ACUTE EFFECTS OF SILVER NANOPARTICLES IN SH-SY5Y CELLS

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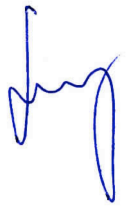
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Nanomaterials are appealing and valuable in various fields, as they are engineered to achieve particular physicochemical properties that are specific for their application. The present study was thus conducted in view of the mounting usage of silver nanoparticles (AgNPs) despite little being known about its toxicity and potential impacts on human health. The most prevalent toxicity mechanism for AgNPs is oxidative stress. Multiple studies have found that AgNPs preferentially disrupt mitochondrial function, since mitochondria are major reactive oxygen species (ROS) producers. Hence, the first part of the study was carried out to examine the acute effects of AgNPs in SH-SY5Y human neuroblastoma mitochondria, and possibly rescue the AgNPs-induced mitochondrial dysfunction through the use of docosahexaenoic acid (DHA) and l-carnitine (LC). Results from various mitochondrial assays revealed that acute exposure of AgNPs to SH-SY5Y cells caused mitochondrial dysfunction, in particular, disrupted mitochondrial membrane potential, and loss of both ATP and ADP, which could be rescued by co-supplementation with DHA and LC.

AgNPs have also demonstrated pro-inflammatory properties, although it is unknown if AgNPs enhance the effects of pro-inflammatory mediators, inhibit anti-inflammatory mediators, or both. Thus, the second part of the study was conducted to examine the relationship between AgNPs and phospholipase A_2 (PLA_2) enzymes, in particular $cPLA_2$ and $iPLA_2$, in SH-SY5Y cells. Real-time RT-PCR and

immunocytochemistry analyses revealed that $iPLA_2$ expression, but not $cPLA_2$, was altered following acute exposure to AgNPs. Interestingly, co-treatment of DHA and/or LC with AgNPs did not result in significant changes in $iPLA_2$ gene expression, suggesting that AgNPs affect $iPLA_2$ expression upstream that of its effects exerted on mitochondria, explaining for the inability of DHA and/or LC to alter $iPLA_2$ expression. Lipidomic analysis also revealed decreased $iPLA_2$ activity after acute exposure to AgNPs.

AgNPs have been reported to produce ROS in various cell lines, although it has not been determined if AgNPs-induced ROS exerts its effects on the inflammatory mediators. Therefore, the third part of the study was conducted to investigate the effects of AgNPs-induced ROS on $iPLA_2$ and to potentially elucidate the mechanism underlying changes observed. Antioxidant pre-treatment prevented the observed decrease in $iPLA_2$ gene expression following AgNPs exposure, indicating that the AgNPs-induced down-regulation of $iPLA_2$ gene involves ROS. Since the $iPLA_2$ promoter contains a sterol regulatory element (SRE) binding site for sterol regulatory element-binding proteins (SREBPs) that is not found on $cPLA_2$, the effect of oxidative stress on SREBP expression was investigated. Real-time RT-PCR analyses suggest that SREBPs are affected by AgNPs-induced ROS formation.

Taken together, the present study's results indicate that AgNPs exposure results in ROS formation causing a down-regulation

of SREBP expression. This potentially reduces the amount of SREBP transcription factors binding to $iPLA_2$ promoter, leading to a decrease in $iPLA_2$ expression and activity, and in turn resulting in mitochondrial dysfunction.

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ABBREVIATIONS

AA	Arachidonic acid
ADP	Adenosine diphosphate
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
AK	Adenylate kinase
AMP	Adenosine monophosphate
ANT	Adenosine nucleotide translocase
ATP	Adenosine triphosphate
AuNPs	Gold nanoparticles
BBB	Blood brain barrier
BSA	Bovine serum albumin
CACT	Carnitine-acylcarnitine translocase
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
Cer	Ceramide
CNS	Central nervous system
CNTs	Carbon nanotubes
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
CPT-1	Carnitine palmitoyltransferase-1
CPT-2	Carnitine palmitoyltransferase-2
CT	Threshold cycle
Cyt C	Cytochrome C

DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
e^-	Electrons
EBSS	Earl's balanced salt solution
ETC	Electron transport chain
H^+	Hydrogen ions
IMP	Inosine monophosphate
$iPLA_2$	Calcium-independent phospholipase A_2
JC-1 dye	5,5',6,6'-tetrachloro-1,1',3,3',-tetraethylbenzimidazolylcarbocyanine iodide
LC	L-carnitine
LOX	Lipoxygenase
LTD	Long-term depression
LTP	Long-term potentiation
LysoPC	Lysophosphatidylcholine
LysoPE	Lysophosphatidylethanolamine
MPTP	Mitochondrial permeability transition pore
NAC	N-acetyl L-cysteine
NPD1	Neuroprotectin D1
NPs	Nanoparticles
NRB	Nucleotide releasing buffer
PBCA	Poly(n-butylcyanoacrylate)
PBN	N-tert-Butyl-α-phenylnitrone
PBS	Phosphate buffered saline

PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
P_i	Inorganic phosphate
PLA_2	Phospholipase A_2
PLGA	Poly(lactic-co-glycolic acid)
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RES	Reticuloendothelial system
ROS	Reactive oxygen species
$sPLA_2$	Secretory phospholipase A_2
SP1	Sp1 transcription factor
SRE	Sterol regulatory element
SREBF1	Sterol regulatory element-binding transcription factor 1
SREBF2	Sterol regulatory element-binding transcription factor 2
SREBPs	Sterol regulatory element-binding proteins
SM	Sphingomyelin
TiO_2	Titanium dioxide

PUBLICATIONS

Different portions of this thesis have been published or are currently in preparation for publication in international referred journals:

1. **Tan JWS**, Ho CFY, Ng YK, Ong WY (2014) Docosahexaenoic acid and l-carnitine prevent ATP loss in SH-SY5Y neuroblastoma cells after exposure to silver nanoparticles. Environ. Toxicol.. doi: 10.1002/tox.22037 [Epub ahead of print].

2. **Tan JWS**, Ng YK, Torta F, Wenk MR, Ong WY (2015) Role of oxidative stress in silver nanoparticles-induced calcium-independent phospholipase A_2 changes. [In preparation for publication].

SECTION I

INTRODUCTION

1. Nanotechnology and Its Applications

1.1. Nanotechnology

Nanoscience and nanotechnology are the understanding and utilization of materials at a nanoscale, with size ranging between 1 and 100 nm, in a broad range of scientific fields including engineering, biology, and material sciences (United States National Nanotechnology Initiative, 2015). The nanotechnology concept was first introduced in 1959 by physicist Richard Feynman. He described a process in which individual atoms and molecules could be manipulated and controlled (United States National Nanotechnology Initiative, 2015). However, modern nanotechnology only begun in the 1980s, after the arrival of the scanning tunneling microscope, an instrument that allowed the viewing and manipulation of materials in the nanoscale (Fahlman, 2007, United States National Nanotechnology Initiative, 2015).

Nanotechnology can be broadly split into three divisions; nanomaterials – structures with at least one dimension smaller than 100 nm, nanodevices – production of nanoscale devices, and nanotools – techniques to synthesize and characterize nanomaterials and nanodevices (Borm et al., 2006). Although nanotechnology is a relatively new field, it has grown rapidly and extensively across wide-ranging industries including biomedical, cosmetic, environmental, and material applications (Taton et al., 2000, Cui et al., 2001, Asz et al., 2006, Chakraborty et al., 2009, Bi et al., 2015, Ongaro et al., 2015, Project on Emerging Nanotechnologies, 2015). Nanotechnology has

also brought about new multidisciplinary fields such as nanomedicine and nanobiotechnology (Moore, 2006, Amini et al., 2014).

Nanotechnology promises benefits to society and the economy. Current treatments for nervous system disorders are often inadequate due to the presence of the blood brain barrier (BBB), which restricts the uptake of neurotherapeutics (Abbott and Romero, 1996, Roney et al., 2005, Martel, 2015). However, nanoneuromedicine could offer plausible solutions. Nanoparticles (NPs) would be efficient drug carriers as the crossing of the BBB depends solely on the physicochemical properties of NPs and not the drug characteristics. Furthermore, manipulation of NP surface confers features that promote BBB targeting or enhances its crossing of the BBB (Masserini, 2013). Chitosan NPs, poly(lactic-co-glycolic acid) (PLGA) NPs, and gelatin nanostructured lipid carriers are promising carriers for nose-to-brain drug delivery (Seju et al., 2011, Md et al., 2014, Zhao et al., 2014b). Table 1.1.1 lists several NPs used as drug-delivery systems to the brain. Nanotherapeutics have also been used clinically, where some examples of FDA-approved drugs include Doxil (anti-cancer drug), Emend (anti-emetic drug), and AmBisome (anti-fungal drug) (Ventola, 2012). Iron oxide NPs have also been studied as a potential contrast agent for bioimaging to gauge brain tumor progression (Neuwelt et al., 2007, Gahramanov et al., 2011). Additionally, nanotechnology can improve the nature of food to meet the needs of a growing population. Crop quality and yield have been suggested to improve by using

appropriate concentrations of fullerol, a carbon-based NP (Kole et al., 2013). Carbon nanotubes (CNTs) are also commonly used to remove organic and inorganic pollutants from contaminated water (Li et al., 2003b, Peng et al., 2005). The attractiveness of nanotechnology has thus brought about global multi-billion dollar investments (Guzman et al., 2006, Oberdörster et al., 2007). The Singapore government has also jumped onto the nanotechnology bandwagon, investing large amounts of money on research and development (Levine, 2014).

Table 1.1.1 NPs as drug-delivery systems to the brain.

Type of NP	Surface modification	Size (nm)	Type of Drug	Type of action	Reference
CBSA	PEG	50 – 58	Aclarubicin	Anti-cancer	Lu et al. (2006)
Chitosan	Non-coated	15.23	Amyloid-β	Vaccine	Zhang and Wu (2009)
PBCA	Tween-80, PEG 20000	100	Hexapeptide dalargin	Anti-nociceptive	Das and Lin (2005)
PBCA	Polysorbate 80	41	Rivastigmine	Anti-Alzheimer's	Wilson et al. (2008)
PBCA	Polysorbate 80	112	Gemcitabine	Anti-cancer	Wang et al. (2009)
PLGA	(R)-g7	143 – 197	Loperamide	Opioid receptor agonist	Tosi et al. (2007)
PLGA	Alginate hydrogel	400 – 600	Dexamethasone	Anti-inflammatory	Kim and Martin (2006)
PLGA	Avidin	120	Leukemia inhibitory factor	Pro-neural, reparative cytokine	Zhao et al. (2014a)
PLGA	Glutathione	326.6	Triiodothyronine	Thyroid hormone	Mdzinarishvili et al. (2013)
Tripalmitin	Non-coated	358 – 362	Etoposide	Anti-cancer	Reddy et al. (2004)

1.2. Nanomaterials

Nanomaterials are defined as structures with at least one dimension smaller than 100 nm, where they can exist in various forms such as fibers, particles, tubes, and wires, of which NPs are considered the building blocks for nanomaterial production (Fahlman, 2007). They can be roughly categorized according to their chemical structures, such as metals (e.g. copper NPs), metal oxides (e.g. titanium dioxide (TiO_2) NPs), carbon-based (e.g. CNTs), and hybrid structures (e.g. quantum dots) (Handy et al., 2008, Smita et al., 2012). Nanomaterials are engineered to achieve particular physicochemical properties that are specific for product application. At the nanoscale, matters display unusual biological, chemical, and physical properties unique from that of bulk materials (Smita et al., 2012, United States National Nanotechnology Initiative, 2015). The reduction in size results in large surface area to volume ratio, rendering elevated reactivity with surrounding surfaces (Fahlman, 2007, Christian et al., 2008, Smita et al., 2012). Changes to nanomaterial's size or structure also result in alterations in fundamental properties, including improved strength, advanced optical characteristics, augmented chemical reactivity, and enhanced thermal or electrical conductivity (Fahlman, 2007, Singh et al., 2009, United States National Nanotechnology Initiative, 2015). These novel features enable nanomaterials to become appealing and valuable in various fields. In the biomedical field, nanomaterials are highly favored for utilization in drug delivery and/or targeting, especially

due to their ability to carry an array of drugs and be targeted to specific organs through manipulation of surface features (Hans and Lowman, 2002, Chouhan and Bajpai, 2009). For instance, rivastigmine is delivered to the brain for treatment of Alzheimer's disease via poly(n-butylcyanoacrylate) (PBCA) NPs coated with polysorbate 80 (Wilson et al., 2008). Commercially, there are close to 2,000 nanoproducts (Fig. 1.1.1) of which majority is made up of health and fitness related products, such as cosmetics, sporting goods, and clothing (Fig. 1.1.2) (Project on Emerging Nanotechnologies, 2015). Researchers are also exploring potentials of nanomaterials in scientific and technical areas such as biomaterials, clinical diagnosis, and tissue engineering (Basu et al., 2004, Stuart et al., 2006, Wang et al., 2014b).

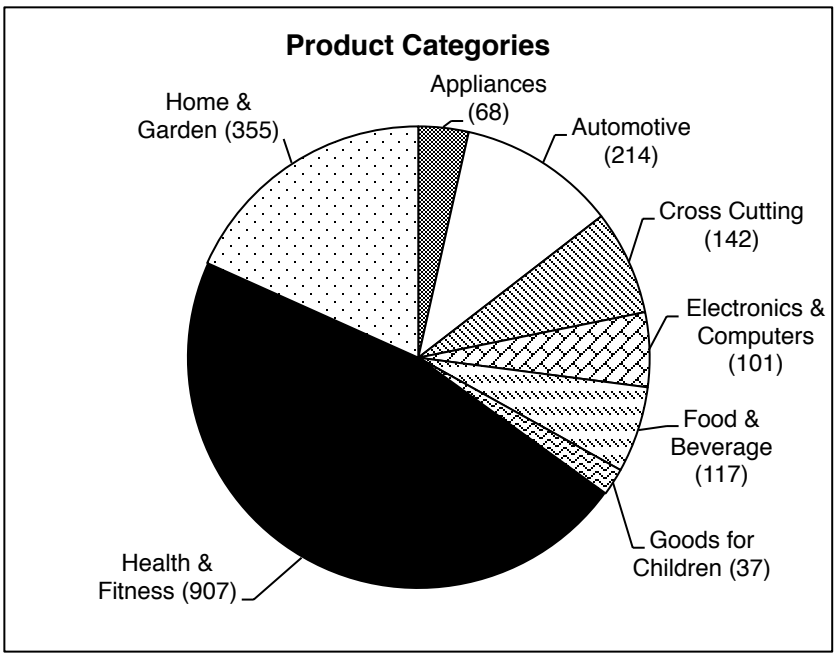


Fig. 1.1.1 Nanoproducts that are currently commercially available. Adapted from Project on Emerging Nanotechnologies (2015).

