Bcl-2) is widely used as an indicator of apoptotic status of a cell (Azimian et al., 2018). Bax/Bcl-2 analysis carried out showed that Lipo-AgNP exposure exhibited the highest
ratio compared to AgNP exposure or non-exposure of the control group (p < 0.05), explaining the observed cell death upon exposure to Lipo-AgNP.

siRNA of Bcl-2 has been demonstrated to make cancer cells sensitive to DNA damage resulting in apoptosis (Knezevic et al., 2007). Based on this the possibility of Lipo-AgNP causing DNA damage which in addition to the low Bcl-2 expression might be the reason for the observed apoptosis was investigated. Activation of H2AX was evaluated in TDMs 24 h post exposure to AgNP or Lipo-AgNP. This is because formation of H2AX foci upon phosphorylation signals increased DNA double strand break (DSB). A significant activation of γH2AX was observed in cells exposed to Lipo-AgNP while AgNP exposed TDMs showed comparable γH2AX fluorescence with control unexposed TDMs (Figure 5-6B). Thus, Lipo-AgNP induced DNA damage in the TDMs may have resulted in apoptosis due to low Bcl-2 expression in the cells.
Figure 5-5. Activation of caspase 3/7 and induction of apoptosis by AgNP and Lipo-AgNP.

TDMs exposed to 2 µg/ml AgNP or Lipo-ANP or 0.5 µM Dox or empty liposome containing DPPC and cholesterol as in 2 µg/ml Lipo-AgNP for 24 h were stained with 500 µM of CellEvent and 1 µM of SytoxAADvanced stain as describe in methods. The fluorescence obtained from confocal microscopy (left) was converted into digital data (right) using imageJ software. Statistical significance was computed using Two-way ANOVA with Tukeys multiple comparison test. Data represents mean ± SD of 3 independent experimental repeats and ***p < 0.001 and ****p < 0.0001.
Figure 5-6. Bax/Bcl-2 ratio and DNA damage in AgNP and Lipo-AgNP exposed TDMs

(A) Lysates from TDMs exposed to AgNP and Lipo-AgNP AgNP or 0.5 µM of Dox or empty liposome containing DPPC and cholesterol as in 2 µg/ml Lipo-AgNP for 24 h were collected and immunoprobed by ELISA for Bax and Bcl-2 and the Bax/Bcl-2 ratios were computed (B) TDMs exposed as above were incubated in anti-phosphoH2AX antibody as described in methods and the H2AX phosphorylation was analysed by flow cytometry. Statistical significance was carried out by One-way ANOVA with Dunnet’s multiple comparison test. Data is represented as mean ± SD for n = 3 independent experimental repeats.
5.1.4 Effect of liposomal encapsulation on AgNP uptake

A time dependent intracellular ROS generation in the TDMs indicated that ROS generation was highest for all exposure groups at 1 h after which the level dropped considerably. This indicates that the AgNP and Lipo-AgNP must have been internalised by the cells prior to 1 h time point. As such the time course uptake of the nanoparticles by flow cytometry was investigated, since the nanoparticles will increase the internal complexity of the cell which can be accurately monitored by the SSC values. The uptake studies indicated that Lipo-AgNP was significantly internalised by the TDMs at 30 mins (p < 0.01) while the AgNP was not internalised at this time point since AgNP exposed TDMs had comparable SSC value when compared with the control unexposed cells (Figure 5-7). At 45 mins, AgNP and Lipo-AgNP uptake had significantly increased (p < 0.0001) but Lipo-AgNP uptake was significantly higher than that of AgNP (p < 0.0001). At 1 h post exposure to the nanoparticles, there was an observed drop in the SSC values of the TDMs which was likely due to the degradation of the nanoparticles. This continued up till 24 h when none of the nanoparticles seem to be in the cells based on the comparable SSC values with control unexposed cells. This finding indicates that entry of the silver nanoparticle at 45 mins must have resulted in ionisation of the nanoparticle causing increased intracellular ROS levels, which may have been suppressed by the liposome shell of Lipo-AgNP.
Figure 5-7. Nanoparticle uptake by TDMs

TDMs were exposed to 2 µg/ml AgNP or Lipo-AgNP for 24 h after which cells were rinsed and analysed by flow cytometry. Statistical analyses were carried out by One-way ANOVA with Dunnet’s multiple comparison test. Data is represented as mean ± SD for n = 3 independent experimental repeats.
5.2 Discussion

In Chapter 4 it was demonstrated in THP-1 monocytes that AgNP encapsulation in liposome enhances the nanoparticle cytotoxicity through steady release of AgNP and induced a caspase-dependent and ROS-independent apoptosis. Based on the different roles of monocytes and macrophages in disease development as well as their varying responses to different stimuli, this study was carried out to probe the molecular mechanism utilised by Lipo-AgNP in inducing cell killing effect in TDMs. As previously observed for THP-1 monocytes, Lipo-AgNP also demonstrated higher cytotoxicity on the TDMs compared with AgNP. However, the IC$_{50}$ for Lipo-AgNP in the TDMs was slightly higher than what was previously recorded for the monocytes. This may be due to the higher uptake rate of the nanoparticle by TDMs because of their active phagocytosis compared to THP-1 monocytes coupled with a more sensitive nature of monocytes. Beduneau et al. (2009) have previously demonstrated that macrophages exhibit higher uptake rate of IgG coated and uncoated super-magnetic iron-oxide nanoparticles compared to monocytes, although the study showed the nanoparticle exhibited similar toxicity in both THP-1 and TDMs which was only after 1 h of exposure. A longer time point might have shown the nanoparticle is more toxic in TDMs. In support of this, Wu et al. (2018) demonstrated the SPIONs induced release of pro-inflammatory cytokines after 24 h exposure of macrophages to the nanoparticles.

ROS generation has been documented to be the main mechanism utilised by AgNP to induce apoptosis in different cell types (Flores-Lopez et al., 2019). Entry of AgNP into the cytoplasm results in ionisation of the nanoparticle by the aqueous environment into silver ions, which subsequently causes generation of intracellular ROS that oxidise cellular proteins and subcellular structures like the mitochondria or induce DNA
damage, culminating in caspase-dependent apoptosis (Mao et al., 2016). Interestingly, the role of macrophages in diseases such as cancer and atherosclerosis are largely modulated on the ability of the immune cell to generate or respond to high intracellular ROS within the tumour microenvironment to suppress anticancer immune response or the atherosclerotic plaque to induce expression of adhesive molecules that facilitate plaque build-up (Bae et al., 2009, Roux et al., 2019). TDMs were employed in this study to investigate Lipo-AgNP effectiveness on macrophages that may be within the tumour microenvironment, especially since Lipo-AgNP possess an anti-inflammatory property in these cells (Chapter 6). As expected AgNP exposure of TDMs resulted in ROS generation supporting findings of other studies (Haase et al., 2014, Haase et al., 2011). Contrary to this and as was previously shown in THP-1 monocytes, Lipo-AgNP suppressed AgNP-induced intracellular ROS generation. This indicates that the encapsulation prevented or delayed the ionisation of the coated AgNP possibly through the anti-inflammatory action of the DPPC liposome or protection of the AgNP within the DPPC liposome once in the cytoplasm. However, this does not explain the enhanced cytotoxicity.

Increased ROS generation is often coupled with reduction in level of GSH. GSH is a tripeptide of glutamine, cysteine and glycine, a biologically active antioxidant against the activities of ROS. Oxidation of GSH by increasing level of ROS results in crosslinking of the two molecules of oxidised glutathione to GSSG causing a drop in the level of GSH and vice versa under low ROS levels. As expected, it was found that exposure to AgNP resulted in reduced GSH level. AgNP is known to induce ROS generation, GSH depletion and activation of caspase 3 (Yin et al., 2013). Interestingly, Lipo-AgNP exposure also resulted in reduced GSH levels which was unexpected since the encapsulation prevented AgNP-induced ROS. The reason for this is currently
unknown but this redox imbalance has been previously demonstrated to induce activation of caspase 3/7 dependent apoptotic pathway (Liu et al., 2014b). It is possible suppression of GSH by Lipo-AgNP is coupled with activity of Bcl-2 since the inhibition of Bcl-2 protein expression was observed. A previous study showed that Bcl-2 overexpressing human leukaemia cells have high level of GSH and another showed that the overexpression of Bcl-2 in a lymphoma cell line aids intranuclear sequestration of GSH (Wright et al., 1998, Voehringer et al., 1998), which may inhibit ROS induced activation of caspase 3/7.

Increased expression and activation of Bax causes destabilisation of mitochondrial outer membrane resulting in release of cytochrome C activating downstream factors preceding activation of caspase 3 and 7. Bcl-2 as an anti-apoptotic factor binds Bax to prevent mitochondrial dysfunction. However, an increased Bax expression relative to a low Bcl-2 expression results in a high Bax/Bcl2 ratio causing Bax to induce mitochondrial outer membrane permeabilization (MOMP), with release of cytochrome C and a subsequent activation of caspases 3 and 7 down in the intrinsic apoptotic pathway (Salakou et al., 2007, Zhu et al., 2015). As such, the Bax/Bcl-2 ratio has been used as a prognostic factor for survival in different cancers and even as predictor of cancer cells response to chemotherapy (Kulsoom et al., 2018, Del Principe et al., 2016, Luo et al., 2015). Interestingly, it has been demonstrated that AgNP induces expression of Bax increasing Bax/Bcl-2 ratio to induce apoptotic cell death in male Wistar rats (Ghooshchian et al., 2017).

Based on the above and to establish a link between Bcl-2 expression and the low GSH level observed in Lipo-AgNP exposed TDMs, Bax and Bcl-2 protein expression in the
cells in response to AgNP and Lipo-AgNP exposure was investigated. Bax protein levels was unaffected in all the exposure groups but Lipo-AgNP was found result in suppression Bcl-2 protein expression. This kept the Bax/Bcl-2 ratio high for Lipo-AgNP exposed TDMs. A high Bax/Bcl-2 ratio has been reported to be associated with increased apoptosis in cells due to high Bax expression and a relatively unaffected Bcl-2 expression in cells exposed to therapeutic agent relative to control cell lines (Azimian et al., 2018). Although this contradicts the finding here where the Bax level is similar in all exposure and control groups, the low Bcl-2 level kept the Bax/Bcl2 levels high such that there is enough free Bax that will induce mitochondria dysfunction and caspase 3/7 activation in Lipo-AgNP exposed TDMs.

In Chapter 4, it was shown that Lipo-AgNP induced DNA fragmentation and cell cycle arrest at the S-phase of THP1 monocytes. This is likely because cells with fragmented DNA cannot successfully proceed through the S-phase due to replication failure. It was further confirmed here that Lipo-AgNP induced DNA damage as the exposure of TDMs to the nanoparticle resulted in significant γH2AX activation. Phosphorylation of H2AX histone result in formation of γH2AX, which recruits DNA damage repair proteins, hence its use as a marker of DNA damage. Bcl-2 overexpression has also been shown to suppress DNA repair, allowing cancer cells accumulate mutations which result in genome instability, a hallmark of cancer (Wang et al., 2008). Deng et al. (1999) also demonstrated that Bcl-2 accelerates recovery of prostate cancer cells from oxidative stress induced nuclear and mitochondrial DNA damage. Taken together, Lipo-AgNP may have induced DNA fragmentation causing H2AX phosphorylation and the low level of Bcl-2 could not allow for Bcl-2 mediated survival of the cell, further making the cells more sensitive to the cytotoxic effect of Lipo-AgNP just as high Bax/Bcl-2 ratio is known to sensitize cancer cells to
chemotherapeutic agents (Luo et al., 2015). This coupled with the high Bax/Bcl-2 ratio could synergistically induce activation of caspase-dependent apoptotic pathway.