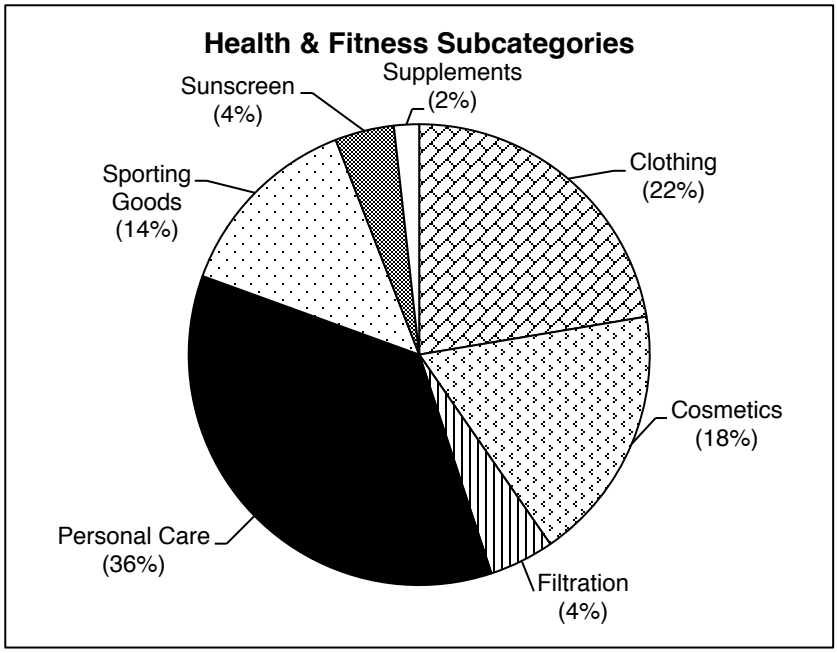


Fig. 1.1.2 Health and fitness nanoproducts that are currently commercially available. Adapted from Project on Emerging Nanotechnologies (2015).

However, these unique properties of nanomaterials could also result in potential health and environmental hazards (Guzman et al., 2006, Oberdörster et al., 2007). *In vitro* and *in vivo* tests have revealed adverse effects of nanomaterials. Nanomaterials have been reported to cause oxidative stress, genotoxicity, and cytotoxicity (Hussain et al., 2005, Li et al., 2008, Nalika and Parvez, 2015). Oxidative stress occurs when the antioxidant defenses in cells are unable to overcome reactive oxygen species (ROS) production (Halliwell, 1996), leading to a range of adverse biological consequences. Nanomaterials induce ROS generation in different manners including its inherent characteristics such as size and composition, and through interactions with cellular components like DNA and lipids, leading to oxidative stress, DNA damage,

mitochondrial dysfunction, and cell death (Li et al., 2008, Sohaebuddin et al., 2010, Chairuangkitti et al., 2013, Manke et al., 2013). Nanomaterials preferentially localize in mitochondria (Foley et al., 2002, Li et al., 2003a), and its deposition could disrupt mitochondrial functions by affecting the electron transport chain (ETC), leading to the formation of ROS and reduced adenosine triphosphate (ATP) production (Arora et al., 2008, AshaRani et al., 2009). Nanomaterials can also enter the bloodstream via multiple routes of exposure including dermal, inhalation, and oral (Table 1.1.2). They then circulate throughout the body, and are taken up by tissues and organs. Over time, it could lead to nanomaterial accumulation in organs, resulting in harmful effects such as tissue dysfunction and inflammation. Generally, nanomaterial biodistribution is size-dependent. Smaller nanomaterials tend to distribute to more organs, exhibit greater accumulation, and induce stronger toxicities than larger nanomaterials (Balasubramanian et al., 2013).

Table 1.1.2 Effects of NPs via various exposure routes in different organisms.

Organism	NP	Size (nm)	Exposure route	Affected organs	Comments	Reference
Sprague-Dawley rat	AgNPs	18 – 19	Inhalation	Lungs, liver, brain, kidneys	Dose dependent translocation, inflammation	Sung et al. (2009)
Hartley albino guinea pig	AgNPs	< 100	Dermal	Skin, liver, spleen	Histopathologic anomalies	Korani et al. (2011)
Wistar rat	AuNPs	20	Intravenous injection	Liver, spleen, kidney, testis	Gene expression changes	Balasubramanian et al. (2010)
Athymic nude mouse	Carbon dots	3	Intravenous, intramuscular, subcutaneous injections	Reticuloendothelial system (RES)	Low retention in RES, rapid clearance through kidneys	Huang et al. (2013)
CD-1 mouse	Ceria NPs	Not reported	Oral	Liver, lungs, blood	Inflammation	Poma et al. (2014)
Wistar rat	Manganese oxide NM	45	Oral	Brain, liver, spleen, blood	Histopathologic anomalies	Singh et al. (2013)

Nanomaterials could also be released into the environment during the production process and its subsequent use, leading to ecotoxicity. For instance, silver NPs (AgNPs) have been found to leach from commercially available products (Benn and Westerhoff, 2008, Kaegi et al., 2010, Farkas et al., 2011). Discharged nanomaterials may interact with the environment resulting in alterations in its physicochemical characteristics, potentially modifying its toxicity profile (Lyon et al., 2005, Klaine et al., 2008). Organisms, including microorganisms, will then take up the released nanomaterials in the ecosystem. Presence of CNTs and TiO_2 NPs in the living environment of rainbow trout has led to organ pathologies, biochemical effects, and respiratory problems (Federici et al., 2007, Smith et al., 2007). Nanomaterials have also shown to be toxic to microbes (Sondi and Salopek-Sondi, 2004, Lyon et al., 2005, Adams et al., 2006), which is critical as microorganisms form the foundation of both aquatic and terrestrial ecosystems (Jafar and Hamzeh, 2013).

1.2.1. Gold Nanomaterials

Gold NPs (AuNPs) have been used in various consumer products including cosmetics, food and beverage, and electronic appliances (Project on Emerging Nanotechnologies, 2015). It is also commonly employed in the development of biomedical applications such as cancer diagnostics and therapeutics (El-Sayed et al., 2006, Huff et al., 2007, Ojeda et al., 2007, Parry et al., 2013), treatment for

rheumatoid arthritis (Tsai et al., 2007, Lee et al., 2013), and cell imaging (Chen et al., 2005, Shukla et al., 2005b). AuNPs are thought to be safe as bulk gold is chemically inert. This allows the manipulation of AuNPs' size and shape without compromising its stability and toxicity profiles (Zhang, 2015). It has been established that AuNPs enter living cells through endocytosis (Shukla et al., 2005a), with several studies demonstrating that AuNPs are normally retained in endosomes (Goodman et al., 2004, Chithrani et al., 2006).

Conflicting reports of AuNPs' toxicity exists. It has been suggested that cytotoxicity of AuNPs is largely dependent on its physical features and cell lines. Several *in vitro* and *in vivo* studies have demonstrated that AuNPs do not show overt toxicity. Despite being taken up into human K562 cells, AuNPs did not cause acute cytotoxicity (Connor et al., 2005). Additionally, there was no inhibition in proliferation of normal peripheral blood mononuclear cells, while the proliferation of three multiple myeloma cell lines were significantly inhibited following AuNPs exposure (Bhattacharya et al., 2007). When exposed to zebrafish embryos (Bar-Ilan et al., 2009, Asharani et al., 2011) and mice (Zhang et al., 2010), AuNPs also did not exhibit significant cytotoxicity. Interestingly, Avalos et al. (2015) did not find consistent changes in cytotoxicity in response to differences in AuNPs size, however, Pan et al. (2007) found that 1 to 2 nm AuNPs were highly toxic, while larger AuNPs, of up to 15 nm, were non-toxic in four representative cell lines. On the other hand, Mironava et al. (2010)

determined that larger AuNPs led to major damage in human dermal fibroblasts. Similarly, AuNPs with cationic side chains were determined to be moderately toxic while anionic particles were nontoxic (Goodman et al., 2004). AuNPs may also be identified as foreign materials by immune cells resulting in acute inflammation and apoptosis (Cho et al., 2009).

Although AuNPs are usually associated with low or no significant cell death, AuNPs may cause serious cellular damage. AuNPs promoted abnormal actin fiber formation in human dermal fibroblasts, leading to decreased cell proliferation, adhesion, and motility (Pernodet et al., 2006). AuNPs could also trigger stress response pathways in cells, including activation of stress-specific kinases, glutathione transferase activities, and chaperone induction (Khan et al., 2007). Multiple studies have reported elevated intracellular ROS levels in response to AuNPs exposure (Taggart et al., 2014). Additionally, Khan et al. (2007) observed activation of more than 30 genes upon AuNPs exposure, indicating that unaffected cell viability does not equate to absence of cellular processes alterations.

1.2.2. Silver Nanomaterials

Silver has been known for its disinfectant properties for centuries. Silver powder was documented as ulcer treatment, while silver-containing cream is still used for serious burn wounds (Chen and Schluesener, 2008). Similarly, AgNPs have been established as

effective biocides against bacteria, fungi, and virii (Elechiguerra et al., 2005, Hernandez-Sierra et al., 2008, Kim et al., 2009a, Marambio-Jones and Hoek, 2010). Due to their innate strong antibacterial characteristic, AgNPs have been utilized in many daily commercial products including cosmetics, kitchenware and textiles. They are also frequently used in medical applications such as wound dressings and antibacterial coatings of medical instruments (Asz et al., 2006, Lee et al., 2007, Eby et al., 2009, Project on Emerging Nanotechnologies, 2015). As such, AgNPs are said to be the most frequently utilized nanomaterial in consumer products (Chen and Schluesener, 2008, Beer et al., 2012), making up approximately 24 % of all nanoproducts (Project on Emerging Nanotechnologies, 2015).

Despite its popularity, severe human health considerations have been brought up. AgNPs can reach the brain in a variety of ways. Inhalation of AgNPs allows AgNPs to reach the olfactory bulb and brain via traveling across the upper respiratory tract (Takenaka et al., 2001, Ji et al., 2007, Sung et al., 2009). AgNPs can also cross the BBB and damage its integrity (Tang et al., 2009, Sharma et al., 2010, Trickler et al., 2010), exposing the brain to previously restricted elements, such as immunological mediators and neurodestructive factors, leading to abnormal cellular reactions and injuries (Sharma et al., 2010). The most common mechanism of toxicity for AgNPs is oxidative stress. Exposure to AgNPs results in elevated ROS production, depletion of glutathione, and reduced superoxide dismutase activity (Hussain et al.,

2005, Arora et al., 2008, Hsin et al., 2008, Kim et al., 2009b, Mukherjee et al., 2012). Additionally, exposure to AgNPs led to obvious alterations in expression of stress response genes involved in oxidative stress, endoplasmic reticulum stress, and apoptosis in an *in vitro* human intestine model (Bouwmeester et al., 2011). Multiple studies have found that AgNPs preferentially disrupt mitochondrial function (AshaRani et al., 2009, Costa et al., 2010, Kang et al., 2012, Mukherjee et al., 2012, Stensberg et al., 2013), since mitochondria are major sites of ROS production. Excessive formation of ROS can result in mitochondrial damage, in turn leading to uncontrolled ROS generation (Guo et al., 2013). Apart from mitochondria, AgNPs also exert genotoxic effects in cells. The generation of ROS from AgNPs exposure causes spontaneous DNA damage, alterations to cell cycle, and ultimately cell death (Cooke et al., 2003, AshaRani et al., 2009, Kim et al., 2009b, Eom and Choi, 2010, Guo et al., 2013).

Exposure of animals to AgNPs also resulted in inflammatory responses. A single intratracheal instillation of AgNPs in rats resulted in distinct inflammatory responses across 28 days (Park et al., 2011). Additionally, a microarray analysis of mice liver RNA revealed alterations in expression of genes involved in apoptosis and inflammation that resulted in phenotypical changes following exposure to AgNPs (Cha et al., 2008). Similarly, rats exposed to AgNPs showed changes in expression of proteins that moderate ROS formation, inflammation and apoptosis (Kim et al., 2010).

2. Mitochondria and Its Related Compounds

2.1. Mitochondria

Mitochondria are commonly known as cellular powerhouses (Correia et al., 2010, Marchi et al., 2012). Apart from the generation of ATP, mitochondria are involved in other essential functions in cells, such as cell cycle regulation, differentiation, calcium homeostasis, and cell death (Raimundo, 2014).

Mitochondrion is made up of an outer and inner membrane enclosing a matrix space, which contains enzymes that are involved in terminal catabolic pathways, such as the citric acid cycle (Schaffer and Suleiman, 2007, Alberts et al., 2008). The inner mitochondrial membrane is a specialized convoluted phospholipid membrane that contains a series of enzymes, known as the ETC. ETC uses electrons to translocate protons across the inner mitochondrial membrane generating an electrochemical gradient, which makes up the mitochondrial membrane potential, for the reformation of ATP (Fig. 1.2.1) (Leuner et al., 2007, Schaffer and Suleiman, 2007, Alberts et al., 2008, Keane et al., 2011, Marchi et al., 2012, Voet et al., 2013). This process is known as oxidative phosphorylation, and accounts for 95 % of energy required by brain (Chang and Reynolds, 2006). ATP is transported out of mitochondria via adenine nucleotide translocase (ANT) for use in various parts of the cell, and in the process is hydrolyzed back to adenosine diphosphate (ADP) and inorganic

phosphate (P_i) (Duchen, 2004, Ballinger, 2005, Alberts et al., 2008, Voet et al., 2013).