It has been shown here that Lipo-AgNP is more cytotoxic than AgNP, inducing a caspase dependent apoptosis. While AgNP was found to be less cytotoxic than the Lipo-AgNP, the uncoated nanoparticle also induced a caspase-dependent apoptosis which was dependent on intracellular ROS generation. This differing mechanism for Lipo-AgNP and AgNP led to the hypothesis that Lipo-AgNP must have a distinct pharmacokinetic mechanism when compared with AgNP. In essence, these nanoparticles must have been metabolised in different ways once they are internalised by the cells. To test this, the uptake of both AgNP and Lipo-AgNP was monitored by evaluating the SSC values of the cells at specific time point. Generally, the SSC value of cells increases with particle uptake due to increase in their internal complexity and there are studies that have successfully applied this technique to measure nanoparticle uptake (Claudia et al., 2017, Zucker and Daniel, 2012, Jochums et al., 2017). Measuring the SSC has several advantages. Firstly, in comparison with fluorescently probing particle uptake, loss of fluorescent intensity will affect the result of particle uptake since SSC measurement does not require cell staining. Secondly, this method is also more valuable in comparison with cell lysis as particles can react with lysis reagent which may confound data from subsequent quantification. Finally, this method is cheap and fast. Measurement of Lipo-AgNP and AgNP uptake showed that Lipo-AgNP was significantly internalised after 30 mins of exposure. AgNP internalisation was not observed until after 45 mins of exposure, which confirms the encapsulation of AgNP in liposome resulted in increased and faster uptake of the nanoparticle. This finding is supported by findings in different studies that reported that liposome encapsulation of pharmacologically active agents enhances drug uptake and delivery,
producing better toxicity profile and increased bioavailability (Fuhrmann et al., 2015, Rafiyath et al., 2012, Ong et al., 2016).

Combining the uptake data with the ROS studies, while Lipo-AgNP were already internalised at 30 mins, it did not result in any rise in intracellular ROS which indicates that the encapsulated AgNP had not been exposed to the aqueous surrounding of the cytoplasm, thus not ionised. In correlation with AgNP uptake by the TDMs, significant intracellular ROS generation was observed at 1 h which was after 15 mins of detected uptake and while the ROS level dropped afterwards, it was still significantly higher than that of other cell groups. While the ROS generated by AgNP would have been responsible for the cytotoxicity observed in AgNP-exposed TDM, the ROS level was likely not high enough to suppress Bcl-2 expression or DNA damage. This also explains why the cell killing effect of AgNP as depicted by Sytox AADvanced staining was significantly less in AgNP-exposed TDMs when compared with Lipo-AgNP exposed cells (Figure 5-5). In addition, the caspase activation induced by Lipo-AgNP must have occurred earlier than that of the AgNP due to the improved uptake causing cell death earlier than in AgNP. This may be the reason why caspase activation was comparable between AgNP and Lipo-AgNP at 24 h but AgNP cell killing effect may occur long after Lipo-AgNP. This is an indication of slower kinetics of AgNP mechanism compared with Lipo-AgNP. On the contrary, Lipo-AgNP maintained comparable intracellular ROS levels as the control unexposed TDMs indicating ROS was not involved in its mechanism. Taken together, it seems the interaction between the lipid bilayer of the liposome and that of the TDMs facilitated uptake of the liposome. However, entry of the liposome prevented ionisation of the AgNP allowing direct delivery of the nanoparticle at concentrations high enough to cause DNA damage which resulted in phosphorylation of H2AX and suppression of Bcl-2
expression. The low level of Bcl-2 in the cells did not match Bax levels allowing free Bax to bind to the mitochondrial membrane causing MOMP and possible release of cytochrome C and subsequent activation of caspase 3/7 to induce apoptosis

5.2.1 Conclusion

AgNP main mechanism of inducing apoptosis has been reported in different studies to involve generation of ROS and offset of the Bax/Bcl-2 ratio causing MOMP. Here it was confirmed that low dose AgNP induce significant generation of intracellular ROS coupled with depletion of GSH but the no significant cell death. ROS generation and inflammation are tightly coupled, and this may be responsible for AgNP induced associated inflammation reported in previous studies. On the contrary, it has been shown that Lipo-AgNP suppressed ROS generation but depleted GSH level creating a redox imbalance. This redox imbalance and DNA damage caused by Lipo-AgNP may be responsible for the suppression of Bcl-2 increasing the Bax/Bcl-2 ration to cause effective MOMP that can induce caspase 3/7 activation and then cell death.
Chapter 6
Silver nanoparticles (AgNP) are widely used as an antibacterial agent and are the active agent in more than 24% of global nanotechnology driven commercialised product, in dermal applications, medical apparels and in sterilisation products for medical equipment (Vance et al., 2015). While the application of AgNP as an antibacterial agent hold promise in the treatment of bacterial infection, their increased commercialisation translates to increased and repeated human exposure. There are reports of skin irritation and permanent discolouration of the eyes and skin as a result of exposure to AgNP (León-Silva et al., 2016). In support of this, several *in vitro* and *in vivo* studies in recent decades have demonstrated AgNP induced inflammatory responses. A repeated-dose toxicity assessment carried out on mice that were orally administered with AgNP showed that the nanoparticle, irrespective of size induced significant expression of IL-1, IL-6 and TNF-α, among other pro-inflammatory cytokines in a dose dependent manner (Park et al., 2010b). In primary rat brain micro-vessel endothelial cells (rBMEC), Trickler et al. (2010) demonstrated that AgNP induced secretion of interleukin 1β (IL-1β), IL-2 and tumour necrosis-α (TNF-α). Murphy et al. (2016) also reported that exposure of THP1 and primary human monocytes to AgNP resulted in increased mRNA expression of IL-1, IL-6 and TNF-α. In addition to this, increased inflammasome activation was suspected due to increased secretion of pro-IL-1β that was observed upon THP1 exposure AgNP.

Inflammation is central to the development and progression of various chronic diseases including cancer, sepsis, cardiovascular, autoimmune, and neurodegenerative diseases (Seol et al., 2017, Pianta et al., 2017, Jones et al., 2003, David et al., 2016,
Findings in the past decades have shown that IL-1β, IL-6 and TNF-α are the major pro-inflammatory cytokines with roles central to promotion and maintenance of systemic inflammation (Mori et al., 2011, Hernandez-Rodriguez et al., 2004). These pro-inflammatory cytokines effect different cell types by inducing secretion of other inflammatory cytokines that activate a complex network of inflammatory pathways especially in disease conditions. Cytokines are mainly produced by immune cells such as monocytes, macrophages and dendritic cells; and stromal cells such as endothelial cells and fibroblasts as a means for intercellular communication in mediating processes like proliferation, differentiation, growth, immune cell activation and migration (Landskron et al., 2014). The maintenance of the delicate balance between anti- and pro-inflammatory cytokines, however, is what defines the line between normal condition and disease state for different chronic diseases. For example, IL-1β, IL-6 and TNF-α are the main cytokines driving the damaging inflammatory responses in rheumatoid arthritis (RA), likely through the perturbation of the balance between anti- and pro-inflammatory cytokines in RA joints (Mori et al., 2011). This perturbation may arise due to the amplification of inflammatory response through the activities of IL-1β, IL-6 and TNF-α in inducing expression of other pro-inflammatory cytokines through activation of transcriptional factors such as Signal Transducer and Activator of Transcription-1 (STAT-1) and STAT-3 (Pugazhenthi et al., 2013, Chung et al., 2017). The Janus kinase/STAT (JAK/STAT) pathway can be activated by IL-6 binding to a receptor tyrosine kinase (RTK) which transactivates JAK which in turn phosphorylate the RTK facilitating the recruitment of STAT to the RTK intracellular domain and subsequent phosphorylation of STAT by JAK. Phosphorylated STAT dimerises and undergo cytoplasmic-nuclear
translocation where it binds to specific DNA sites to facilitate transcription of cytokine inducible genes, amplifying inflammation (Morris et al., 2018).

In the same manner, the anti-tumoral response of immune cells like monocytes and macrophages or pro-tumoral activities of tumour associated macrophages (TAM) during chronic inflammation are also regulated by the balance between the anti- and pro-inflammatory cytokine subsets. Chronic inflammation induced and sustained by expression of IL-1β, IL-6 and TNF-α in TAMs are known to induced tumour progression and even increase susceptibility of individuals with Crohn’s disease and ulcerative colitis in developing neoplasia (Poh and Ernst, 2018). During chronic inflammation, cancer cells are known to also utilise IL-8 and its receptor to facilitate the recruitment of immune cells with pro-tumoral and immunosuppressive activities to offset the activities of anti-tumour cytotoxic immune cells (David et al., 2016). Investigations have shown that AgNP are capable of inducing expression and secretion of IL-8 to stimulate neutrophil or stem cell activation and proliferation which if left unregulated may provoke pathogenic inflammation and disease (Fraser et al., 2018, Jung et al., 2014).

Taken together, repeated human exposure to AgNP may result in chronic inflammation that may favour autoimmunity or cancer development. In this study, it was shown that liposomal encapsulation of AgNP suppresses AgNP induced inflammation in both THP1 monocytes and THP1 cells differentiated into macrophages and inhibits STAT3 expression possibly suppressing any STAT3-dependent perpetuation of inflammatory response in both immune cells.
6.1 Results

6.1.1 Time dependent release of IL-1β in Pro-THP1, monocytic THP1 and TDMs

THP1 cells are pro-monocytic cell line that grow in suspension. To induce maturation to monocytes (monocytic THP1) or TDMs, the pro-monocytic THP1 (pro-THP1) were stimulated with 100 ng/ml PMA for 24 h or 72 h respectively and then allowed to rest in a PMA free RPMI medium for another 24 h as recommended by Chanput et al. (2014). In other to understand the effect of AgNP and Lipo-AgNP on the inflammatory response of these cells, the cells were primed with LPS from *E. coli* 0111:B4, which is known to induce release of pro-inflammatory cytokines in THP1 cells (Lackman and Cresswell, 2006). The cells (pro-THP1, monocytic THP1 and TDMs) were primed with 10 µg/mL LPS after which the cells were also stimulated with 5 mM ATP prior to collection of culture supernatant at 0 h. A time-response analysis of IL-1β release in cell culture supernatant from pro-THP1 indicated very low release of IL-1β (< 25 pg/ml) in both LPS primed and non-primed cells between 0 and 4 h, except for LPS stimulated pro-THP1 at 4 h where significantly higher IL-1β concentration was found (78 pg/ml, *p* < 0.0001) (Figure 6-1A). At 0 h, both unprimed and LPS primed monocytic THP1 cells secreted similar but low IL-1β level (14 pg/ml and 12 pg/ml respectively). This level significantly increased at 0.5 h to 118 pg/ml and 146 pg/ml for unprimed and LPS primed monocytic THP1 respectively. IL-1β release in the monocytic THP1 cells continues to rise at 1 hr to 165 pg/ml and 192 pg/ml respectively for unprimed and LPS-primed monocytic THP1. This level peaked at 2 hrs up to 4 h for both unprimed and LPS-primed monocytic THP1 not increasing above 198 pg/ml (Figure 6-1C). Unlike the monocytic THP1, the TDMs showed gradual increase in level of IL-1β secreted from 0 h (9 pg/ml) peaking at 3 h (199 pg/ml) for the unprimed...
cells. This level remained unchanged at 4 h. The same trend was observed for the LPS-primed TDMs which also showed gradual increase from 0 h (15 pg/ml) peaking at 2 h (213 pg/ml). This level slightly reduced at 3 h and 4 h to 212 pg/ml and 206 pg/ml respectively (Figure 6-1D). At 4 h, both monocytic THP1 and TDM secreted similar concentrations of IL-1β, which was 25 folds and 3 folds IL-1β release of unprimed and LPS-primed pro-THP1 respectively. Figure 6-1B shows IL-1β release by pro-THP1 on the same scale as those of monocytic THP1 and TDM indicating less IL-1β release.
Figure 6-1. Time dependent IL-1β release in unprimed and LPS primed proTHP1, monocytic THP1 and TDMs:

(A) pro-THP1 (C) monocytic THP1 and (D) TDM were either primed with 10 µg/mL LPS or unprimed and then challenged with 5 mM ATP. Culture supernatant was collected at time shown for IL-1β analysis by sandwich ELISA. (B) IL-1β secretion by pro-THP1 in the same scale as monocytic THP1 and TDMs. Data was presented as mean ± SD of 3 independent experiments. Statistically significant differences were analysed by Two-way ANOVA Sidak multiple comparison test. *** p < 0.001, **** p < 0.0001.
6.1.2 Role of caspase-1 in release of IL-1β upon ATP and/or LPS challenge of THP1 and TDMs

Matured IL-1β is produced after proteolysis of pro-IL-1β by the activity of caspase-1 also referred to as interleukin 1 converting enzyme (ICE) in an independent event from the release of matured IL-1β by immune cells. LPS, used to mimic trigger of inflammation during infection/sepsis, and ATP, which activates the P2X7 receptor, are commonly used to trigger release of matured IL-1β in immune cells, both acting in independent pathways (Stoffels et al., 2015). As such IL-1β release in both monocytic THP1 and TDMs in response to ATP and/or LPS was investigated and whether this release was affected while caspase-1 was inhibited. This was necessary to understand the influence of both AgNP and Lipo-AgNP on LPS-induced and basal-level cytokine release. Both monocytic THP1 and TDMs were cultured in RPMI media containing 30 µg/ml of polymyxin B for 1 h to inhibit exogenous LPS contamination. The cells were then primed with 10 µg/mL LPS for 3 h after which they were stimulated with 5 mM ATP for another 1 h with or without 20 µM ICE\textsubscript{inh}. As controls, unprimed monocytic THP1 and TDMs were also subjected to the same conditions. It was found that ATP stimulation of unprimed monocytic THP1 without caspase-1 inhibition by ICE\textsubscript{inh} resulted in significantly higher IL-1β release (199.77 pg/ml) compared to when caspase-1 was inhibited by ICE\textsubscript{inh} in the presence of ATP (101.62 pg/ml) ($p < 0.0001$). The IL-1β release consequent of only ATP stimulation was also significantly higher than the basal IL-1β release (103.85 pg/ml), i.e. when the cells were neither stimulated with ATP nor exposed to ICE\textsubscript{inh}. Inhibition of caspase-1, was found to have no significant effect on IL-1β release (99.75 pg/ml) (Figure 6-2A). This finding that IL-1β release in monocytic THP1 is not affected in the absence of ATP or when caspase-1 is inhibited suggests that the role of ATP upstream of caspase-1 is
solely for stimulation of caspase-1 into activity in THP1 monocytes. Conversely, LPS priming of the monocytes without ATP stimulation was found to induce significant increase in basal IL-1β release when caspase-1 was not inhibited ($p < 0.0001$). Even when caspase-1 was inhibited, ATP stimulation and LPS priming of the monocytic THP1 with LPS was found to be sufficient to significantly enhance IL-1β release ($p < 0.0001$). This may suggest that LPS priming may induce resistance of caspase-1 to the inhibitory activity of ICE$_{inh}$ (Figure 6-2B).

Contrary to the finding in the monocytes, ATP stimulation of TDMs was found to significantly enhance IL-1β release ($p < 0.0001$), irrespective of caspase-1 activity in proteolyzing pro-IL-1β. IL-1β release in TDMs that were neither stimulated with ATP nor treated with ICE$_{inh}$ (16.69 pg/ml) was 6-fold smaller than in monocytic THP1 of the same treatment condition (103.85 pg/ml) ((Figure 6-2C). This suggests that TDMs do not readily secret IL-1β likely due to low caspase-1 activity and ATM stimulation only resulted in release of low amount of IL-1β that is proteolyzed by caspase-1 basal activity. Priming of TDMs by LPS on the other hand, was found to induce significant enhancement of IL-1β release ($p < 0.0001$) except in TDMs that were not stimulated with ATP, which were also exposed to ICE$_{inh}$ (> 185 pg/ml) (Figure 6-2D). This indicates that TDMs are more responsive to LPS priming in secreting IL-1β except when pro-IL-1β processing by caspase-1 is blocked and there is no ATP stimulation. This finding suggests that caspase-1 activity in TDMs is sensitive to ATP stimulation especially LPS priming even in the presence of ICE$_{inh}$. 
Figure 6-2. LPS priming effect IL-1β secretion in both monocytic THP1 and TDM.

(A) unprimed monocytic THP1 (B) monocytic THP1 primed with 10 µg/mL LPS (C) unprimed TDM and (D) TDMs primed with 10 µg/mL LPS were incubated for 2 h followed by exposure to 20 µM ICEinh for another hour and challenged with 5 mM ATP in the last hour before collection of culture supernatant for IL-1β analysis by ELISA. Data was presented as mean ± SD of 3 independent experiments. Statistically significant differences were analysed by Two-way ANOVA Tukeys multiple comparison test. *p < 0.05, **** p < 0.0001.
6.1.3 Inflammatory response in monocytic THP1 exposed to AgNP and Lipo-AgNP

IL-1β plays a central role in inflammatory responses throughs its involvement in regulating its own release in addition to that of IL-6 and its crosstalk with TNF-α during inflammation (Mori et al., 2011, Di Paolo et al., 2015). As such, whether exposure of the monocytic THP1 and TDMs to AgNP or Lipo-AgNP would provoke an inflammatory response in these cell types was investigated by evaluating the release of IL-1β, IL-6, IL-8 and TNF-α upon exposure to the nanoparticles. Prior to nanoparticle exposure or LPS priming, the cells were exposed to polymyxin B for 1 h to inhibit any LPS contamination that have occurred in procedures prior to this stage. After this, LPS-primed and unprimed monocytic THP1 and TDMs were exposed to 1 µg/ml and 2 µg/ml of AgNP or Lipo-AgNP for 3 h after which the cells were stimulated with 5 mM ATP for another 1 h when supernatant from the cells was collected for ELISA analysis of IL-1β release. From Figure 6-3A, analysis of IL-1β measurement in monocytic THP1 indicated that both 1 µg/ml and 2 µg/ml of AgNP significantly induced IL-1β release compared to untreated and unprimed monocytic THP1 (p < 0.05 and 0.0001 respectively). While Lipo-AgNP maintained no significant induction of IL-1β compared to the unprimed and untreated monocytic THP1 at both 1 µg/ml and 2 µg/ml, Lipo-AgNP significantly suppressed AgNP induced IL-1β release at both concentrations (p < 0.05 and 0.0001 respectively). At 1 µg/ml concentration, both AgNP and Lipo-AgNP were able to significantly suppress LPS induced IL-1β release (p < 0.05) but at 2 µg/ml, the nanoparticles showed no effect on LPS induction of IL-1β release.

Given that IL-1β and TNF-α interact during inflammation to upregulate transcription and expression of pro-inflammatory cytokines, release of TNF-α upon monocytic
THP1 exposure to both AgNP and Lipo-AgNP was also investigated following the same procedure used for measuring IL-1β levels. As observed for IL-1β release, Figure 6-3B shows that exposure of unprimed monocytic THP1 to 1 µg/ml AgNP resulted in significant induction of TNF-α compared to untreated and unprimed monocytic THP1 ($p < 0.05$). Coupled with this, exposure of the monocytes to 1 µg/ml Lipo-AgNP did not induce any rise in TNF-α release compared to the untreated and unprimed monocytic THP1. This coupled with the observation that both 1 µg/ml and 2 µg/ml Lipo-AgNP maintained IL-1β levels similar to that of untreated and unprimed indicates that Lipo-AgNP mediate suppression of AgNP mediated IL-1β and TNF-α release. At 2 µg/ml concentration, both AgNP and Lipo-AgNP induced similar levels of TNF-α as that in untreated and unprimed control monocytes. The contrary was observed for LPS primed monocytic THP1 as exposure to AgNP but not Lipo-AgNP at 1 µg/ml and 2 µg/ml resulted in suppression of LPS mediated induction of TNF-α ($p < 0.01$ and $p < 0.0001$ respectively). Also, Lipo-AgNP exposure resulted in significantly higher levels of TNF-α release compared to AgNP exposed monocytic THP1 at 1 µg/ml and 2 µg/ml in the presence of LPS ($p < 0.001$).

IL-1β and TNF-α are known regulators of IL-6 release (Palmqvist et al., 2008) and based on this, the effect of AgNP and Lipo-AgNP on IL-6 release was next investigated. Findings revealed that IL-6 release was not influenced by LPS priming of monocytic THP1 cells even in the cells that were exposed to 1 µg/ml AgNP and Lipo-AgNP alone or with LPS (Figure 6-3C). Conversely, exposure of the monocytic THP1 to 2 µg/ml AgNP alone or with LPS priming, induced highly significant increase in IL-6 release in comparison to untreated unprimed THP1 monocytes ($p < 0.0001$ and $p < 0.001$ respectively). An interesting finding here was the suppression of AgNP induced IL-6 release by Lipo-AgNP both at 1 µg/ml and 2 µg/ml monocytes ($p <$
It is noteworthy that LPS priming of monocytic THP1 resulted in suppression of IL-6 release although not significantly (p = 0.5875). In the same manner, exposure of LPS-primed monocytic THP1 to 2 µg/ml AgNP resulted in significantly lower release of IL-6 when compared to cells exposed to 2 µg/ml AgNP alone. Taken together, these findings might be indicative of an antagonistic role of LPS on release of IL-6 in the monocytes.

IL-8 is a pro-inflammatory cytokine with tumour promoting roles (Long et al., 2016). IL-8 release upon exposure of unprimed and LPS-primed monocytic THP1 to either AgNP or Lipo-AgNP was investigated. As shown in Figure 6-3D, there was significant induction of IL-8 release in LPS primed monocytic THP1 (1422.65 pg/ml) compared with unprimed THP1 monocytes (505 pg/ml) (p < 0.0001). As observed for IL-1β, exposure of the THP1 monocytes to 1 µg/ml and 2 µg/ml of Lipo-AgNP resulted in similar IL-8 release profile with those that were untreated and unprimed with LPS (498.36 pg/ml and 354.48 pg/ml respectively). Conversely, AgNP at both 1 µg/ml and 2 µg/ml resulted in significant induction in released IL-8 levels (1191.17 pg/ml and 1371.61 pg/ml respectively) (p < 0.0001), while Lipo-AgNP significantly suppressed AgNP induced IL-8 release (p < 0.0001). However, both AgNP and Lipo-AgNP exposure did not suppress LPS induced release of IL-8 at both concentrations evaluated.
Figure 6-3. Lipo-AgNP suppress AgNP mediated secretion of IL-1β, IL-6, IL-8 and TNF-α in monocytic THP1 cells.

Monocytic THP1 cells that were either primed with 10 µg/mL LPS or unprimed were exposed to 1 µg/ml or 2 µg/ml of AgNP and Lipo-AgNP and incubated for 3 h. The cells were challenged with 5 mM ATP for 1 h before culture supernatant was collected for ELISA analysis of (A) IL-1β (B) TNF-α (C) IL-6 and (D) IL-8 release. Data was presented as mean ± SD of 3 independent experiments. Statistically significant differences within same exposure group were analysed by Two-way ANOVA Tukeys multiple comparison test while differences between different exposure groups were analysed by Sidak multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
6.1.4 Inflammatory response in TDMs exposed to AgNP and Lipo-AgNP

Following the discovery that Lipo-AgNP was able to suppress AgNP and LPS-mediated cytokine release in the monocytic THP1 cells, the cytokine release experiment was replicated in TDMs upon their exposure to AgNP and Lipo-AgNP. This was necessary because monocytes and macrophages are both important components of the innate immune system in processes leading up to inflammation especially in the release of pro-inflammatory cytokines either during injury, disease development or infection. As such, release of the same set of cytokines measured in the monocytic THP1 were examined in TDMs under the same conditions.

Analysis of IL-1β release upon LPS priming of TDMs indicated a significant increase or enhancement of IL-1β release by TDMs (188.43 pg/ml) compared to the untreated and unprimed control TDMs (174.45 pg/ml) (p < 0.05). Exposure of the unprimed TDMs to 1 µg/ml of AgNP and Lipo-AgNP resulted in significant suppression of IL-1β release (28.41 pg/ml and 41.28 pg/ml respectively) (p < 0.0001), although AgNP significantly suppressed the IL-1β release compared to Lipo-AgNP (p < 0.05). In a similar manner, 2 µg/ml of AgNP and Lipo-AgNP significantly suppressed IL-1β release in unprimed TDMs (23.73 pg/ml and 48.59 pg/ml respectively) (p < 0.0001). Unfortunately, both AgNP and Lipo-AgNP did not suppress LPS mediated release of IL-1β in TDMs (Figure 6-4A).

The effect of the nanoparticles on TDM response in terms of TNF-α release was next examined. As shown in Figure 6-4B, the first observation was that TNF-α release in TDMs was more than twice that secreted by monocytic THP1s under all exposure conditions. It was found that LPS priming of TDM induced significantly high level of TNF-α release (3458 pg/ml) compared to unprimed TDM (593.33 pg/ml) (p < 0.0001). Exposure of the TDM to 1 µg/ml and 2 µg/ml of Lipo-AgNP resulted in significant
suppression of TNF-α release (399.43 pg/ml and 258.33 pg/ml respectively). AgNP exposure on the other hand only resulted in significant suppression of TNF-α release at 2 µg/ml (341.9 pg/ml) (p < 0.01). In TDMs that were primed with LPS, exposure to 2 µg/ml AgNP and Lipo-AgNP resulted in significant suppression of LPS induced TNF-α release in the TDMs (3270 pg/ml and 3068 pg/ml respectively) (p < 0.05 and p < 0.0001 respectively). Furthermore, 2 µg/ml Lipo-AgNP significantly mediated suppression of TNF-α release in comparison with AgNP at the same concentration.

As observed in monocytic THP1, LPS priming of TDMs induced significant release of IL-6 (42.86 pg/ml) when compared with the untreated and unprimed TDMs (0.76 pg/ml) (p < 0.0001). In addition, AgNP and Lipo-AgNP at 1 µg/ml and 2 µg/ml did not induce further IL-6 release as the TDMs exposed to the nanoparticles maintained similar IL-6 release as the control. While 1 µg/ml of AgNP and Lipo-AgNP did not affect LPS induce IL-6 secretion in TDMs, exposure of the LPS-primed TDM to 2 µg/ml AgNP resulted in significantly higher release of IL-6 (54.39 pg/ml) compared to 1 µg/ml AgNP (42.59 pg/ml) (p < 0.05). The reverse was observed for 2 µg/ml Lipo-AgNP exposure which induced significantly lower IL-6 release compared to 1 µg/ml Lipo-AgNP (37.86 pg/ml to 49.21 pg/ml respectively) (p < 0.005) (Figure 6-4C). In TDMs, observed IL-8 release upon exposure to 1 µg/ml AgNP and Lipo-AgNP (1118.51 pg/ml and 1033.22 pg/ml respectively) was significantly higher compared to control untreated group (566 pg/ml) (p < 0.0001). The same was observed for the nanoparticles at 2 µg/ml concentration but AgNP induced significantly higher IL-8 release (1273 pg/ml) compared to Lipo-AgNP (1099.57 pg/ml) (p < 0.01) (Figure 6-4D). LPS priming of the TDMs also resulted in significantly higher release of IL_8 at both 1 µg/ml and 2 µg/ml of both AgNP and Lipo-AgNP (p < 0.0001).
**Figure 6-4.** Lipo-AgNP suppress AgNP mediated secretion of IL-1β, IL-6 and TNF-α in TDMs.

TDMs that were either primed with 10 µg/mL LPS or unprimed were exposed to 1 µg/ml or 2 µg/ml of AgNP and Lipo-AgNP and incubated for 3 h. This was followed by 5 mM ATP challenge for 1 h before culture supernatant was collected for ELISA analysis of (A) IL-1β (B) TNF-α (C) IL-6 and (D) IL-8 release. Data was presented as mean ± SD of 3 independent experiments. Statistically significant differences within same exposure group were analysed by Two-way ANOVA Tukeys multiple comparison test while differences between different exposure groups were analysed by Sidak multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.
6.1.5 Cell viability of monocytic THP1 and TDMs at 4 h post-exposure to AgNP and Lipo-AgNP

It has been shown that Lipo-AgNP exhibit immunosuppressive role, even AgNP to some extent. To ensure that the suppressed cytokine release was not due to reduced cell viability or reduction in number of cells that are able to release the cytokine upon exposure to AgNP and Lipo-AgNP, a cell viability assay was carried out. Both LPS primed and unprimed monocytic THP1 and TDMs were exposed to 1 µg/ml and 2 µg/ml of either AgNP or Lipo-AgNP for 3 h after which the cells were stimulated with 5 mM ATP for another 1 h, as done for cytokine release. The cell viability by Alamar blue was then carried out. As shown in Figure 6-5.A and B, exposure conditions for AgNP and Lipo-AgNP did not affect the cell viability of both monocytic THP1 and TDM at 4 h when the cytokine release was evaluated.
Figure 6-5. Cell viability of monocytic THP1 and TDMs.

(A) monocytic THP1 and (B) TDMs that were either primed with 10 µg/mL LPS or unprimed were also exposed to 1 µg/ml and 2 µg/ml AgNP and Lipo-AgNP for 3 h followed by 5 mM ATP challenge for 1 h. The cells were then exposed incubated in 10% Alamar blue containing RPMI media for 2 h. Data is presented as mean ± SD.
6.1.6 Expression of STAT-3 in AgNP and Lipo-AgNP exposed monocytic THP1 and TDMs

Data obtained from this study have shown that AgNP provokes inflammation in both monocytic THP1 and TDMs. The effect of AgNP on STAT-3 expression was next evaluated. STAT-3 is a transcriptional factor known to regulate expression of different inflammatory cytokines, which can result in uncontrolled inflammation (Kasembeli et al., 2018). Monocytic THP1 and TDMs that were either primed with LPS or unprimed were exposed to 1 µg/ml of either of AgNP or Lipo-AgNP for 24 h. The lysate collected was subjected to sandwich ELISA analysis and result obtained showed that exposure of LPS primed monocytic THP1 to AgNP resulted in significant induction of STAT-3 expression compared to LPS primed and untreated control monocytes ($p < 0.05$) (Figure 6-6A). It was also found that AgNP induced significantly higher STAT-3 expression when compared with Lipo-AgNP exposure, both in LPS-primed monocytic THP1 cells ($p < 0.01$). Contrastingly in TDMs, exposure of the cells to AgNP induced significant expression of STAT-3 in both LPS primed and unprimed TDMs when compared to primed and unprimed untreated controls ($p < 0.05$ and $p < 0.001$) as well as LPS primed and unprimed Lipo-AgNP exposed TDMs ($p < 0.05$ and $p < 0.001$) (Figure 6-6B). However, Lipo-AgNP exposure did not affect STAT-3 expression in THP1 monocytes or TDMs in both LPS primed and unprimed cells.
Figure 6-6. AgNP induced STAT-3 expression in monocytic THP1 and TDMs. (A) Monocytic THP1 and (B) unprimed TDMs or TDMs primed with 10 µg/mL LPS were also exposed with either of 1 µg/ml AgNP or Lipo-AgNP for 24 hrs. Lysates were collected and analysed by sandwich ELISA for STAT-3 expression. Statistically significant differences within same exposure group were analysed by Two-way ANOVA Tukeys multiple comparison test while differences between different exposure groups were analysed by Sidak multiple comparison test. *p < 0.05, *** p < 0.001.
6.2 Discussion

In Chapter 4, it was shown that Lipo-AgNP at very low dose induced significant caspase 3/7 dependent cell death compared to uncoated AgNP in THP1 cells in a ROS independent manner (Yusuf et al., 2018). Here, the immunomodulatory role of Lipo-AgNP was investigated using THP1 monocytes and TDMs as models for human
monocyte and macrophages respectively. Monocytes and macrophages are central to the functioning of the innate and adaptive immune system in response to infection, injury and tissue repair or in autoimmune and inflammatory diseases such as rheumatoid arthritis (RA) (Roberts et al., 2015, Ogle et al., 2016). As monocytes and macrophages functions are mainly modulated by inflammatory cytokines in above conditions, the response of these immune cells to AgNP and Lipo-AgNP in normal condition and during induced inflammation was investigated.

In this study, pro-THP1 were stimulated with PMA for 24 h to induce maturation into monocytic cells because THP1 cells, referred to as pro-THP1 here, have been described to have phenotypic characteristic of pro-monocytes which are the progenitor cells from which monocytes develop (Daigneault et al., 2010, Li et al., 2013b). Coupled with this, pro-THP1 are known to secrete significantly less concentration of inflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-8 compared to matured monocytes (Jones et al., 2003, Shalit et al., 2005). This may be indicative of a less active role of promonocytes during inflammation or tissue repair. The level of cytokine generated by promonocytes may also be difficult to quantify accurately, subjective to method of measurement and type of equipment used. This low response was observed in this study where it was found that pro-THP1 produced significantly less IL-1β compared to monocytic THP1 and TDMs at all time points (p < 0.0001). This was also the case for the highest recorded IL-1β release at 4 h post LPS priming of pro-THP1 and was 2.5 folds less than IL-1β release in both monocytic THP1 and TDMs at same time point. CD14, toll-like receptor 2 (TLR2) and TLR4 are major toll like receptors expressed by monocytes and macrophages to sense and identify pathogen associated molecular patterns (PAMPs) to activate pathways for secretion of either cytokines or chemokines that mediate inflammation or recruitment of more immune cells to either
fight infection or induce perpetuation of immune response that causes autoimmunity (Liu et al., 2014d, Mukherjee et al., 2016, Zoccal et al., 2014). Interestingly, expression of CD14, TLR2 and TLR4 have been reported to be higher in monocytes compared to pro-THP1 (Parker et al., 2004), which may explain why cytokine expression in promonocytes is less than in monocytes and macrophages.