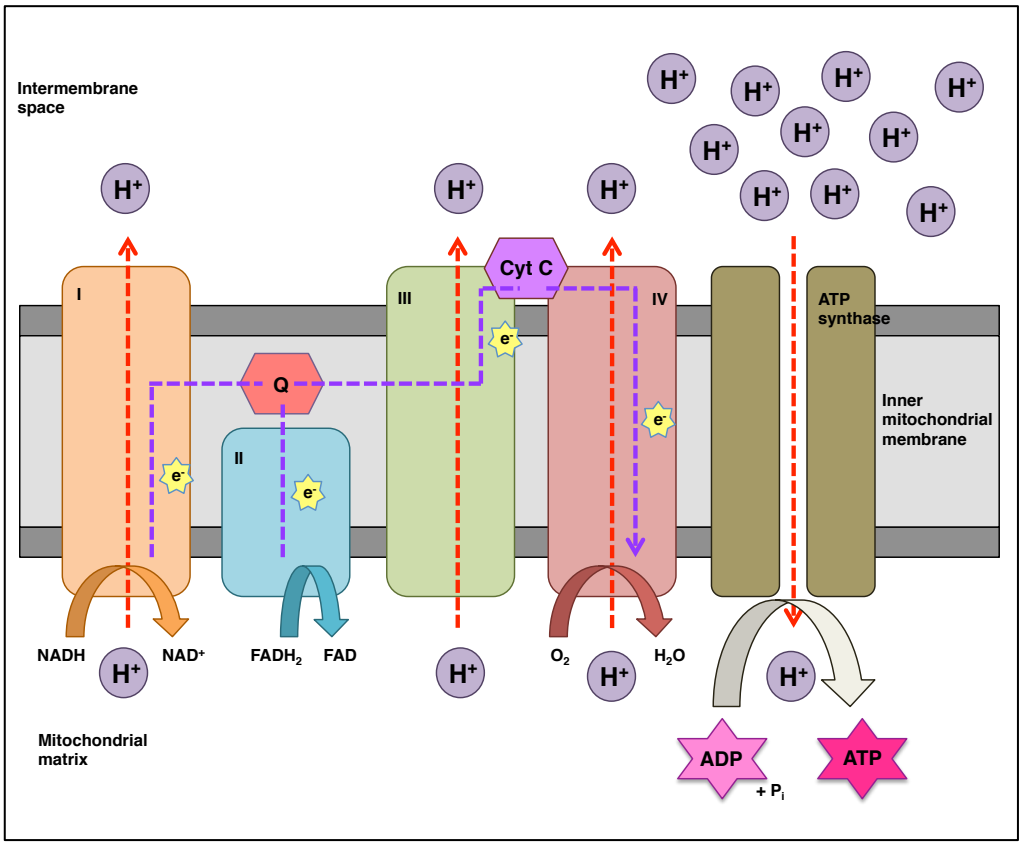


Fig. 1.2.1 Schematic diagram of the ETC. Electrons are transferred across mitochondrial complexes I to IV, resulting in the corresponding pumping of H^+ from the matrix (low concentration of H^+) to intermembrane space (high concentration of H^+), across the inner mitochondrial membrane. This electrochemical gradient is used for the reformation of ATP from ADP and P_i, via ATP synthase. Dotted red lines denote movement of H^+, while dotted purple lines denote movement of electrons (e^-). Abbreviations: Cyt C: cytochrome C; e^-: electrons; H^+: hydrogen ions; I – IV: mitochondrial complexes I to IV; Q: coenzyme Q. Adapted from Keane et al. (2011).

As a consequence to being the leading energy producer in cells, mitochondria are primary sites of ROS generation (AshaRani et al., 2009, Marchi et al., 2012). Electrons readily interact with oxygen during the oxidative phosphorylation process producing ROS, which

then interact with DNA, lipids, and proteins leading to oxidative damage (Marchi et al., 2012). Mitochondria destroy ROS using endogenous antioxidants such as vitamin E, coenzyme Q and glutathione, or enzymatically through activation of superoxide dismutase and catalase. However, when the antioxidant defenses cannot keep up with the production of ROS, ROS would accumulate in mitochondria (Mukhopadhyay and Weiner, 2007). Mitochondria are major targets of ROS as mitochondrial DNA, lipids, and proteins are susceptible to oxidative damage (Leuner et al., 2007, Pieczenik and Neustadt, 2007, Marchi et al., 2012). Additionally, mitochondria play crucial roles in cell death where the breakage of the outer mitochondrial membrane via the opening of mitochondrial permeability transition pore (MPTP) and the dissipation of the electrochemical gradient could result in apoptosis and/or necrosis (Kroemer et al., 1998, Green and Kroemer, 2004, Orrenius, 2004, Montero et al., 2010). Mitochondrial dysfunction has been associated with pathological development of various chronic neurodegenerative disorders, such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease (Cozzolino et al., 2015, Ferrigno et al., 2015, Long et al., 2015).

2.2. Docosahexaenoic Acid

Docosahexaenoic acid (DHA, 22:6) (Fig. 1.2.2), a long-chain polyunsaturated fatty acid (PUFA), is the most abundant fatty acid in brain and is a structural component of neuronal membranes (Horrocks

and Farooqui, 2004). However, neurons do not have the capability to produce DHA. Instead, it is taken up through the diet, and subsequently transported across the BBB and inserted into the sn-2 position of glycerol backbone of membrane phospholipids (Glomset, 2006, Lukiw and Bazan, 2008, Nguyen et al., 2014). Due to its high number of double bonds, DHA helps to increase membrane fluidity, hence moderating membrane organization and function (Yang et al., 2011). Supplementation of DHA in HL-60 and Neuro-2A cells led to its incorporation into neural cell membranes, resulting in increased resistance against excitotoxic damage (Horrocks and Farooqui, 2004). High intake of DHA also resulted in its incorporation into mitochondrial membrane phospholipids, and was coupled to a resistance to Ca^{2+}-induced MPTP opening (O'Shea et al., 2009, Khairallah et al., 2010, Khairallah et al., 2012).

DHA is crucial in neuroprotection as it contains anti-oxidative stress, anti-inflammation, and anti-apoptotic properties (Eady et al., 2012, Tanaka et al., 2012). However, as the most common PUFA, DHA is a target for lipid peroxidation (Stillwell et al., 1997). Prolonged oxidative stress can oxidize DHA into prostaglandin-like substances, triggering the production of ROS leading to further oxidative stress, a vicious cycle that will result in extensive damage (Porter et al., 1995, Montine et al., 2004, Lukiw and Bazan, 2008).

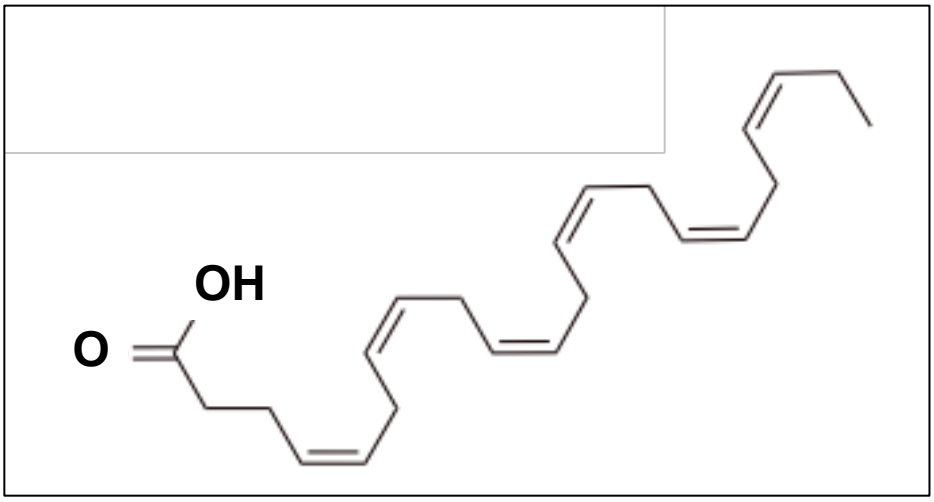


Fig. 1.2.2 Structure of DHA. Adapted from Rajnavolgyi et al. (2014).

2.3. L-Carnitine

L-carnitine (LC) (Fig. 1.2.3) is an endogenous compound found in all mammalian species, which exists mainly as the unesterified form (Walter and Schaffhauser, 2000, Jones et al., 2010). It can be transported and accumulated in brain (Bresolin et al., 1982, Jones et al., 2010). LC is essential for brain function, where the absence of LC results in major detrimental consequences in brain, such as development of metabolic encephalopathy (Kimura and Amemiya, 1990, Jones et al., 2010).

The main function of LC is to facilitate fatty acid transport into mitochondrial matrix for the production of ATP via β-oxidation (Kerner and Hoppel, 2000, Walter and Schaffhauser, 2000, Hoppel, 2003). This occurs via a three-step process known as the carnitine shuttle (Fig. 1.2.4). Acyl-CoA found in the cytosol is transferred onto LC by carnitine palmitoyltransferase-1 (CPT-1), which is subsequently

transported into mitochondrial matrix for β-oxidation (Virmani and Binienda, 2004, Sharma and Black, 2009, Houten and Wanders, 2010). The carnitine shuttle is critical as elevated levels of free fatty acids can result in mitochondrial dysfunction such as membrane damage, leading to mitochondria-dependent cell death (Virmani et al., 1995, Luo et al., 1999, Furuno et al., 2001, Sharma and Black, 2009). LC effectively protects mitochondria and cells against oxidative stress by inhibiting mitochondrial membrane depolarization and ROS formation, increasing mitochondrial functions, and preventing cell death in serum- or glucose-deprived medium (Ishii et al., 2000, Virmani and Binienda, 2004, Hino et al., 2005, He et al., 2011, Geier and Geier, 2013). Additionally, LC actively scavenges for ROS, playing a potential antioxidant role in cells (Derin et al., 2004, Augustyniak and Skrzydlewska, 2010, Mescka et al., 2011).

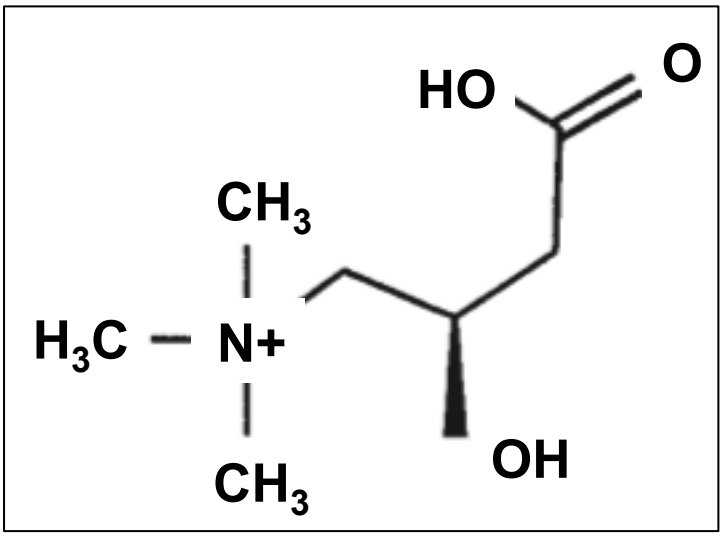


Fig. 1.2.3 Structure of LC. Adapted from Pettegrew et al. (2000)

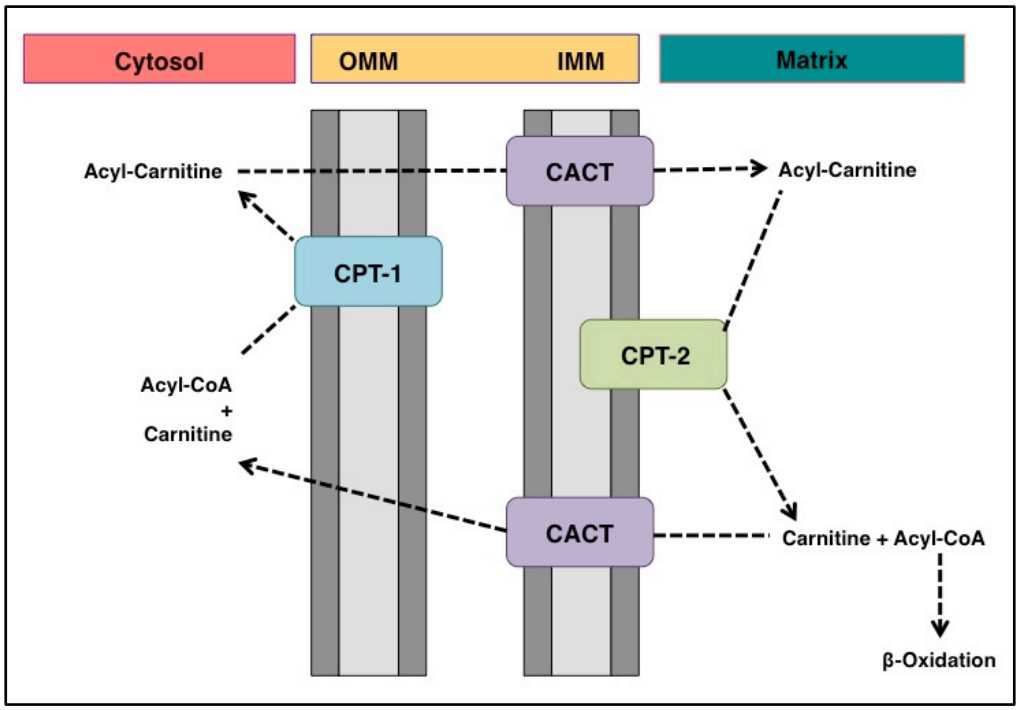


Fig. 1.2.4 Schematic diagram of the carnitine shuttle. CPT-1 transfers an acyl-CoA onto carnitine to form acyl-carnitine, which is then translocated across the inner mitochondrial membrane via CACT. In the mitochondrial matrix, CPT-2 catalyzes the conversion of acyl-carnitine back to acyl-CoA and carnitine, where acyl-CoA can then undergo β-oxidation. Carnitine is then brought out from mitochondria via CACT. Dotted black lines denote movement of various compounds. Abbreviations: CACT: carnitine-acylcarnitine translocase; CPT-1: carnitine palmitoyltransferase-1; CPT-2: carnitine palmitoyltransferase-2. Adapted from Sharma and Black (2009).

3. Phospholipase A_2

3.1. Phospholipase A_2

Phospholipase A_2 (PLA_2) is a superfamily of enzymes that cleaves the acyl ester bond of glycerophospholipids at the sn-2 position, in turn releasing free fatty acids and lysophosholipids (Dennis, 1994, Takenaka et al., 2001, Burke and Dennis, 2009, Ong et al., 2015). Almost 20 PLA_2 enzymes have been identified (Kudo, 2004, Sun et al., 2014) based on structural and biochemical features including sequence similarity and subcellular localization (Dennis,

1997, Tang et al., 1997). These enzymes have been grouped into several broad families such as calcium-independent PLA_2 ($iPLA_2$), cytosolic PLA_2 ($cPLA_2$), and secretory PLA_2 ($sPLA_2$), which are then subdivided further into various isoforms (Dennis, 1994, Farooqui et al., 1997, Sun et al., 2004, Ong et al., 2015).

PLA_2s play many integral roles in brain such as modulating neurotransmitter release, long-term potentiation (LTP), and behavioral and cognitive functions, and are crucial for signal transduction, production of eicosanoids and lipid mediators, inflammation, membrane remodeling, and lipid metabolism (Dennis, 1997, Farooqui et al., 1997, Farooqui et al., 2000, Farooqui et al., 2006, Schaloske and Dennis, 2006, Ong et al., 2010, Murakami et al., 2011, Sun et al., 2014). These enzymes work as critical regulators of cell membrane phospholipids via the deacylation-reacylation cycle, by working as the initiation point of activation, releasing fatty acids and lysophospholipids (Fig. 1.2.5) (Sun and MacQuarrie, 1989, Sun et al., 2014). Under normal conditions, PLA_2s maintain structure and function of membranes via removing oxidized fatty acids from phospholipids, hence preventing membrane lipid peroxidation (Tan et al., 1984, Sevanian and Kim, 1985, McLean et al., 1993, Ong et al., 2015). In pathological conditions however, elevated activation of PLA_2 enzymes results in increased production of fatty acids and lysophospholipids that will be metabolized to form second messengers and metabolites that could lead to alterations in membrane permeability, stimulation of lipolytic enzymes, and

inflammation (Sun et al., 2005, Sun et al., 2014, Ong et al., 2015). Hence, PLA_2 enzymes are said to be major components involved in oxidative stress and inflammation in brain, and have been implicated in a range of neurological disorders (Farooqui et al., 1997, Liu and Xu, 2010, Ong et al., 2015).

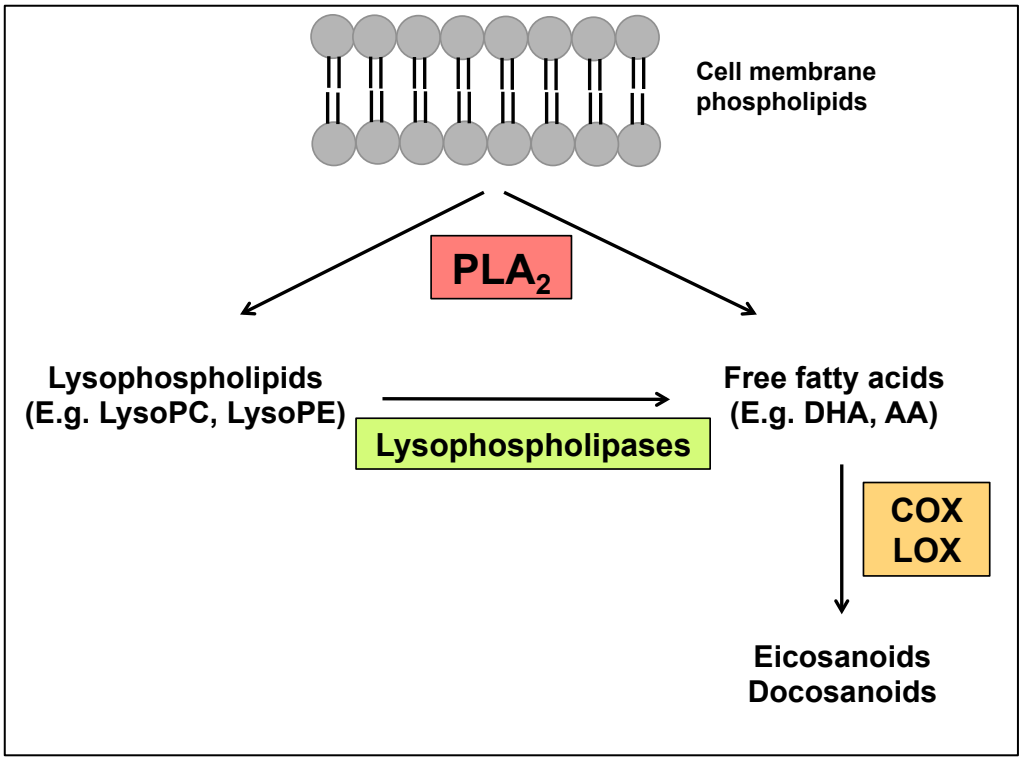


Fig. 1.2.5 Schematic diagram of the PLA_2 enzymatic pathway. PLA_2 acts on cell membrane phospholipids, releasing fatty acids and lysophospholipids, which can be further metabolized to form second messengers and metabolites. Abbreviations: AA: arachidonic acid; COX: cyclooxygenases; DHA: docosahexaenoic acid; LOX: lipoxygenases; LysoPC: lysophosphatidylcholine; LysoPE: lysophosphatidylethanolamine; PLA_2: phospholipase A_2.

3.2. Secretory Phospholipase A_2

$sPLA_2$ enzymes have low molecular weights of 14 – 19 kDa, and comprise of sPLA2-IB, sPLA2-IIA, $sPLA_2$-IIC, $sPLA_2$-IID, $sPLA_2$-

IIE, $sPLA_2$-IIF, $sPLA_2$-III, $sPLA_2$-V, $sPLA_2$-X, $sPLA_2$-XIIA, and $sPLA_2$-XIIB (Kudo and Murakami, 2002). In the rat brain, $sPLA_2$ isoforms are found in all regions, with highest activities in medulla oblongata, pons, and hippocampus, and lowest in olfactory bulb and cerebellum (Thwin et al., 2003). It is synthesized intracellularly, and in its mature form is secreted from the cytoplasm to the extracellular space to exert its effects on glycerophospholipids (Gentile et al., 2012, Sivaprakasam and Nachiappan, 2015).

$sPLA_2$ is dependent on Ca^{2+} for its functions, and lacks specificity for cleavage of fatty acids at the sn-2 position of glycerophospholipids (Murakami and Kudo, 2002, Sun et al., 2004, Schaloske and Dennis, 2006, Burke and Dennis, 2009, Yagami et al., 2014, Ong et al., 2015). $sPLA_2$ has been shown to release arachidonic acid (AA), oleic acid, and linoleic acid, as well as other pro-inflammatory mediators from glycerophospholipids (Rosengren et al., 2006). $sPLA_2$-IIA, $sPLA_2$-V, and $sPLA_2$-X have been reported to amplify eicosanoid production by either modulating $cPLA_2$ activity or directly releasing AA (Han et al., 2003, Kikawada et al., 2007). Studies reveal that pro-inflammatory cytokines and lipopolysaccharides induce $sPLA_2$ mRNA expression (Li et al., 1999, Shen et al., 2005, Sun et al., 2007). $sPLA_2$ activity has been recorded in various inflammatory conditions such as sepsis, arthritis, acute lung injury, and neutrophilic inflammation (Suzuki et al., 2000, Munoz et al., 2009, Boilard et al., 2010). Elevated gene and protein expressions of $sPLA_2$ have also

been reported in neurodegenerative diseases including Alzheimer's disease (Moses et al., 2006), cerebral ischemia (Lin et al., 2004), and multiple sclerosis (Cunningham et al., 2006).

Apart from its PLA_2 activity, $sPLA_2$ is involved in the release of neurotransmitters (Matsuzawa et al., 1996) and neurite outgrowth (Ikeno et al., 2005, Burke and Dennis, 2009). $sPLA_2$ is also found in mitochondrial fractions, playing a role in the regulation of mitochondrial phospholipid composition and the proper functioning of the ETC (Zhang et al., 1999, Adibhatla et al., 2003, Gentile et al., 2012).

3.3. Cytosolic Phospholipase A_2

$cPLA_2$ enzymes have high molecular weights of 85 – 110 kDa, and consist of $cPLA_2\alpha$, $cPLA_2\beta$, $cPLA_2\gamma$, $cPLA_2\delta$, $cPLA_2\varepsilon$, and $cPLA_2\zeta$ (Molloy et al., 1998, Balboa et al., 2002). In normal rats, basal mRNA expression of $cPLA_2$ is kept very low in brain (Pardue et al., 2003). $cPLA_2$ protein is found uniformly across various regions of rat brain, with relatively high levels of expression in hypothalamus, brainstem, cerebellum, and spinal cord (Ong et al., 1999).

$cPLA_2$ requires Ca^{2+} for its activities and preferentially releases AA from glycerophospholipids (Murakami and Kudo, 2002, Sun et al., 2004, Dennis et al., 2011, Murakami et al., 2011, Ong et al., 2015). AA plays a critical role in multiple physiological functions. It serves as a substrate for cyclooxygenases (COX) and lipoxygenases (LOX) to become precursors for eicosanoids and prostanoids, which

mediate inflammatory responses in cells (Kramer and Sharp, 1997, Uozumi et al., 1997, Phillis et al., 2006, Calder, 2008, Farooqui, 2012, Sun et al., 2014). Discrepancies in eicosanoid production could lead to chronic inflammation, disturbing signaling pathways that could result in irregular immune functions (Harizi et al., 2008, Serhan, 2010). Additionally, $cPLA_2$ can be activated by ROS (Xu et al., 2003, Zhu et al., 2006, Sun et al., 2007) and AA metabolism produces ROS as a by-product (Gentile et al., 2012). Hence, elevated $cPLA_2$ activity is often associated with oxidative stress and neuroinflammation (Arai et al., 2001, Ong et al., 2003, Farooqui and Horrocks, 2006, Tanaka et al., 2012). For instance, $cPLA_2$ has been associated with a range of inflammatory neurological disorders including Alzheimer's disease (Gentile et al., 2012, Sagy-Bross et al., 2014), Parkinson's disease (Klivenyi et al., 1998), multiple sclerosis (Nomura et al., 2011, Yang et al., 2014), and spinal cord injury (Liu and Xu, 2010). It is therefore crucial to regulate activity of $cPLA_2$ stringently so as to preserve appropriate levels of AA and lysophospholipases for cellular homeostasis (Katsuki and Okuda, 1995, Farooqui et al., 2006, Sun et al., 2010, Gentile et al., 2012).

Although attention on $cPLA_2$ is primarily due to its preference for AA release leading to inflammation, it is also involved in other functions in cells. Like other PLA_2 enzymes, $cPLA_2$ moderates membrane fluidity, and affect downstream signaling pathways via the action of released fatty acids, their metabolites, and lysophospholipids.

It also plays a role in regulation of membrane trafficking (San Pietro et al., 2009, Ha et al., 2012, Leslie, 2015), and synaptic plasticity via LTP and long-term depression (LTD) (Bazan, 2005, Le et al., 2010, Wang et al., 2014a, Leslie, 2015). Apart from its phospholipase activity, $cPLA_2$ exerts lysophospholipase and transacetylase activities although the physiological relevance is currently unknown (Ghosh et al., 2006, Dennis et al., 2011, Gentile et al., 2012, Leslie, 2015).

3.4. Calcium-Independent Phospholipase A_2

$iPLA_2$ has a molecular weight of 85 – 88 kDa, and exists as a multimeric complex (Ackermann et al., 1994, Tang et al., 1997, Winstead et al., 2000). Multiple splice variants have been discovered, including $iPLA_2\alpha$, $iPLA_2\beta$, $iPLA_2\gamma$, $iPLA_2\delta$, $iPLA_2\varepsilon$, $iPLA_2\zeta$, and $iPLA_2\eta$ (Mancuso et al., 2000, Tanaka et al., 2000, Balboa et al., 2002, van Tienhoven et al., 2002, Jenkins et al., 2004, Glynn, 2005). $iPLA_2$ is found in all regions of brain, with high expression in cerebral cortex, basal ganglia, brainstem, and cerebellum, and is localized in the cytoplasm (Ong et al., 2005).

Unlike $cPLA_2$ and $sPLA_2$, $iPLA_2$ has been postulated to show preference for DHA release at the sn-2 position of glycerophospholipids (Murakami and Kudo, 2002, Strokin et al., 2003, Farooqui and Horrocks, 2006, Strokin et al., 2007, Green et al., 2008, Basselin et al., 2010, Ong et al., 2015). Both DHA and its derivative neuroprotectin D1 (NPD1) have revealed neuroprotective roles in the

central nervous system (CNS) (Bazan et al., 2013). DHA is metabolized by 15-LOX enzyme to docosanoids such as resolvins and neuroprotectins, which antagonize effects of AA-derived pro-inflammatory eicosanoids thus exhibiting anti-inflammatory and anti-apoptotic characteristics (Hong et al., 2003, Mukherjee et al., 2004, Serhan, 2005, Bazan, 2009, Orr et al., 2013). Under conditions of oxidative stress and inflammation, NPD1 has been shown to up-regulate anti-apoptotic proteins while down-regulating pro-apoptotic proteins, resulting in an overall pro-survival situation (Mukherjee et al., 2004, Lukiw et al., 2005, Bazan, 2007).

$iPLA_2$ is an integral 'housekeeping' enzyme, highly expressed in brain under normal conditions (Ong et al., 2010). Similar to other PLA_2 enzymes, $iPLA_2$ is heavily involved with lipid regulation, cell signaling, and transcriptional regulation (Balsinde et al., 1997, Isenovic and LaPointe, 2000, Williams and Ford, 2001, Moran et al., 2005, Hooks and Cummings, 2008). In the brain, $iPLA_2$ has been associated with neurotransmitter release (St-Gelais et al., 2004), LTP and memory (Schaeffer and Gattaz, 2005, Shalini et al., 2014). The remodeling and repair of membrane phospholipids by $iPLA_2$ could also protect mitochondrial integrity and function, since mitochondria are key ROS-generating organelles in cells. Seleznev et al. (2006) demonstrated a reduction in caspase-3 activation, fragmentation of DHA, and phosphatidylserine (PS) externalization upon $iPLA_2$ expression in INS-1 cells, preventing mitochondria-induced apoptosis.

Moreover, $iPLA_2$ has been reported to repair oxidized mitochondrial membrane components, and modulate opening of MPTP (Gadd et al., 2006, Zhao et al., 2010). Mutations in $iPLA_2$ gene have been associated with development of neurologic childhood disorders due to iron accumulation in brain (Sun et al., 2010, Illingworth et al., 2014). Abnormal $iPLA_2$ has also been observed in cerebellar atrophy, dystonia-parkinsonism, and increased expression of Lewy bodies and neurofibrillary tangles (Kurian et al., 2008, Paisan-Ruiz et al., 2009, Sun et al., 2010).

Table 1.1.3 Key characteristics of PLA_2 isoforms.

$sPLA_2$	**$cPLA_2$**	**$iPLA_2$**
14 – 19 kDa	85 – 110 kDa	85 – 88 kDa
Synthesized intracellularly, with mature form secreted to extracellular space	Requires Ca^{2+} for activities and preferentially releases AA	Preference for DHA release
Lacks specificity for cleavage of fatty acids	Elevated $cPLA_2$ activity often associated with oxidative stress and neuroinflammation	Integral 'housekeeping' enzyme, highly expressed in brain under normal conditions

SECTION II

AIMS OF THE PRESENT STUDY

The introduction of nanotechnology in recent years has brought about multiple benefits to society and the economy, where novel features of nanomaterials enable them to become appealing and valuable in wide-ranging fields. AgNPs are said to be the most frequently utilized nanomaterial in consumer products (Chen and Schluesener, 2008, Beer et al., 2012), making up approximately 24 % of all nanoproducts (Project on Emerging Nanotechnologies, 2015). Much research has been conducted on the health effects of AgNPs with respect to organs such as the lungs and liver, however, little is known about the impacts on the human brain. Recent studies revealed the ability of AgNPs to readily cross the BBB, exerting its effects on the CNS (Tang et al., 2009, Sharma et al., 2010, Trickler et al., 2010). Additionally, multiple studies have found that AgNPs preferentially disrupt mitochondrial function (AshaRani et al., 2009, Costa et al., 2010, Kang et al., 2012), since mitochondria are major sites of ROS production. Apart from disrupting mitochondrial function, nanotoxicity is often associated with the triggering of inflammatory responses (Park and Park, 2009, Xue et al., 2012, Panas et al., 2013).