Unprimed monocytic THP1 exhibited significant increase in IL-1β release at 0.5 h, while this type of increase was not observed in unprimed TDMs until at 2 h. During infection or tissue repair, monocytes are recruited to the site of infection or tissue damage where they then differentiated into either of macrophages or dendritic cells (Yang et al., 2014). Upon recognition of microbial PAMP, evidence in the literature has shown that IL-1β is one of the first set of cytokines that kickstart the differentiation of monocytes into macrophages (Schenk et al., 2014). This possibly explains the reason why the monocytic THP1 released high amount of IL-1β upon ATP challenge which may be sensed by the cells as stimuli to induce inflammatory response, eventually resulting in either their differentiation into macrophages or phagocytosis of foreign body. This may also account for the reason why TDMs had slow onset of IL-1β release. During hypoxia induced inflammation, expression of IL-1α precursor is upregulated which is then released by the dying cells. This allows the recruitment of neutrophils to the site to clear up the cells. Expression of IL-1β precursor and release of matured IL-1β on the other hand occurs as a late signal for migration of macrophages (Rider et al., 2011), indicating a time lag in macrophage requirement for IL-1β.

Release of matured IL-1β is regulated by caspase-1 in both monocytes and macrophages. Caspase-1 proteolytically cleave pro-IL-1β into the mature IL-1β but this process is not always spontaneous in vitro especially in THP1 derived cells. In
vitro, extracellular ATP is thus used to challenge monocytes or macrophages to activate the P2X7 receptor, a purinergic receptor that mediates huge efflux of potassium ion (K⁺). This K⁺ drives proteolytic activation of caspase-1 by the NLRP3 inflammasome, subsequently influencing the processing and release of IL-1β (Wiley et al., 2011, Brough and Rothwell, 2007, Amores-Iniesta et al., 2017). This supports the finding of this study that THP1 monocytes exhibited enhanced IL-1β release after ATP stimulation except when caspase-1 activity was inhibited. ATP may be acting upstream of caspase-1 in processing of IL-1β for release. Thus, the inhibition of IL-1β release when the monocytes were stimulated with ATP while caspase-1 was inhibited may be as a result of the failure of ATP to stimulate a non-functioning caspase-1. LPS priming on the other hand, seem to induce a resistance in the monocytes against caspase-1 inhibition. Consequently, higher ICEinh concentration may be required to bring about the observed inhibition in unprimed monocytic THP1. LPS binding to TLR2 and TLR4 induce activation of NF-κβ resulting in increased transcription of NLRP3 and more pro-IL-1β release (Gaidt et al., 2016). As such, it could indicate that increased level of NLRP3 available for formation of NLRP3 inflammasome tilts the steady state kinetics of ICEinh interaction with caspase-1 due to the formation of more pro-caspase 1 that is activated to caspase-1 molecule at levels significantly higher than the ICEinh concentration.

Macrophages unlike monocytes do not readily secrete IL-1β based on the finding here and those from other studies (Carta et al., 2011, Madej et al., 2017), this may be attributed to the tissue resident role of macrophages which is less involved promoting inflammation rather than secretion of chemokine required for recruitment of immune cells (Madej et al., 2017). This line of evidence supports the finding here that TDMs used in this study secrete significantly higher IL-8 than monocytic THP1. IL-8 is a
chemokine that facilitates recruitment of immune cells like neutrophils to site of inflammation and different lines of evidence now exist in the literature for its tumour promoting role based on its chemokine activities (Turner et al., 2014, David et al., 2016). The finding here indicated that AgNP also induced higher release of IL-8 compared to Lipo-AgNP, although both nanoparticles induced release of IL-8 higher than the basal levels in TDMs. In addition to this, AgNP induction of IL-8 release in THP1 monocytes was similar to that induced by LPS and this may indicate a possible contribution of AgNP mediated inflammation that may favour tumour development. LPS has been demonstrated to induced cancer development and promote tumour invasion and metastasis (Kurago et al., 2008, Seol et al., 2017), which may be linked to the IL-8 secretion and the chemokine activity. As such, AgNP stimulation of IL-8 release may facilitate IL-8 chemokine activity in the same manner. This may also explain the hormetic effect of AgNP in stimulating THP1 cell proliferation (data not shown) and in HepG2 and A549 cancer cells (Sthijns et al., 2017, Jiao et al., 2014). Interestingly, HepG2 cell proliferation have been shown in another study to be inhibited by gallic acid through inhibition of IL-8 secretion (Lima et al., 2016). Although, IL-1β release in the TDMs was significantly less than in THP1 monocytes, LPS priming of TDM alone or with ATP stimulation resulted in IL-1β release comparable to that exhibited by the monocytic cell even under caspase-1 inhibition. Priming of TDMs might have resulted in much more higher transcription rate of NLRP3 resulting in no net effect of caspase-1 inhibition of IL-1β release upon ATP stimulation. Furthermore, monocytes response is heavily reliant on the formation NLRP3 inflammasome while macrophages utilise AIM2 inflammasome in addition to NLRP3. As AIM2 pathway has been previously shown to be activated by LPS for induction of IL-1β release (Turner et al., 2014), this pathway in addition to that of
NLRP3 may have been activated by LPS to induce activation of caspase-1 to process release of IL-1β.

Inflammation is tightly associated with activities of reactive oxygen species (ROS). Excessive generation of ROS such as that generated from the mitochondria during oxidative stress or even subtle changes in endogenous ROS level can induce cellular responses that activate redox sensitive proteins like NF-κβ causing inflammation due to upregulation of NF-κβ target genes such as IL-1β, IL-6 and TNF-α (Mittal et al., 2014, Forrester et al., 2018, Wang et al., 2007). Likewise, inflammatory response can induce generation of ROS. Human retinal pigment epithelial cells exposed to TNF-α, IL-1β and IL-6 exogenously were shown to exhibit increased ROS generation in a time and dose dependent manner from NADPH oxidase and mitochondrial induced oxidative stress (Yang et al., 2007). Many studies have documented ROS generation to be AgNP main mechanism of action as an antibacterial, even as anticancer (Yuan et al., 2017, Xu et al., 2012, El-Hussein and Hamblin, 2017, Gurunathan et al., 2013), as such it was hypothesised Lipo-AgNP will suppress AgNP induced inflammation as it does AgNP ROS generation.

In this study, exposure of the THP1 monocytes to AgNP resulted in significant release of IL-1β, IL-6, IL-8 and TNF-α but the nanoparticle did not influence inflammatory response of TDMs except suppression of IL-1β release. In support of this, evidence that AgNP induce inflammation is largely present in the literature. Sweeney et al. (2016) showed that AgNP induced secretion of IL-6 and IL-8 in mouse neuronal cells human alveolar type-I-like epithelial cells. In the same manner, Murphy et al. (2016) demonstrated increased gene expression of IL-1, IL-6 and TNF-α and increased secretion of pro-IL-1β upon exposure of THP-1 and primary human blood monocytes to AgNP. These observations have also been recorded in non-human models. Park et
al. (2011a) demonstrated that intra-tracheal instillation of silver nanoparticle in mice resulted in a time and dose dependent increase in release of IL-1, IL-2, IL-6 and TNF-α. It was also found that AgNP resulted in upregulation of genes associated with inflammation and tissue damage. Huang et al. (2015) also showed in mouse nerve cells that AgNP induced increased release of IL-1β as well as increased gene expression of CXCL13 chemokine and glutathione synthetase likely due to induced oxidative stress. Thus, this observed inflammation may be linked to ROS generation by AgNP. Interestingly, Lipo-AgNP suppressed both AgNP-mediated inflammation and basal cytokine release by significantly suppressing the release of IL-1β, IL-6, IL-8 and TNF-α in THP1 monocyte. In TDMs, Lipo-AgNP suppressed IL-1β and TNF-α release while maintaining basal level of IL-6 which was aggravated by AgNP. Coupled with the finding from the previous study that Lipo-AgNP suppresses generation of ROS (Yusuf et al., 2018), DPPC which is the major component of the liposome encapsulating AgNP in Lipo-AgNP is known to also possess an immunosuppressive feature (Murphy et al., 2015b). Sweeney et al. (2016) had shown that a DPPC containing commercial surfactant prevented release of IL-6 and IL-8 in addition to the near abolishment of ROS generation in human alveolar type-I-like epithelial cells. As such, Lipo-AgNP may have suppressed the inflammatory response by preventing generation of ROS, supporting the finding here that AgNP generated ROS induced the observed inflammation. The immunosuppressive activity of Lipo-AgNP opens up an application in treatment of RA and other inflammatory diseases. Involvement of bacteria and bacterial-induced inflammation have been reported in the development of RA due to the microbe exacerbation of inflammation and oxidative stress, and sometimes induced autoimmunity (Pretorius et al., 2017, Olsen-Bergem et al., 2016). As such, Lipo-AgNP can serve as a double-edged sword to suppress the induced
inflammation at the joint and to exert its antibacterial effect on the pathogens within the RA joint.

As a major PAMP responsible for bacterial induced inflammation during infection, LPS binding to CD14, TLR2 and TLR4 are prominent to initiation of cytokine release. The application of AgNP as antibacterial is fared to drive inflammation into a chronic state that may favour development of diseases like cancer and autoimmunity. It was discovered that AgNP supressed LPS-induced IL-1β (at 1 µg/ml) and TNF-α release (at both concentration) while Lipo-AgNP (at 1 µg/ml) only suppressed release of IL-1β in THP1 monocytes. While AgNP significantly enhanced LPS induced-IL-6 release, Lipo-AgNP exposure of THP1 monocytes did not induce IL-6 release. In TDMs, only 2 µg/ml Lipo-AgNP mediated suppression of LPS-induced TNF-α. The reason for suppression of LPS induced IL-1β and TNF-α release by either of AgNP or Lipo-AgNP in monocytic THP1 or TDM is not known but it could be that LPS interaction with AgNP modifies the surface chemistry of both AgNP/Lipo-AgNP and LPS resulting in alteration in interaction between LPS and the TLRs. Thus, subsequently modulating expected inflammatory response. However, further studies are required to probe this possibility. Another possible explanation could be that both AgNP and Lipo-AgNP at the right concentrations can inhibit iNOS (inducible nitric oxide synthase) which may have resulted in suppression of LPS-induced IL-1β and TNF-α. In a study by Sarkar et al. (2008), allylpyrocatechol obtained from crude extract of from piper beetle leaf was shown to inhibit iNOS mRNA expression resulting in suppression of LPS-induced secretion of TNF-α, COX-2 and IL-12p40 in RAW 264.7 macrophages. Other compounds such as neocryptotanshinone and naringenin, which are plant extracts were also shown in different studies to inhibit LPS-induced mRNA and protein expression of IL-1β, IL-6 and TNF-α in RAW 264.7
macrophages upon inhibition of iNOS (Kumar and Abraham, 2017, Wu et al., 2015). A study supporting the activity of AgNP in suppression of LPS induced inflammation through suppression of iNOS and nitric oxide was that of Haase et al. (2014), who showed that AgNP suppressed nitric oxide synthesis in human neutrophils and macrophages. Another study also reported that AgNP attenuated the expression of iNOS inhibiting production of nitric oxide in Hep-G2 cells (Zuberek et al., 2017). Although findings of other studies have shown that AgNP can induce expression of iNOS due to increased ROS generation such as in pancreatic cancer and osteoblastic cell lines (Zielinska et al., 2016, Barcinska et al., 2018), this may be as a result of differing cellular responses to AgNP because of the different genetic backgrounds.