The present study was conducted in view of the mounting usage of AgNPs despite little being known about its toxicity and potential impacts on human and environmental health. Hence, several issues would be examined in this study. Firstly, the current study aims to investigate the effect of AgNPs on human neuronal mitochondria, as mitochondria are crucial organelles in cells (Raimundo, 2014), and

mitochondrial dysfunction often leads to cell death (Montero et al., 2010). AgNPs have been reported to target mitochondria (AshaRani et al., 2009), although little is known about its effects in human neuronal cells. Additionally, I wanted to determine if any mitochondrial dysfunction observed could be rescued through the use of endogenous compounds, such as DHA and/or LC, since these compounds have shown to be essential for healthy mitochondrial function (He et al., 2011, Khairallah et al., 2012).

AgNPs have also demonstrated inflammatory properties (Kim et al., 2010). However, it is unknown if AgNPs enhances the effects of pro-inflammatory mediators, inhibit anti-inflammatory mediators, or both. Thus, the current study aimed to examine the effect of AgNPs on inflammatory mediators, in particular the pro-inflammatory mediator, $cPLA_2$, and the anti-inflammatory mediator, $iPLA_2$. Furthermore, AgNPs have been reported to produce ROS in various cell lines (Mukherjee et al., 2012), although it has not been determined if AgNPs-induced ROS exerts its effects on the inflammatory mediators. Hence, aims 4 and 5 of the present study were to investigate effects of AgNPs-induced ROS on inflammatory mediators and to elucidate the mechanism underlying changes observed.

Due to the increasing usage of AgNPs in consumer products, it is crucial to highlight and understand the potential toxicities of AgNPs on human health. It is hoped that the present study could provide additional insights with respect to AgNPs' toxicity in human

neuronal cells, and possibly the biochemical mechanisms underlying the said nanotoxicity.

SECTION III

EXPERIMENTAL STUDIES

CHAPTER 1

DOCOSAHEXAENOIC ACID AND L-CARNITINE PREVENT ACUTE SILVER NANOTOXICITY

1.1. Introduction

Nanomaterials are appealing and valuable in various fields, as they are engineered to achieve particular physicochemical properties that are specific for their application. However, serious health considerations have been considered. The most prevalent toxicity mechanism for AgNPs is oxidative stress. Multiple studies have found that AgNPs preferentially disrupt mitochondrial function, since mitochondria are major ROS producers. AgNPs exposure led to damaged mitochondrial membrane, reduced mitochondrial membrane potential, opening of MPTP, disruption of ETC, reduced ATP production, and eventually cell death (AshaRani et al., 2009, Costa et al., 2010, Piao et al., 2011, Teodoro et al., 2011, Mukherjee et al., 2012, Chairuangkitti et al., 2013, Stensberg et al., 2013). On the other hand, there are conflicting reports of AuNPs' toxicity. Several studies have demonstrated that AuNPs do not show overt toxicity (Connor et al., 2005, Zhang et al., 2010, Asharani et al., 2011). However, Li et al. (2008) determined that AuNPs inhibited cell proliferation, and induced oxidative damage and cytotoxicity in human lung fibroblasts. It has been suggested that the cytotoxicity of AuNPs is largely dependent on its physical properties (Goodman et al., 2004, Pan et al., 2007). When compared to AgNPs, AuNPs are noticeably less toxic (Bar-Ilan et al., 2009).

DHA is the major fatty acid in brain, and is a structural component of neuronal (Horrocks and Farooqui, 2004) and

mitochondrial membranes (O'Shea et al., 2009, Khairallah et al., 2012). DHA is crucial in neuroprotection as it contains anti-oxidative stress, anti-inflammation, and anti-apoptotic properties (Eady et al., 2012, Tanaka et al., 2012). Fatty acids are transported into mitochondrial matrix for ATP production via the carnitine shuttle, involving LC (Walter and Schaffhauser, 2000, Hoppel, 2003). Essential for brain function, the lack of LC results in major detrimental consequences including the development of metabolic encephalopathy (Kimura and Amemiya, 1990, Jones et al., 2010). LC effectively protects mitochondria and cells against oxidative stress, by inhibiting mitochondrial membrane depolarization and ROS formation, increasing mitochondrial functions, and preventing cell death in serum- or glucose-deprived medium (Virmani and Binienda, 2004, Hino et al., 2005, He et al., 2011, Geier and Geier, 2013).

In this chapter, the relationship between AgNPs and mitochondria was examined. The possibility of DHA and/or LC supplementation preventing AgNPs toxicity in SH-SY5Y cells was also determined.

1.2. Materials and Methods

1.2.1. Cell Culture

SH-SY5Y cells (CRL-2266™, ATCC®, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium containing 10 % heat-inactivated fetal bovine serum and 1 % penicillin-streptomycin (Gibco®, Life Technologies, Carlsbad, CA, USA). The combined medium is defined as complete growth medium in this thesis. SH-SY5Y cells were grown in 100 mm dishes, incubated under standard conditions of 37 °C and 5 % CO_2, and regularly passaged with 0.25 % Trypsin-EDTA (Gibco®, Life Technologies, Carlsbad, CA, USA).

1.2.2. Synthesis of AgNPs

5 mL 2 mg/mL silver nitrate ($AgNO_3$) solution (Merck Millipore, Billerica, MA, USA) was added to 75 mL reagent-grade water, and heated in a 65 °C silicone oil bath. The solution was stirred at 1,000 rpm. A separate solution of 3.1 mL 1 mg/mL tannic acid solution (Sigma-Aldrich, St. Louis, MO, USA), 4 mL 10 mg/mL sodium citrate dihydrate solution (Sigma-Aldrich, St. Louis, MO, USA), and 12.9 mL reagent-grade water was warmed for 20 minutes in the oil bath, and subsequently combined with the $AgNO_3$ solution. Temperature of oil bath was increased to 100 °C, and the solution was heated for an additional 20 minutes, during which solution color turned golden yellow (Fig. 3.1.1). Synthesized AgNPs were cooled to room temperature prior to storing at 4 °C in the dark.

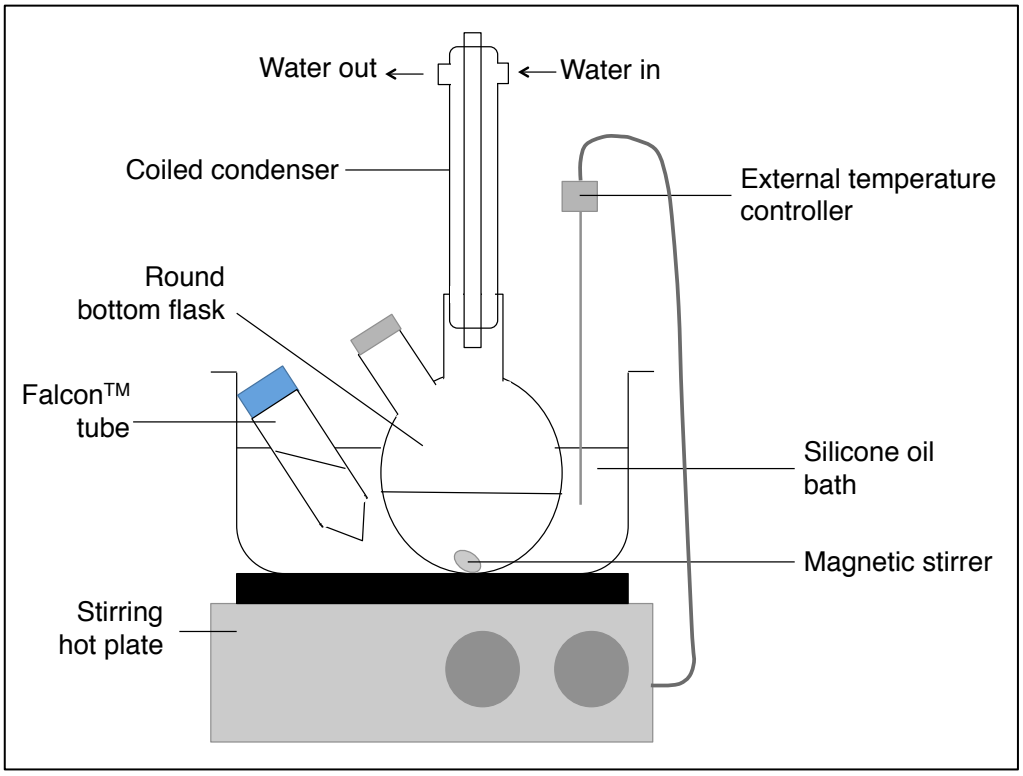


Fig. 3.1.1 Schematic diagram of the set up used for NPs synthesis (not drawn to scale). Solutions were heated in a temperature controlled silicone oil bath. A condenser was fitted onto the set up to prevent loss of solution due to vapor formation during the heating process.

1.2.3. Purification of AgNPs

8 mL synthesized AgNPs suspension was centrifuged at 5,000 *g* for 30 minutes at 4 °C. Supernatant (S1) was separated and centrifuged with identical parameters to retrieve remaining AgNPs. AgNPs pellet (P1) was resuspended with 8 mL reagent-grade water. After S1 centrifugation, supernatant (S2) was decanted and AgNPs pellet (P2) added to existing AgNPs suspension. The process was repeated, and final pellets (P3 and 4) were resuspended in 8 mL complete growth medium (Fig. 3.1.2).

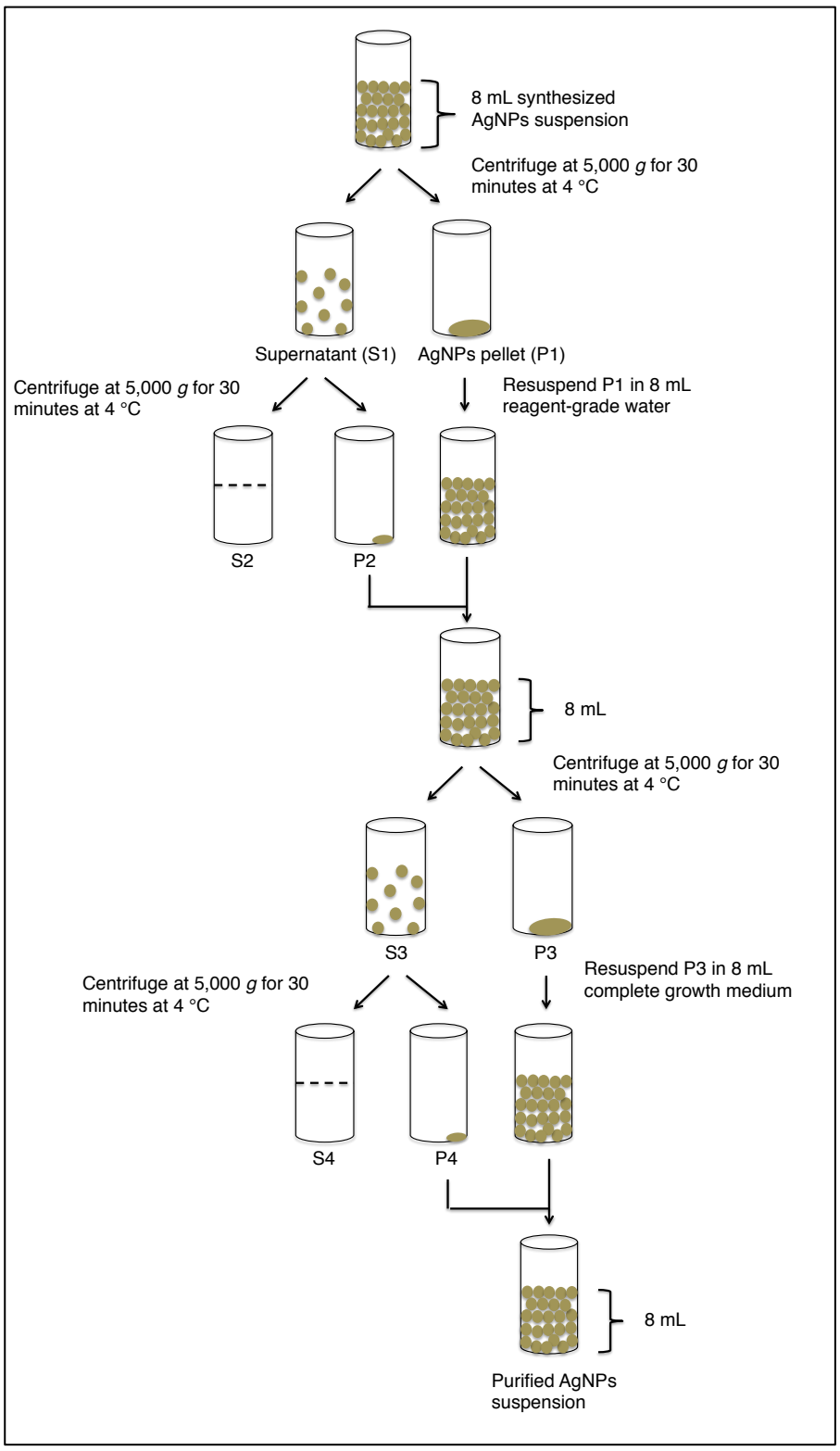


Fig. 3.1.2 Schematic diagram of AgNPs purification process. AgNPs suspension was purified twice to remove potential contaminants, using the following conditions: 5,000 *g* at for 30 minutes 4 °C. Adapted from Balasubramanian et al. (2010).

1.2.4. Synthesis of AuNPs

AuNPs synthesis was conducted as previously described by Turkevich et al. (1951). 100 µL 10 % (w/v) gold chloride hydrate solution (Sigma-Aldrich, St. Louis, MO, USA) was added to 95 mL reagent-grade water, warmed in a 100 °C silicone oil bath, and stirred at 1,000 rpm. A separate solution of 5 mL 1 % sodium citrate dihydrate solution was heated for 20 minutes in the oil bath, and combined with the gold chloride hydrate solution. The solution was heated for an additional 20 minutes, during which solution color turned wine-red. Synthesized AuNPs were cooled to room temperature prior to storing at 4 °C in the dark.

1.2.5. Purification of AuNPs

540 µL synthesized AuNPs suspension was centrifuged at 7,000 *g* for 20 minutes at 4 °C. Supernatant (S1) was separated and centrifuged with identical parameters to retrieve remaining AuNPs. AuNPs pellet (P1) was resuspended in 540 µL reagent-grade water. After S1 centrifugation, supernatant (S2) was decanted and AuNPs pellet (P2) added to existing AuNPs suspension. The process was repeated, and final pellets (P3 and 4) were resuspended in 540 µL complete growth medium.

1.2.6. Characterization of AgNPs and AuNPs

Concentration, hydrodynamic size, and polydispersity of NPs were analyzed via dynamic light scattering measurements, using the Zetasizer Nano ZS (Malvern Instruments, Worcestershire, England, UK). General morphology, size, and possible aggregation of NPs were determined using Philips CM120 BioTwin transmission electron microscope (FEI Philips, Hillsboro, OR, USA).