It has been demonstrated that AgNP induced inflammatory responses, which is suppressed by encapsulation in DPPC based liposome. Particular, AgNP induced release of IL-1β, IL-6 and TNF-α indicating that it may induce persistent inflammation through STAT-3. To investigate this possibility, the effect of AgNP on the expression of STAT-3, a transcriptional factor in the JAK/STAT pathway known to be involved in sustained inflammatory response through its regulation especially by IL-6 family of protein (Wang et al., 2013b), was examined. It was found that exposure of LPS-monocytic THP1 to AgNP resulted in significant induction of STAT-3 expression. On the other hand, TDMs exposure to AgNP was found to induce significant expression of STAT-3 irrespective of LPS priming. This finding in addition to those existing in the literature suggests AgNP may sustain inflammatory response by at least indirectly activating STAT-3 expression. For example, IL-1β, IL-6 and TNF-α as found to be induced by AgNP in this study have been previously shown to induce STAT3 phosphorylation and activation causing prolonged inflammation and joint destruction in RA mice (Mori et al., 2011). IL-6 binding with its receptor, IL-6R, is known to
indirectly induce activation of STAT-3 through IL-6R interaction with epidermal growth factor receptor (EGFR) or activation of janus kinase (JAK) leading to STAT-3 activation (Figure 6-7). This STAT-3 activation subsequently results in prolonged inflammation through continued expression of proteins like the MAP kinase (MAPK) and interleukin-1 receptor associated kinase binding protein-1 (IRAK1BP1) that play crucial role in inflammation and cancer (Wang et al., 2013b). The fact that Lipo-AgNP maintained STAT-3 expression within the level observed in untreated control THP1 monocytes and TDMs may be indicative of the suppressive role of the encapsulation on AgNP mediated inflammation. Taken together, Lipo-AgNP may be a potential in treatment of RA and the pannus formation in RA joint. Like a double-edged sword, Lipo-AgNP may treat the RA killing the often-colonised bacteria and suppressing the provoked chronic inflammation. In addition, Lipo-AgNP may be useful in treating other bacterial diseases that are characterised with heightened inflammation like ulcerative colitis and Crohn’s disease or even cancer like inflammatory breast cancer.
Figure 6-7. Mechanism of AgNP sustenance of perpetual inflammation.

Entry of AgNP into the cell can result in generation of ROS through mitochondrial membrane disruption and cytochrome C release or through ionisation of the nanoparticle by the aqueous milieu of the cytoplasm. The generated ROS could activate IKKβ releasing it from the IKK complex. IKKβ then activates NFκβ re-pathway which in turns activates IKK which in turns activate NF-κβ facilitating its translocation into the nucleus to initiate transcription of pro-inflammatory cytokines like IL-1β, IL-6 and TNF-α. ROS can also activate NFκβ through the MAP kinase (MAPK) pathway. Release of IL-6 and its subsequent interaction with IL-6R can then activate the JAK/STAT3 pathway leading to phosphorylation and activation of STAT3 which facilitates transcription of factors like MAPK that can further activate upstream signaling cascade in the NFκβ pathway further creating a continuous loop of cytokine release.
6.3 Conclusion

While AgNP suppression of LPS-induced IL-1β and TNF-α may be indicative of a somewhat favourable contribution of the nanoparticle during LPS triggered inflammation, AgNP provocation of inflammatory response in the absence of LPS indicates otherwise. AgNP is mainly used in preventing bacterial infection which means human exposure to AgNP is highly likely under sterile conditions such as in sterilised items like garments, cosmetics and medical equipment that contain AgNP with intent of preventing rather than treating infection. As such, the increased contact in such conditions may likely drive inflammation that are favourable for chronic diseases like cancer and inflammatory diseases. On the other hand, It has been shown that Lipo-AgNP effectively suppressed AgNP mediated inflammatory responses in both monocytic THP1 and TDMs. Based on the evidence presented here and that in Chapter 4, Lipo-AgNP application will not only translate to lower AgNP concentration required to achieve effective cytotoxicity, but will likely mitigate AgNP-mediated inflammation, preventing vicious cycle of chronic inflammation that favours disease development and progression. This will prove especially useful in treating inflammation induced bacterial disease such as RA and the associated pannus development, ulcerative colitis and Crohn’s disease, all of which are characterised with bacterial infection and chronic inflammation since AgNP is an antibacterial coupled with the immunosuppressive properties of Lipo-AgNP.
Chapter 7
7 General Discussion

AgNPs are the most researched and most commercialised nanoparticles primarily owing to their antibacterial properties against a wide range of bacteria strains (Vance et al., 2015). The main mechanism of AgNP induced bactericidal effect has been demonstrated to be due to intracellular ROS generation upon ionisation of the nanoparticle within the aqueous environment of the cell (El-Hussein and Hamblin, 2017, Abdal Dayem et al., 2017). Furthermore, research has now shifted into the investigation of AgNP as an anticancer agent since it is now known that AgNP has some anticancer properties. As an antibacterial, AgNP is present in various everyday consumable product such as cosmetics, food items and apparels in very high concentrations (discussed in section 1.1.1), which in addition to the potential anticancer applications will result in increased human exposure. Not only will this repeated human exposure to AgNP pose an impending risk of adverse effects development to human, AgNP that leaches into the ecosystem poses great risk to marine organisms and wildlife.

Many studies have demonstrated the cytotoxic effects of AgNP in different organisms ranging from marine organisms such as *Daphna magna* and *Chaetoceros curvisetus* to mammals such as mice and Guinea pig as well as several human cell lines (Korani et al., 2013, Lodeiro et al., 2017, Kawata et al., 2009, Yusuf et al., 2018, Hou et al., 2017). While there have been no studies directly evaluating the toxicity of AgNP on human subjects, there are reports in the literature indicating that exposure to high concentrations of AgNP is linked to cardiotoxicity, hepatotoxicity and nephrotoxicity (Tacar et al., 2013). In addition, AgNP at high concentration is also linked to less toxic conditions such as agyria, eye and skin discolouration (Tak et al., 2015). In addition to the reports on possible AgNP toxicity in humans, several *in vitro* and *in vivo* studies
have reported inflammatory response induced by exposure of specific cells or animals to AgNP (Murphy et al., 2016, Fraser et al., 2018, Park et al., 2010b, Park et al., 2011a, Park et al., 2011b).

To circumvent the toxic effect AgNP and the potential of the adverse effect, it was hypothesised that improving the intracellular delivery of AgNP will enhance its cytotoxicity and possibly the induced inflammation, resulting in lessened concentration of the nanoparticle in consumable products and mitigated risk of adverse effects. This step is also hoped to reduce the amount of AgNP that will eventually be released into the ecosystem. Unfortunately, there are no studies till date that have researched the possible ways of enhancing AgNP toxicity as well as mitigating AgNP-induced inflammation. Hence, this thesis aimed to enhance AgNP delivery and subsequently its cytotoxicity by encapsulating the nanoparticle in a DPPC-based liposome for improved delivery and suppression of AgNP-induced inflammation owing to the anti-inflammatory role of DPPC (Sweeney et al., 2016, Murphy et al., 2015b).

7.1 AgNP encapsulation in DPPC based liposome by extrusion method produced stable nanocapsules and enhances nanoparticle cytotoxicity

The first step in this thesis was to synthesise AgNP by chemical reduction of AgNO₃ by NaBH₄ in a cold environment following the equation in section 3.9. Two different encapsulation methods, which are probe sonication and extrusion techniques, were then trialled for preparation of Lipo-AgNP to yield PB-Lipo-AgNP and Ex-Lipo-AgNP respectively. Data from DLS analysis showed PB-Lipo-AgNP and Ex-Lipo-AgNP to be of 143 nm and 140 nm respectively. However, PB-Lipo-AgNP had a second peak of agglomerated nanoparticle of roughly 5 µm in size which made up about 14% of the nanoparticle (Table 3-1) which was also confirmed by SEM and
STEM. The uptake rate nanoparticles is affected by their sizes (Foroozandeh and Aziz, 2018), giving the first insight into the acceptance of Ex-Lipo-AgNP over PB-Lipo-AgNP. Contrastingly, Ex-Lipo-AgNP formed distinct population of spherical liposomes. The STEM showed that AgNP in the Ex-Lipo-AgNP to be present within the liposome but some AgNPs were found on the surface of the PB-Lipo-AgNP. This indicated free unencapsulated AgNP which was supported by the EE found to be 67.8% for PB-Lipo-AgNP and 86.5% for Ex-Lipo-AgNP. In addition, the UV-Vis spectra of the PB-Lipo-AgNP was similar to that of uncoated AgNP most likely due to the free unencapsulated AgNP in the PB-Lipo-AgNP. This was also apparent in the golden yellow colour of the uncoated AgNP and PB-Lipo-AgNP (Figure 3-2). The free AgNP on the PB-Lipo-AgNP may be responsible for the drastic increase in the average nanoparticle size in culture media in a similar fashion to uncoated AgNP.

Nanoparticles such as AuNPs are known to interact with the protein corona in culture media as a consequence of its reactive surface (Sabuncu et al., 2012). Contrastingly, this aggregation with protein was not observed for Ex-Lipo-AgNP, likely because most of the AgNP are present within the liposome causing no net change in the nanoparticle size from water to culture media.

To further evaluate the applicability of both Lipo-AgNP preparations for biological studies, the stability and drug release profile of both PB-Lipo-AgNP and Ex-Lipo-AgNP were carried out. Findings indicated that Ex-Lipo-AgNP was more stable over a 6-month period with minimal increase in size and reduction in zeta potential compared with PB-Lipo-AgNP at both 4°C and 24°C (Table 3-2). DLS analysis of temperature-dependent size change indicated that Ex-Lipo-AgNP was more stable in RPMI culture media compared with PB-Lipo-AgNP which exhibited a time dependent increase in size over a 6 h period from 20°C at 0 h to 37°C at 6 h. This reduction in
size was attributed to the loss of the liposomal water content due to osmosis since the culture media is of higher osmolarity compared with the aqueous content of the liposome (Monteiro et al., 2014). This data also supports the finding here that PB-Lipo-AgNP possess a burst release of content at both pH 6.5 and 7 unlike the Ex-Lipo-AgNP which showed a steady load release. Based on this, PB-Lipo-AgNP is suggested to have a less stable liposome resulting in quick loss of liposomal content.

The large size of PB-Lipo-AgNP due to agglomeration, its less stable liposome both in water and culture media, and the poor drug release profile all suggest that it is of less biological potential compared with Ex-Lipo-AgNP. A 24 h cytotoxicity profile of both Lipo-AgNP preparations were then evaluated on THP1 monocytes. Data from Alamar blue, flow cytometry and confocal microscopy all showed that Ex-Lipo-AgNP was more cytotoxic than PB-Lipo-AgNP. In fact, PB-Lipo-AgNP had similar cytotoxicity profile compared with uncoated AgNP which is likely due to high quantity of unencapsulated AgNP in the PB-Lipo-AgNP. The less cytotoxic nature of PB-Lipo-AgNP was suggested to be due to the large size affecting endocytosis of the nanoparticle as larger particles exhibit poor intracellular uptake. The stability of Ex-Lipo-AgNP and the uniform size must have allowed increased intracellular uptake and delivery of the nanoparticle in a manner that allowed efficiently load release.

7.2 Ex-Lipo-AgNP (Lipo-AgNP) induced ROS-independent and caspase dependent apoptosis

AgNP mechanism of antibacterial and anticancer activity has been widely investigated to involve ROS generation, degradation on intracellular structures, lipid peroxidation and inhibition of bacterial cell growth (Qing et al., 2018, Hsueh et al., 2015, Oves et al., 2018). However, there has been no study investigating encapsulation of AgNP in a liposome and the effect this modification will have on the mechanism of AgNP
antibacterial and anticancer activities. Based on the superior biological activity of Ex-Lipo-AgNP, this Lipo-AgNP preparation was selected for the subsequent studies and its biological activities were compared with uncoated AgNP to evaluate how the encapsulation modifies AgNP activities in THP1 monocytes. The rationale behind the employment of THP1 monocyte in this research is because of the following;

1. THP1 monocytes are a leukemic (cancer) cell line, thus allowing investigation of the toxicological profile of Lipo-AgNP on a cancer cell line.