1.2.7. Trypan Blue Exclusion Cell Viability Assay

SH-SY5Y cells were plated in a 6-well plate at 0.5 X 10^6 cells/well, and allowed to attach overnight. Four groups of cells were treated with the following reagents: (1) vehicle, water, (2) 78.7 µg/mL $AgNO_3$, (3) 50 µg/mL AgNPs, (4) 91.3 µg/mL AuNPs, and incubated for one hour. Phosphate buffered saline (PBS, pH 7.4) was used to wash the cells. SH-SY5Y cells were then exposed to 0.4 % trypan blue solution (Sigma-Aldrich, St. Louis, MO, USA) for five minutes, and viable cells percentage determined.

1.2.8. Mitochondrial Membrane Potential Assay

SH-SY5Y cells were plated in a 24-well plate at 1.0 X 10^5 cells/well, and allowed to attach overnight. Three groups of cells were treated with the following reagents: (1) negative control: vehicle, water, (2) positive control: vehicle, water, (3) 50 µg/mL AgNPs, and incubated for one hour. PBS was used to wash the cells. SH-SY5Y cells were

then incubated with 0.5 µM 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1 dye) (Molecular Probes®, Life Technologies, Carlsbad, CA, USA) at 37 °C for 20 minutes in Earl's balanced salt solution (EBSS) (Sigma-Aldrich, St. Louis, MO, USA). PBS was used to wash the cells. EBSS with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was supplemented to negative control and AgNPs-treated cells, while positive control was given 5 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Santa Cruz Biotechnology, Dallas, TX, USA). Samples were read with the Tecan Infinite® 200 microplate reader (Männedorf, Switzerland) using excitation and emission wavelengths of 488 nm and 535 nm for green fluorescence and 590 nm for red fluorescence. Cells were analyzed at two-minute intervals for 30 minutes. Red/green fluorescence (590/535) ratios were taken as an expression of mitochondrial membrane potential.

1.2.9. ADP/ATP Ratio Assay

1.2.9.1. Treatment with AgNPs, DHA and LC

SH-SY5Y cells were plated in a 96-well plate at 1.0 X 10^4 cells/well, and allowed to attach overnight. Five groups of cells were treated with the following reagents: (1) vehicle, ethanol and water, (2) 50 µg/mL AgNPs, (3) 50 µg/mL AgNPs and 10 µM DHA (Cayman Chemical, Ann Arbor, MI, USA), (4) 50 µg/mL AgNPs and 100 µM LC (Sigma-Aldrich, St. Louis, MO, USA), (5) 50 µg/mL AgNPs, 10 µM

DHA, and 100 µM LC, and incubated for one hour. PBS was used to wash the cells. The assay was then carried out according to the manufacturer's instructions (Abcam, Cambridge, UK). Briefly, SH-SY5Y cells were exposed to nucleotide releasing buffer (NRB) for 10 minutes with gentle agitation. Concurrently, a reaction mix containing NRB and ATP monitoring enzyme was added into each well of a 96-well white-walled luminometer plate. After 10 minutes, the lysed cells were transferred into the white-walled luminometer plate, and allowed to sit for an additional 10 minutes. Samples were then read using the Tecan Infinite® 200 microplate reader (Data A). Samples were analyzed again after 10 minutes (Data B). ADP converting enzyme was added to the samples, and samples were analyzed after another 10 minutes (Data C). Intracellular ADP/ATP ratios were determined using the following equation:

$$\frac{\text{Data C - Data B}}{\text{Data A}}$$

1.2.9.2. Treatment with AgNPs and AuNPs

SH-SY5Y cells were plated in a 96-well plate at 1.0 X 10^4 cells/well, and allowed to attach overnight. Three groups of cells were treated with the following reagents: (1) vehicle, water, (2) 50 µg/mL AgNPs, (3) 91.3 µg/mL AuNPs, and incubated for one hour. PBS was used to wash the cells. The assay was conducted as per above.

1.2.10. Statistical Analyses

Mean and standard error of values were determined for each experimental group, and possible significant differences among various groups were analyzed using one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $p < 0.05$ was deemed significant.

1.3. Results

1.3.1. Characterization of AgNPs and AuNPs

AgNPs and AuNPs were roughly spherical, with an approximate diameter of 26 nm for AgNPs (Fig. 3.1.3) and 20 nm for AuNPs (Fig. 3.1.4). AgNPs and AuNPs suspension concentrations were determined to be 50 µg/mL and 91.3 µg/mL respectively. Number of particles was equivalent in both suspensions, at 2.79 X 10^{17} particles/mL, signifying that subsequent experiments were centered on number of particles, which is a better design.

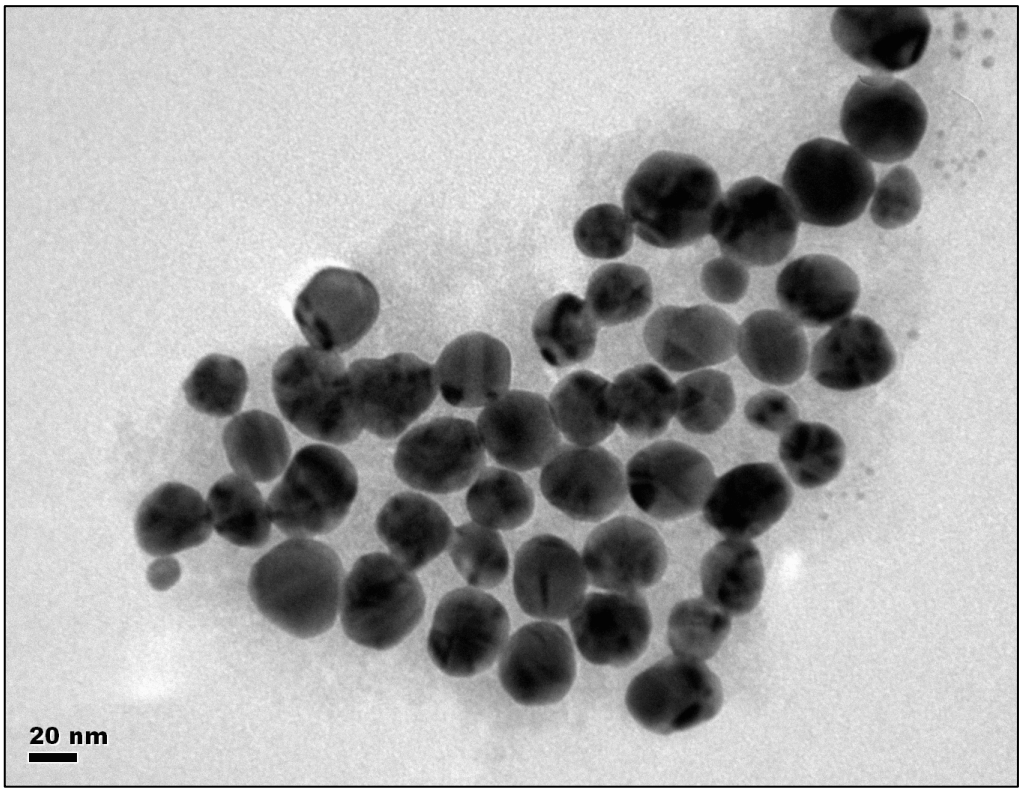


Fig. 3.1.3 TEM micrograph of AgNPs used in the present study. AgNPs are roughly spherical, with an average diameter of 26 nm. Scale: 20 nm.

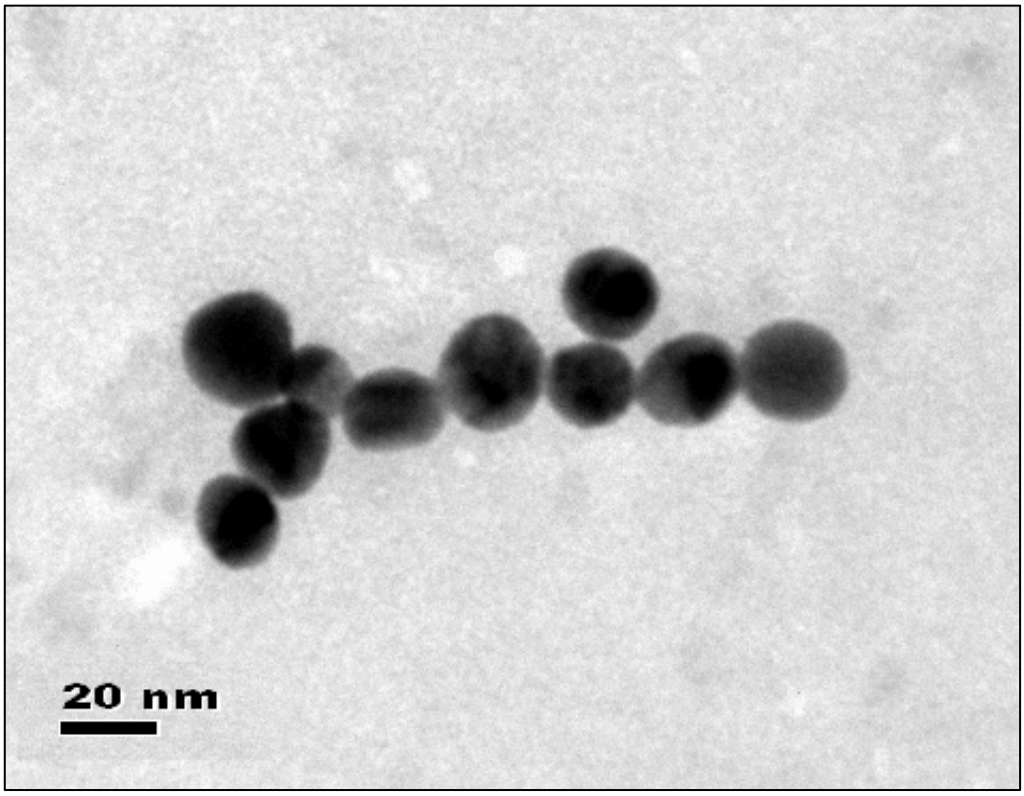


Fig. 3.1.4 TEM micrograph of AuNPs used in the present study. AuNPs are roughly spherical, with an average diameter of 20 nm. Scale: 20 nm.

1.3.2. Trypan Blue Exclusion Cell Viability Assay Analysis

One hour incubation of SH-SY5Y cells with 78.7 µg/mL $AgNO_3$ resulted in rapid cell death, with an observed cell viability of 0.99 % (Fig. 3.1.5). Cell viabilities of vehicle control, 50 µg/mL AgNPs-, and 91.3 µg/mL AuNPs-treated SH-SY5Y cells after one hour incubation were 92.98 %, 94.52 %, and 92.77 % respectively. Significant differences were observed between 78.7 µg/mL $AgNO_3$-treated cells and vehicle control, 50 µg/mL AgNPs-, and 91.3 µg/mL AuNPs-treated cells ($p < 0.001$).

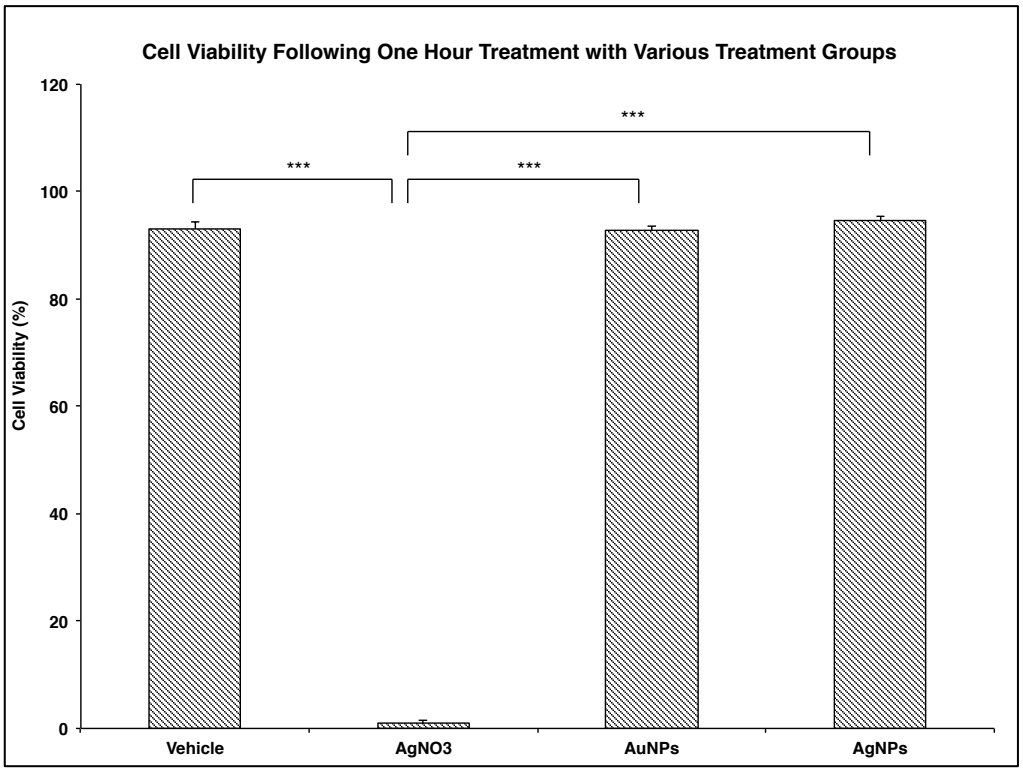


Fig. 3.1.5 Cell viability of SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 78.7 µg/mL $AgNO_3$; 50 µg/mL AgNPs; 91.3 µg/mL AuNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate significant differences in cell viability by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. *** $p < 0.001$.

1.3.3. Mitochondrial Membrane Potential Assay Analysis

One hour incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a significant reduction in 590/535 ratio compared to negative control over time ($p < 0.001$) (Fig. 3.1.6). Similarly, positive control showed a significant decrease in 590/535 ratio compared to negative control over time ($p < 0.001$).

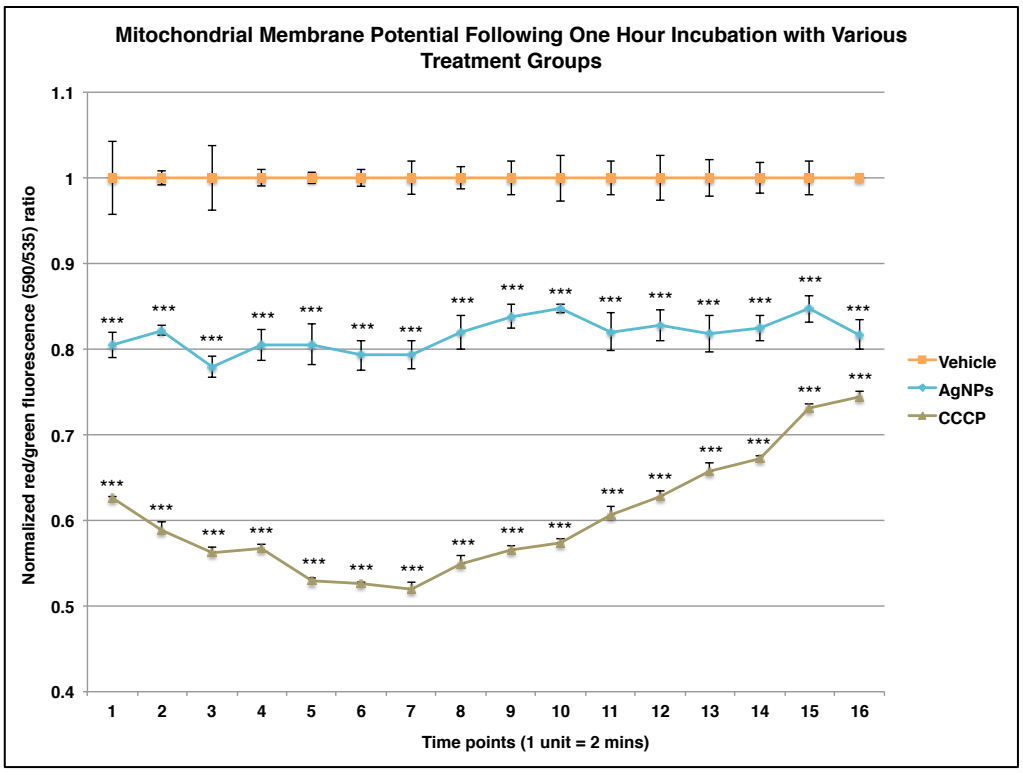


Fig. 3.1.6 Mitochondrial membrane potential of SH-SY5Y cells following one hour incubation with various treatment groups – negative control; 50 µg/mL AgNPs; 5 µM CCCP (positive control) (n = 4 in each group). Each time point represents a two-minutes interval. Asterisks (*) indicate significant differences in 590/535 ratio compared to negative control by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. *** $p < 0.001$.

1.3.4. ADP/ATP Ratio Assay Analyses

1.3.4.1. Treatment with AgNPs, DHA, and LC

1.3.4.1.1. ATP Levels

One hour incubation of SH-SY5Y cells with 50 μg/mL AgNPs resulted in a 0.63-fold ($p < 0.001$) statistically significant change in ATP levels compared to vehicle control (Fig. 3.1.7). There was a 0.71-fold ($p < 0.001$), 0.79-fold ($p < 0.001$), and 1.05-fold ($p = 1.00$) change in ATP levels in cells treated with the various rescue efforts – 50 μg/mL AgNPs + 10 μM DHA, 50 μg/mL AgNPs + 100 μM LC, and 50 μg/mL AgNPs + 10 μM DHA + 100 μM LC – compared to vehicle control. Compared to 50 μg/mL AgNPs-treated SH-SY5Y cells, significant increases in ATP levels were observed in cells treated with 50 μg/mL AgNPs + 10 μM DHA ($p = 0.033$), 50 μg/mL AgNPs + 100 μM LC ($p < 0.001$), and 50 μg/mL AgNPs + 10 μM DHA + 100 μM LC ($p < 0.001$). Statistically significant increases in ATP levels were also observed in cells treated with 50 μg/mL AgNPs + 10 μM DHA + 100 μM LC compared to 50 μg/mL AgNPs + 10 μM DHA ($p < 0.001$) and 50 μg/mL AgNPs + 100 μM LC ($p < 0.001$).

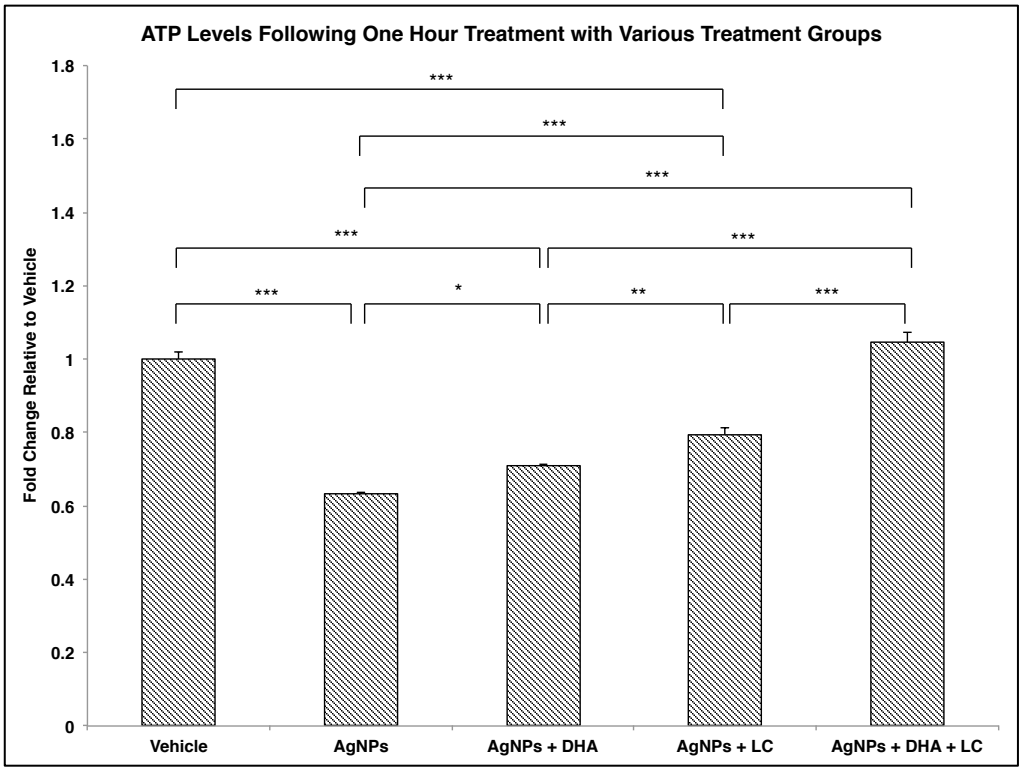


Fig. 3.1.7 Fold change in ATP levels of SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 50 µg/mL AgNPs + 10 µM DHA; 50 µg/mL AgNPs + 100 µM LC; 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC (n = 6 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. * $p < 0.05$, $p < 0.01$, *** $p < 0.001$.

1.3.4.1.2. ADP Levels

One hour incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 0.79-fold ($p < 0.001$) statistically significant change in ADP levels compared to vehicle control (Fig. 3.1.8). There was a 0.80-fold ($p < 0.001$), 0.78-fold ($p < 0.001$), and 0.88-fold ($p = 0.003$) change in ADP levels in cells treated with the various rescue efforts – 50 µg/mL AgNPs + 10 µM DHA, 50 µg/mL AgNPs + 100 µM LC, and 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC – compared to vehicle control. Statistically significant increases in ADP levels were also observed in cells treated with 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC compared to 50 µg/mL AgNPs ($p = 0.021$) and 50 µg/mL AgNPs + 100 µM LC ($p = 0.011$).

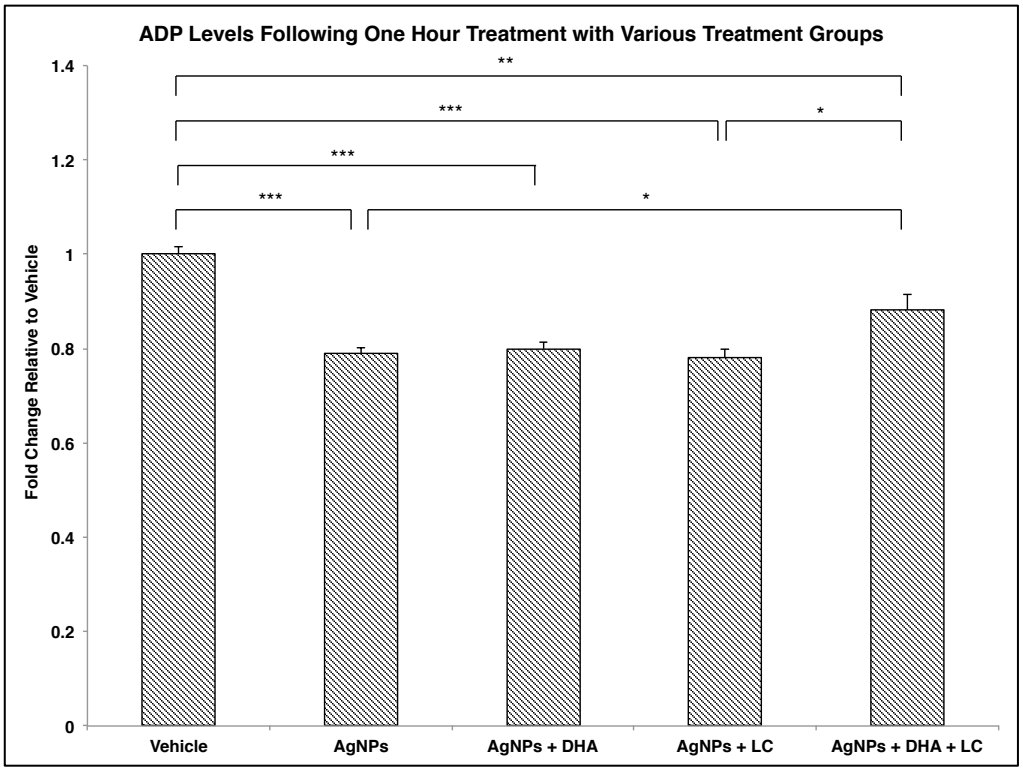


Fig. 3.1.8 Fold change in ADP levels of SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 50 µg/mL AgNPs + 10 µM DHA; 50 µg/mL AgNPs + 100 µM LC; 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC (n = 6 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

1.3.4.1.3. ADP/ATP Ratio

One hour incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 1.25-fold ($p < 0.001$) statistically significant increase in ADP/ATP ratio compared to vehicle control (Fig. 3.1.9). There was a 1.13-fold ($p < 0.001$), 0.98-fold ($p = 1.00$), and 0.84-fold ($p < 0.001$) change in ADP/ATP ratio in cells treated with the various rescue efforts – 50 µg/mL AgNPs + 10 µM DHA, 50 µg/mL AgNPs + 100 µM LC, and 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC – compared to vehicle control. Compared to 50 µg/mL AgNPs-treated SH-SY5Y cells, significant decreases in ADP/ATP ratio were observed in cells treated with 50 µg/mL AgNPs + 10 µM DHA ($p = 0.002$), 50 µg/mL AgNPs + 100 µM LC ($p < 0.001$), and 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC ($p < 0.001$). Statistically significant decreases in ADP/ATP ratio were also observed in cells treated with 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC compared to 50 µg/mL AgNPs + 10 µM DHA ($p < 0.001$) and 50 µg/mL AgNPs + 100 µM LC ($p < 0.001$).

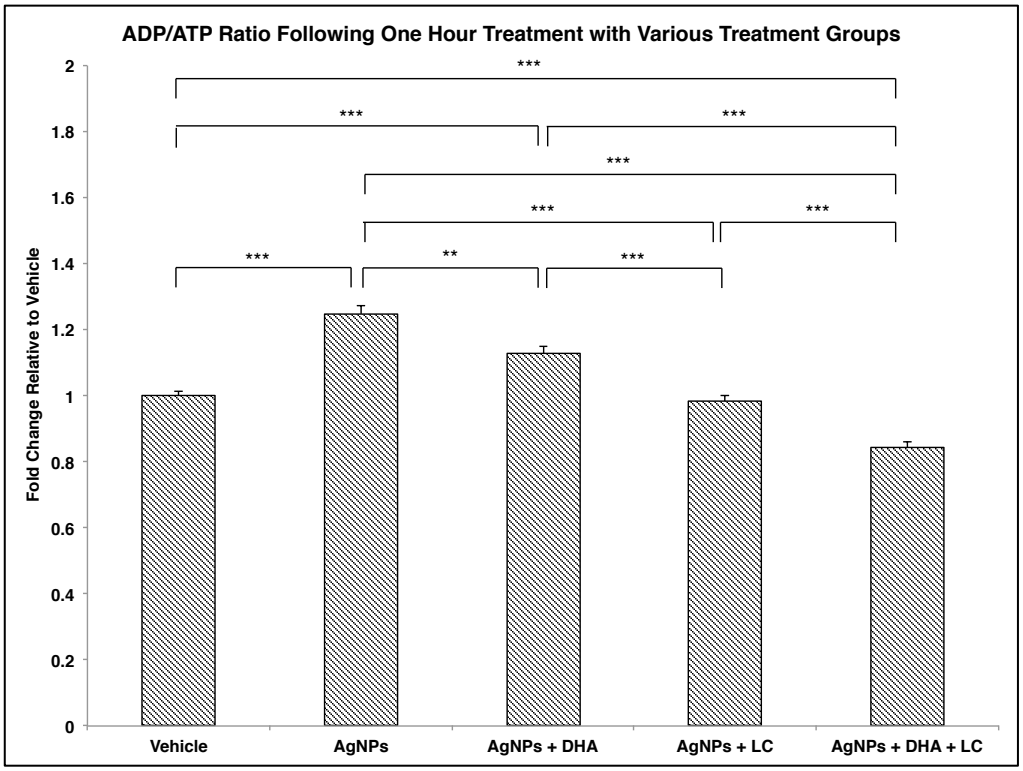


Fig. 3.1.9 Fold change in ADP/ATP ratio of SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 50 µg/mL AgNPs + 10 µM DHA; 50 µg/mL AgNPs + 100 µM LC; 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC (n = 6 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$.

1.3.4.2. Treatment with AgNPs and AuNPs

1.3.4.2.1. ATP Levels

One hour incubation of SH-SY5Y cells with 91.3 µg/mL AuNPs resulted in a 1.36-fold ($p < 0.001$) statistically significant increase in ATP levels compared to vehicle control (Fig. 3.1.10). Similar to previous findings, 50 µg/mL AgNPs-treated SH-SY5Y cells led to a 0.47-fold ($p < 0.001$) statistically significant change in ATP levels compared to vehicle control.