2. Monocytes and similar immune cells such as macrophages are important component of the innate and adaptive immune response to foreign objects including nanoparticles upon human exposure (Mrakovcic et al., 2014, Rueda-Romero et al., 2016, Robbins et al., 2015), making the cell line a perfect model to also study the effect of the nanoparticle on the innate immune system which is relevant in the case of nanoparticle induced cytokine release.

The 24 h IC$_{50}$ of Lipo-AgNP was first estimated for better comparison of the nanoparticle toxicological profile. Lipo-AgNP was found to have a 24 h IC$_{50}$ of 3.045 μg/mL compared to AgNP with 4.991 μg/mL. Exposure of the THP1 monocytes to 0.625 μg/mL of either of AgNP or Lipo-AgNP, a concentration that is considerably lower than the IC$_{50}$ of both nanoparticles, indicated from flow cytometric data, that Lipo-AgNP possessed significant cytotoxic effect on the cells compared to unexposed and AgNP-exposed THP1 monocytes. The possible explanation for this was either the liposome was toxic, or the encapsulation must have enhanced the toxicity of the nanoparticle. Exposure of the cells to equivalent concentration of empty liposome of the same size was found to show no effect on the cell viability, suggesting the encapsulation must have enhanced the toxic effect of AgNP.
In determining cell viability upon exposure to toxic agents, analysis of the cell cycle profile and investigations of apoptotic hallmarks are crucial to the determination of cytotoxic impact of any pharmacological agent (Orrenius et al., 2010). The cell cycle profile of THP1 monocytes exposed to 0.625 µg/mL of either AgNP or Lipo-AgNP showed that Lipo-AgNP induced interruption in the cell cycle at the S-phase causing less THP1 monocytes progressing to the G2/M phase. Interestingly, the cells that failed to progress to the G2/M phase were found present in the Sub-G1. Since the Sub-G1 phase cells are cells with fragmented DNA, these data suggest that Lipo-AgNP might have induced DNA fragmentation preventing DNA replication in the S-phase causing the observed cell death.

AgNP ionisation into Ag\(^+\) which may cause oxidation of cellular proteins and lipids releasing ROS which may cause DNA damage (Marnett, 2002). It was thus hypothesised that Lipo-AgNP might have induced significant ROS generation that resulted in DNA damage. Investigation into the ROS induction by both AgNP and Lipo-AgNP showed that while AgNP at 0.625 µg/ml did not cause significant cell death or interruption of the cell cycle of THP1 monocytes, it induced some level of ROS. On the contrary, Lipo-AgNP at the same concentration resulted in ROS suppression, suggesting that ROS generation was not responsible for the DNA damage in the THP1 monocytes. The activity of ROS upstream of caspase 3 and 7 will suggest that Lipo-AgNP induced cell death may not involve the executioner caspases. However, the activation of the executioner caspases 3 and 7 was investigated to confirm the type of apoptosis involved. Contrary to expectation, it was found that exposure of the THP1 monocytes to Lipo-AgNP resulted in significant caspase 3 and 7 activation and cell death in comparison to AgNP and control unexposed cells.
The data here suggested that Lipo-AgNP may have caused significant improvement in the intracellular uptake of the encapsulated AgNP which must have remained within the liposome and protected the nanoparticles from ionisation by the aqueous intracellular environment. The entry via the endo-lysosomal pathway may have caused nuclear delivery of the AgNP resulting in DNA damage and subsequent activation of the executioner caspases causing ROS independent cell death.

7.3 Lipo-AgNP improved intracellular nanoparticle delivery and causes redox imbalance to induce cell death

It has been established that Lipo-AgNP can induce significant cytotoxic effect on THP1 cell at very low concentration and the induced cell death is ROS independent but caspase dependent. The mechanistic detail of Lipo-AgNP cytotoxicity was further probed in TDMs obtained from THP1 monocytes. As stated earlier, both monocytes and macrophages are important to the functioning of the innate immune response. In addition, macrophages play important role in development and progression of chronic diseases such as cancer and arthritis (Lameijer et al., 2013), making them a potential therapeutic targets in treatment of these diseases. Cell viability studies carried out on the TDMs post-exposure to AgNP and Lipo-AgNP showed a similar toxicological profile for both nanoparticles to what was observed in the monocytes, although the IC560 in the TDMs were higher than in the monocytes. The 24 h IC₅₀ values of AgNP and Lipo-AgNP was found to be 5.71 µg/ml and 3.98µg/ml respectively indicating a higher dose requirement in the TDMs to achieve same toxicity levels as in the THP1. This differential response of THP1 monocytes and TDMs to AgNP and Lipo-AgNP may be dependent on the nanoparticle uptake rate of the cells which is known to be different for monocytes and macrophages. Unlike monocytes which only uptake particles by endocytosis, macrophages are specialised cells for phagocytosis in
addition to endocytosis (Lunov et al., 2011), causing the TDMs to exhibit higher nanoparticle uptake.

Based on the higher IC$_{50}$ of the nanoparticles in TDMs, a higher working concentration was implemented in the TDMs. While 0.625 µg/mL was used in THP1 monocytes, 2 µg/mL was used in the TDMs for all biological studies. Cell viability studies by confocal microscopy aided with calcein-AM stain showed that AgNP at 2 µg/mL resulted in considerable but non-statistically significant reduction in cell viability after 24 h. On the contrary, Lipo-AgNP induced significant reduction in cell viability ($p < 0.01$). Investigation of ROS generation in the TDMs after exposure to the nanoparticles revealed that not only did Lipo-AgNP significantly suppressed AgNP-induced intracellular ROS generation for the 4 h period considered, it maintained the ROS level at level similar to that in control untreated cells. Macrophages are known to generate significant levels of intracellular ROS and the ROS induction plays is an important mechanism in their roles in diseases such as cancer and cardiovascular disease (Roux et al., 2019, Bae et al., 2009).

Data obtained here for cell viability and ROS studies were similar to that observed in THP1 monocytes (section 4.1.5). This led to the investigation of the GSH level in the TDMs after nanoparticle exposure. It was expected that high ROS would result in a low GSH level and vice versa. This was true for AgNP, as the high ROS level led to significant reduction in the GSH level. Unexpectedly, a significantly low GSH level which interprets to a redox imbalance was observed in the TDMs exposed to Lipo-AgNP. Conventionally, the only specific function attributed to intracellular ROS accumulation has been known to be damage to cellular structures and subsequent cellular dysfunction as seen in chronic pathologies such as cancer, neurodegenerative diseases and premature ageing (Sena and Chandel, 2012). Furthermore, ROS has been
considered to be a waste from aerobic metabolism and consequence of cellular toxicity. However, studies in the last decade have shown that low ROS level acts as redox activator to signal proliferation and cellular differentiation (Zhu et al., 2012, Hernández-García et al., 2010). On the contrary, the finding here and that of Liu et al. (2014a) showed association between low ROS level and cellular apoptosis post exposure to cytotoxic agents. Liu et al. (2014a) demonstrated that the survival of HepG2 cancer cell was dependent on a balance between the ROS and antioxidant level. The sharp reduction in ROS level induced by the dihydromyricetin used in the study resulted in imbalance redox state perturbing the ROS signalling further resulting in apoptosis. The low ROS level induced by Lipo-AgNP may have used the same mechanism.

The cellular redox state is also often used to describe the GSSG/GSH level and under normal physiological condition, the this is maintained within narrow range just as pH is regulated. In pathological conditions, the redox state can be abnormally high or low (Flohé, 2011, Liu et al., 2014a). Low GSH levels have been shown to be associated with mitochondrial dysfunction and apoptosis, which can sensitize tumour cells to chemotherapy (Nie et al., 2009). The data indicated that Lipo-AgNP dose dependently decreased GSH levels in the TDMs with activation of caspase 3 and 7. The low GSH level as such may have resulted in mitochondrial dysfunction, release of cytochrome C and the observed caspase 3 and 7 activation, causing apoptosis. Bax and Bcl-2 are pro- and anti-apoptotic proteins regulating cell death and survival respectively, both of which maintain mitochondrial stability. A balanced Bax and Bcl-2 level is required for cell survival and high Bcl-2 expression results in inhibition of caspase-dependent apoptosis while high Bax expression induces apoptosis and vice versa (Luo et al., 2015). Bax binding with the mitochondrial membrane causes MOMP and release of
cytochrome C then caspase-dependent apoptosis. But this is prevented by inhibition of Bax through its binding with Bcl-2. In this study, the data showed that Lipo-AgNP caused suppression of Bcl-2 protein expression while Bax expression was unaffected. This tipped the Bax and Bcl-2 balance towards Bax causing it to exert its apoptotic effect on the TDMs through induction of MOMP. MOMP coupled with the low GSH level led to a possible release of cytochrome C and subsequent activation of caspase 3 and 7 dependent apoptosis (Figure 7-1).

Both AgNP and Lipo-AgNP induced caspase activation at 24 h but only Lipo-AgNP caused significantly higher cell death in comparison with the control untreated TDMs. This was an indication of difference in the kinetics of the nanoparticle uptake by the TDMs. To confirm this, the uptake of both AgNP and Lipo-AgNP were monitored for 4 h by flow cytometry and it was found that Lipo-AgNP had been internalised by the TDMs by 30 mins while AgNP internalisation was detectable after 45 mins. The improvement in Lipo-AgNP uptake can be attributed to the similarity between the cell membrane and the liposome shell of the Lipo-AgNP. Coupled with this, the 15 mins difference in uptake coupled with the redox imbalance may have enhanced the cytotoxic effect of Lipo-AgNP making it exert a more deleterious effect which was not observed for AgNP. In essence, improved uptake of AgNP within the liposome and the protection of the liposomal content form the cytosol changes the overall pharmacokinetic of the nanoparticle, thus altering the pathway utilised from that which AgNP would activate. From this, AgNP ionisation was prevented early on in the cytosol and the entry of Lipo-AgNP through the endo-lysosomal pathway must have provided a way for delivery of the AgNP content close to the nucleus for interaction with the DNA causing the observed cell death.
**Figure 7-1. Proposed mechanism of action of Lipo-AgNP.**

Lipo-AgNP is endocytosed and within the endosome which prevents early release of AgNP within the liposome preventing AgNP from ionisation in the cytosol. The AgNP from the liposome is thus released in the late endosome where it undergoes nuclear translocation to cause DNA fragmentation. This signals activation of ATM which phosphorylates CHK2 and subsequently activate p53. P53 in addition with the redox imbalance caused by suppressed ROS and GSH level prevents interaction of Bcl-2 inhibition and Bax causing Bax to form a membrane pore on the mitochondria resulting in cytochrome C release. This is followed by APAF1 oligomerisation and interaction with cytochrome C to form the apoptosome which proteolytically activate pro-caspase 9 forming caspase 9 that then induce activation of caspase 3 and 7 (Galluzzi et al., 2018).
7.4 Lipo-AgNP mitigates AgNP-induced inflammation

While AgNP and Lipo-AgNP has both been shown to activate caspase 3 and 7, Lipo-AgNP was found to significantly higher apoptosis when compared with AgNP at the same concentration after 24 h. Here, it was shown that AgNP induced ROS generation as previously demonstrated in several other studies (El-Hussein and Hamblin, 2017, Xu et al., 2012, Lodeiro et al., 2017). However, Lipo-AgNP with its enhanced cytotoxicity at the same AgNP concentration exhibited a ROS suppressive activity both in THP1 monocytes and TDMs. Intracellular ROS generation that is induced by internal factors such as metabolic processes or external factors such as pharmacological agents is tightly coupled to inflammation, playing crucial role in inflammatory diseases such as atherosclerosis, arthritis, diabetes and cancer (Forrester et al., 2018). AgNP has been reported and demonstrated in various studies to induce inflammation (Murphy et al., 2016, Fraser et al., 2018, Trickler et al., 2010), implicating AgNP-induced ROS in the nanoparticle mediated inflammation. Although there have been no human studies directly linking AgNP exposure to development of inflammatory disease, AgNP-induced inflammation is still a cause for concern.

Suppression of ROS generation by Lipo-AgNP led to the hypothesis that Lipo-AgNP may also suppress AgNP mediated inflammation. To test this hypothesis, THP1 monocytes and TDMs were exposed to AgNP and Lipo-AgNP. Monocytes and macrophages are important component of the innate and adaptive immune system in response to cellular invasion by pathogens and foreign particles. Upon recognition of a pathogen or foreign particle, monocytes and macrophages secrete pro-inflammatory cytokines which aids clonal expansion of specific subset of the immune system like lymphocytes, recruitment of inflammatory cells like T-cells or activation of others such as neutrophils to facilitate endocytosis, phagocytosis and cell killing of such
pathogens (Arango Duque and Descoteaux, 2014). Macrophages generate high level of ROS during chronic inflammation and in inflammatory diseases such as cancer and the ROS generation is implicated in the release of cytokines seen in these diseases (Kamp et al., 2011). Based on this, THP1 monocytes and TDMs were exposed to either of AgNP or Lipo-AgNP to study the effect of these nanoparticles on cytokine release. In THP1 monocytes, findings showed that AgNP induced significant release of IL-1β, IL-6, IL-8 and TNF-α in comparison with control unexposed THP1 monocytes. On the contrary, Lipo-AgNP maintained cytokine release level that was similar to that of control untreated THP1 monocytes for all cytokines. This is an indication that Lipo-AgNP was able to suppress AgNP mediated release of the cytokines in a similar fashion to what was observed in the ROS studies. Furthermore, AgNP and Lipo-AgNP did not influence LPS-induced cytokine release except Lipo-AgNP inhibition of LPS-induced IL-6 release which was maintained at the level of control unexposed monocytes.

In TDMs, both AgNP and Lipo-AgNP exposure resulted suppression of IL-1β and TNF-α when compared to the control untreated cells, but it was found that IL-1β release was more suppressed in AgNP exposed TDMs while TNF-α was more suppressed in TDMs exposed to Lipo-AgNP. It is interesting to note that TNF-α interaction with TNFR is a known activator of the extrinsic cell death pathway (Rauert et al., 2011). As such, TNF-α dependent apoptosis induction is unlikely the pathway activated by Lipo-AgNP especially as the endocytosis of Lipo-AgNP may have also resulted in endocytosis of TNFR death receptor preventing activation of this pathway. Unfortunately, the limitation of ELISA used in determining expression of these cytokines in this study prevent determination of their cellular localisation.
For IL-6, while the release was not significantly higher, AgNP exposure caused a considerable release of the IL-6 in TDMs (p = 0.5875). Just as observed in the monocytes, AgNP exposure cause enhanced the release of IL-8 in comparison to Lipo-AgNP exposure which caused similar release levels as that in control untreated cells. As observed in the monocytes, both AgNP and Lipo-AgNP did not suppress LPS induced cytokine release in the TDMs. Monocytes generally require cytokines like IL-1β for activation and maturation, while macrophages produce IL-1β to recruit immune cells to cite of inflammation. The difference in cytokine release pattern in the monocytes and TDMs may thus be attributable to difference in functional cytokine requirement and protein expression pattern of both cell lines. In addition, method by which the TDMs were obtained via PMA differentiation may also impact the cytokine release profile of the TDMs.

Lipo-AgNP successfully inhibited AgNP-induced release of IL-8 both in THP1 monocytes and TDMs. IL-8 is a chemokine that serves as chemoattractant for immune cells towards site of infection or inflammation, and it also has a tumour promoting role (David et al., 2016). Not only was AgNP induced IL8 release higher than that of control unexposed cells, it was similar to the levels both in THP1 monocytes and TDMs, suggesting AgNP might enhance both favourable and unfavourable chemokine activities of IL-8.

IL-1β, IL-6 and TNF-α play central role in chronic inflammation especially in tissue injury, cancer and inflammatory diseases like arthritis and atherosclerosis (Mori et al., 2011, Poh and Ernst, 2018). IL-1β, IL-6 and TNF-α may activate NF-κβ or STAT-3, transcriptional factors that activate transcription of inflammatory genes, thus perpetuating inflammation. In fact, there is evidence that TNF-α induced activation of NF-κβ can induce IL-6 secretion which in turn activates STAT-3 and induce
expression of STAT-3 target genes (McFarland et al., 2013). There is also a report of NF-κβ interaction with STAT-3 to induce expression of myriad of inflammatory cytokines such as IL-1β and IL-6 as well as transcriptional factors like STAT-3 and JAK-2 creating a feedback loop for STAT-3 activities in the IL-6/STAT-3 pathway (Ji et al., 2019). It was demonstrated here that exposure to AgNP in both TDMs and THP1 monocytes correlates with STAT-3 expression while Lipo-AgNP exposure did not affect STAT-3 protein expression. Put together, it is not implausible that repeated AgNP exposure may favour chronic inflammation and subsequent disease development or progression due to activities of IL-6/STAT-3 pathway. On the other hand, the inhibitory effect of Lipo-AgNP on the cytokine release both in THP1 monocytes and TDMs highlights the potential of the nanoparticle in suppression of chronic inflammation preventing progression of inflammatory diseases. Due to the limitations of the ELISA methods as to its indifference in identifying the different forms of STAT-3 (active and inactive), an overestimation of STAT-3 expression might have resulted.
8 Conclusions

This thesis has demonstrated that AgNP can be successfully encapsulated within DPPC-based liposome using both probe sonication and extrusion methods. However, data from characterisation studies carried out on both encapsulates obtained by both preparation methods indicated the extrusion method produced a more stable liposomal AgNP, which also exhibited a significantly more cytotoxic effect on THP1 monocytes in comparison with that obtained by probe sonication method or the free unmodified AgNP in very low concentrations. A probe into the mechanistic detail of Lipo-AgNP revealed that low concentration of Lipo-AgNP unlike AgNP as employed in this study suppressed ROS generation but activated caspase-dependent cell death in both THP1 monocytes and TDMs. From the data obtained in this study, the observed ROS-independent caspase activation was believed to be caused by DNA damage, Bcl-2 inhibition and redox imbalance caused by suppression of GSH upon exposure to Lipo-AgNP. While Lipo-AgNP was found to be more cytotoxic on both THP1 monocytes and TDMs, findings into the effect Lipo-AgNP on AgNP-dependent cytokine release indicated that Lipo-AgNP suppressed AgNP induced inflammation especially in THP1 monocytes. In TDMs however, Lipo-AgNP generally suppressed cytokine release but only AgNP-dependent release of IL-6 and IL-8 were suppressed by encapsulation while both AgNP and Lipo-AgNP suppressed IL-1β and TNF-α release in the TDMs.

In summary, this study has demonstrated Lipo-AgNP could be used to enhance AgNP cytotoxicity while at the same time suppress AgNP induced inflammation in THP1 monocytes and TDMs. Considering the role of macrophages for instance in cancer and arthritis, Lipo-AgNP may serve as a potential therapeutic that not only target inflammation induced within the tumour microenvironment and the inflamed pannus...
of rheumatoid arthritis. In addition, Lipo-AgNP has an established antibacterial activity on wide range of bacteria as alluded to in earlier chapters. As such, Lipo-AgNP may be a novel way of treating inflammatory diseases aggravated by bacterial infection such as ulcerative colitis and Crohn’s disease tackling both the bacterial colonisation as well as the chronic inflammation.

One major limitation of the methods and approach of the studies in this thesis was identification of the possible mechanistic processes involved in Lipo-AgNP and AgNP mode of action within the cell, but not sorting to proof these processes. For instance, IL-1β, IL-6 and TNF-α were investigated based on the suspicion of the inflammatory roles of AgNP which might involve these inflammatory cytokines. It is quite possible that other cellular factors might be involved in the mechanism of action of AgNP and Lipo-AgNP which may be further probed within the context of various experimental models to yield an in-depth information on the possible biological role of this nanoparticle in health and disease state.

8.1 Future studies

One of the main aims of this thesis was to encapsulate AgNP in a DPPC-based liposome to improve its delivery and enhance its cytotoxicity and study the mechanism of action of Lipo-AgNP. A probe into the mechanistic detail of Lipo-AgNP action revealed that exposure to Lipo-AgNP concentration lower than the IC50 caused significant DNA damage to interrupt the cell cycle progression, inhibited Bcl-2 expression and suppressed ROS and GSH levels subsequently causing activation of caspase 3 and 7. Lipo-AgNP was also found to suppress AgNP induced inflammation in the same manner as the ROS. One of the things to further investigate might be to investigate if AgNP induced ROS is directly responsible for the inflammation by
exposing Lipo-AgNP exposed cells to an ROS inducer such as hydrogen peroxide and evaluate the cytokine release.

This thesis highlighted some factors in the intrinsic apoptosis pathway that were modulated by Lipo-AgNP. It was shown here that Lipo-AgNP suppressed ROS, a key stimulus of caspase dependent apoptosis, followed by inhibition of Bcl-2 indicating possibility of a compromise of mitochondrial integrity leading to cytochrome C release. As such, the effect of Lipo-AgNP on mitochondria integrity need to be evaluated by examining the mitochondrial membrane and evaluating cytochrome C release in addition to apoptosome formation. While the GSH level of exposed cell was evaluated in this thesis, there is need for caution in interpreting the results. Evaluating the GSSG to GSH ratio in combination with the ROS levels may paint a more accurate picture of the redox status of the cells.

Lipo-AgNP has been demonstrated to have anti-inflammatory properties, it’s in vivo efficacy needs to be studied. Several animal models of RA have been developed (Fischer et al., 2017) and these can be utilised to investigate anti-inflammatory properties of Lipo-AgNP. For example, Lipo-AgNP could hypothetically be targeted at the inflamed joint especially RA joint that has formed pannus where aggressive macrophages and other inflammatory cells have localised. Lipo-AgNP could therefore exert its cytotoxic effect on the immune cells while at the same time suppress the inflammation.

Cytotoxicity of Lipo-AgNP was only tested on THP1 monocytes and TDMs as such there is a need to test Lipo-AgNP on a panel of cancer cell lines to investigate if it possesses enhanced activity and efficacy on the cancer cells in comparison to AgNP especially using cells which AgNP has been tested on. In addition, in vivo potential of
Lipo-AgNP need to be evaluated as different animal models are available for cancer studies (Cekanova and Rathore, 2014). Lipo-AgNP can be administered locally at tumour site to assess resection in tumour size. One major setback in the *in vivo* application of Lipo-AgNP will be the lack of specificity during systemic administration, making the nanoparticle potentially cytotoxic to normal cells. Here, it will be worth considering improving Lipo-AgNP to a targeted delivery system. This may involve conjugating moieties such as ligands like peptides or antibodies against receptors highly expressed on cancer cells or macrophages known to be present within the tumour microenvironment or RA joint. Furthermore, Lipo-AgNP can be trialled on a tumour model for targeted delivery to the tumour tissue.
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## Appendices

### Appendix A1: Table of materials and reagents

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List of Publications