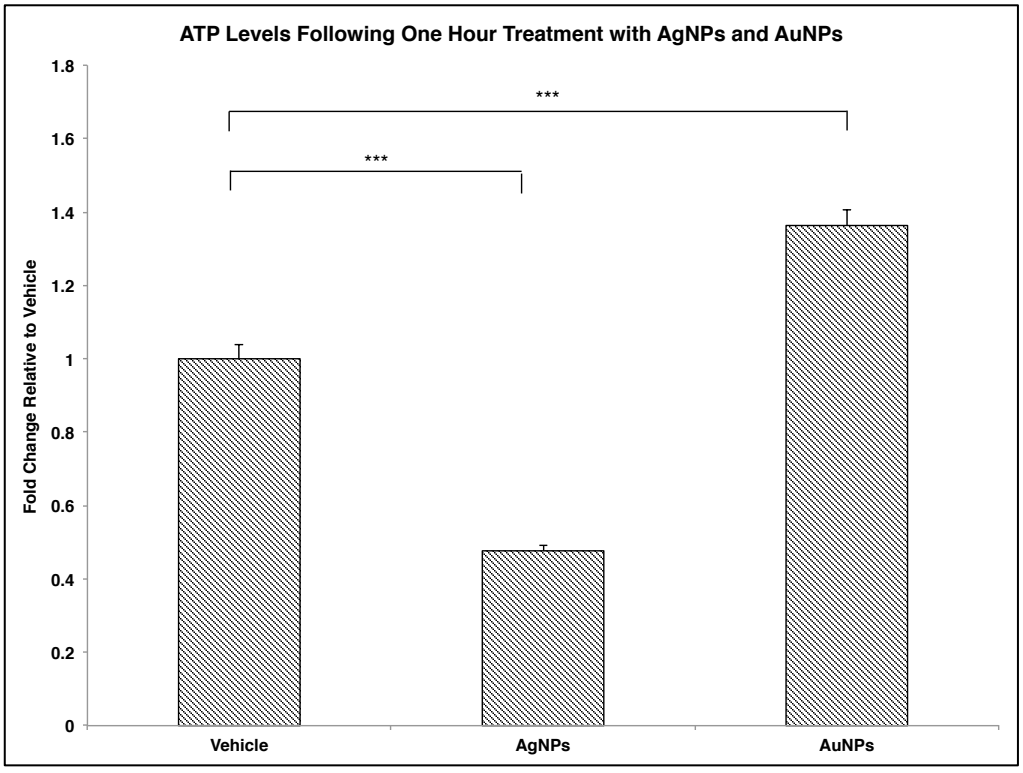


Fig. 3.1.10 Fold change in ATP levels of SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 91.3 µg/mL AuNPs (n = 6 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. *** $p < 0.001$.

1.3.4.2.2. ADP Levels

One hour incubation of SH-SY5Y cells with 91.3 µg/mL AuNPs resulted in a 0.96-fold (p = 1.00) change in ADP levels compared to vehicle control (Fig. 3.1.11). Similar to previous findings, 50 µg/mL AgNPs-treated SH-SY5Y cells led to a 0.70-fold ($p < 0.001$) statistically significant change in ADP levels compared to vehicle control.

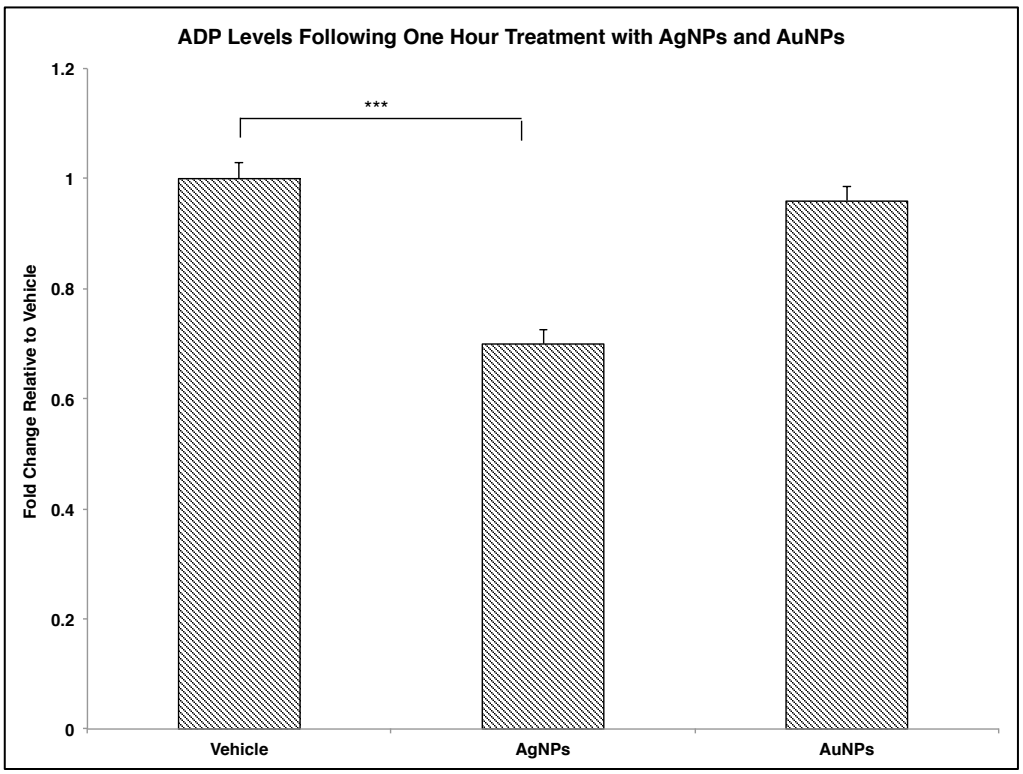


Fig. 3.1.11 Fold change in ADP levels of SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 91.3 µg/mL AuNPs (n = 6 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. *** $p < 0.001$.

1.3.4.2.3. ADP/ATP Ratio

One hour incubation of SH-SY5Y cells with 91.3 µg/mL AuNPs resulted in a 0.70-fold ($p = 0.004$) statistically significant change in ADP/ATP ratio compared to vehicle control (Fig. 3.1.12). Similar to previous findings, 50 µg/mL AgNPs-treated SH-SY5Y cells led to a 1.46-fold ($p < 0.001$) statistically significant increase in ADP/ATP ratio compared to vehicle control.

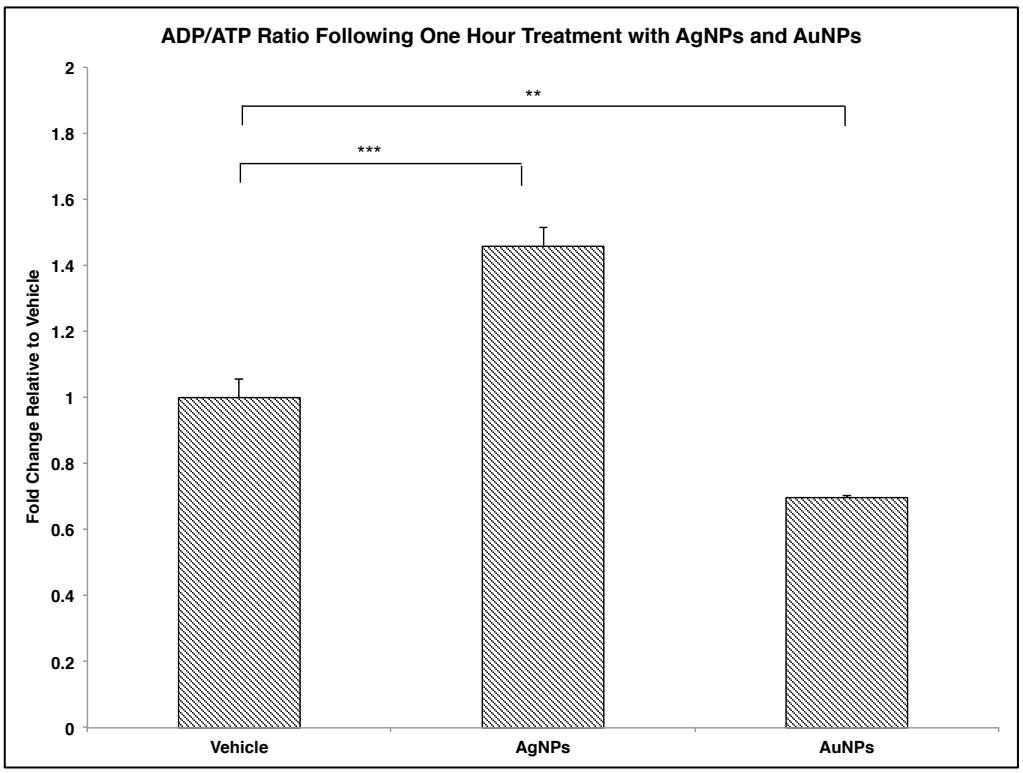


Fig. 3.1.12 Fold change in ADP/ATP ratio of SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 91.3 µg/mL AuNPs (n = 6 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$.

1.4. Discussion

This chapter was conducted to investigate the acute effects of AgNPs in SH-SY5Y human neuroblastoma mitochondria, and possibly rescue the AgNPs-induced mitochondrial dysfunction through the use of DHA and LC. SH-SY5Y is a subclone from SK-N-SH, an uncloned neuroblastoma line (Biedler et al., 1978, Lopes et al., 2010, Yusuf et al., 2013). Due to its capacity to acquire neuron-like phenotypes, SH-SY5Y is frequently used as a neuronal model (Lopes et al., 2010, Yusuf et al., 2013). Additionally, cell lines are easy to manipulate, with infinite lifespans when handled appropriately (ATCC, 2012). Therefore, SH-SY5Y human neuroblastoma cells were selected to investigate the effects of AgNPs.

The selected AgNPs concentration, 50 µg/mL, is based on previous cell culture studies that utilized AgNPs, where changes in mitochondrial function and cell morphology were detected (Hussain et al., 2005, Arora et al., 2008, Hsin et al., 2008). $AgNO_3$ and AuNPs concentrations were chosen to match the number of Ag and Au particles in the respective solutions to Ag particles in 50 µg/mL AgNPs suspension, at approximately 2.79 X 10^{17} particles/mL. AgNPs have been reported to release Ag ions over time (AshaRani et al., 2009, Kittler et al., 2010, Liu and Hurt, 2010, Yu et al., 2013). Hence, $AgNO_3$ solution was used as a control, to distinguish between effects of Ag nanoparticulates and Ag ions in solution. By determining number of AgNPs in suspension, total amount of Ag atoms was calculated and

converted to the concentration of $AgNO_3$ solution required, in the event that all atoms went into solution as Ag ions. After one hour incubation, $AgNO_3$-treated cells exhibited 0.99 % cell viability, while 94 % of AgNPs-treated cells survived. This reveals that AgNPs are much less hazardous as compared to Ag ions in solution, and indicates that results obtained from AgNPs-treated SH-SY5Y cells in subsequent assays are mainly due to the actions of AgNPs. Since incubation with $AgNO_3$ led to death of almost all SH-SY5Y cells, it was not used in other experiments.

Mitochondrial membrane potential is generated from the presence of an electrochemical gradient across the inner mitochondrial membrane (Alberts et al., 2008, Voet et al., 2013). JC-1 dye is used to indicate status of mitochondria as it accumulates as red fluorescence aggregates in healthy cell mitochondria but stays in its cytoplasmic green monomeric form when mitochondrial membrane depolarizes (Ma et al., 2011, Guo et al., 2013). Acute exposure of AgNPs to SH-SY5Y cells led to a drop in 590/535 ratio, similar to CCCP, the positive control, suggesting mitochondrial membrane damage. CCCP is a well-characterized protonophore. Exposure of CCCP to cells causes rapid loss of mitochondrial membrane potential and mitochondrial morphological swelling (Minamikawa et al., 1999, Lim et al., 2001, Perry et al., 2011). This corroborates with results from previous studies where AgNPs affected mitochondria of non-neuronal cells, determined by TPP+ – selective electrode (Teodoro et al., 2011) and rhodamine

123 uptake (Hussain et al., 2005). Mitochondrial membrane depolarization and oxidative stress are important cellular occurrences that often precede the induction of apoptosis (Taggart et al., 2014). However, the incubation period of one hour in the current study was probably too short for apoptosis to occur, as demonstrated by the lack of significant cell death in the trypan blue exclusion cell viability assay.

ATP levels decreased in AgNPs-treated cells indicating possible damage to mitochondrial respiratory chain (AshaRani et al., 2009, Costa et al., 2010). AgNPs have been reported to cause oxidative stress and calcium dysregulation in cells (Haase et al., 2012). In order to restore intracellular calcium levels, the Ca^{2+} ATPase pump or Na^{+}/Ca^{2+} exchanger could be activated, leading to increased intracellular sodium and Na^{+}/K^{+} ATPase pump activity to maintain resting potential (Wang et al., 2003, Alberts et al., 2008). Therefore, usage of these pumps could in turn result in lowered ATP levels in cells. Another reason for the observed reduction in ATP levels could be due to cell lysis and release of intracellular ATP (Suszynski et al., 2008). Nevertheless, this is unlikely as negligible cell death was observed for AgNPs-treated cells as compared to vehicle control in the current study. As ATP levels decline, a corresponding rise in ADP levels is expected since ATP hydrolyzes to form ADP (Alberts et al., 2008, Myhill et al., 2009, Voet et al., 2013). Conversely, a decrease in ADP levels was detected. This could possibly be due to the action of adenylate kinase (AK), a phosphotransferase that catalyzes the

conversion of ADP to ATP and adenosine monophosphate (AMP) (Alberts et al., 2008, Voet et al., 2013). Under conditions of low ATP levels, AK compensates by using two ADP molecules to generate more ATP and in the process produce AMP, which can be degraded by AMP deaminase to give rise to inosine monophosphate (IMP), preventing the recycling of AMP to ADP and ATP (Myhill et al., 2009). This decreases the adenine nucleotide source and diminishes ADP and ATP stores.

Studies have demonstrated protective properties of DHA and LC against mitochondrial dysfunction and neuronal injury (Horrocks and Farooqui, 2004, Virmani and Binienda, 2004, Alves et al., 2009, He et al., 2011). Co-incubation of SH-SY5Y cells with AgNPs and DHA led to greater ATP and ADP levels compared to AgNPs-treated cells only, suggesting that DHA was capable of partially reversing damage caused by acute AgNPs exposure. DHA is incorporated in both cell (Horrocks and Farooqui, 2004) and mitochondrial membrane phospholipids (O'Shea et al., 2009, Khairallah et al., 2012), and could repair AgNPs-induced damage to these membranes. Moreover, co-supplementation of DHA and LC was even more effective at preventing AgNPs-induced drop in ATP levels. Statistically significant differences were also detected between cells treated with AgNPs + DHA and AgNPs + DHA + LC, and cells treated with AgNPs + LC and AgNPs + DHA + LC, possibly indicating a synergistic association between DHA and LC. The main function of LC

is to facilitate fatty acid transport, such as DHA, into mitochondrial matrix for the production of ATP via β-oxidation (Kerner and Hoppel, 2000, Walter and Schaffhauser, 2000, Hoppel, 2003). LC is also crucial for protection against mitochondrial dysfunction (Ishii et al., 2000, Virmani and Binienda, 2004, Hino et al., 2005, He et al., 2011, Geier and Geier, 2013). It is hypothesized that the effectiveness of co-supplementation of DHA and LC in diminishing AgNPs' harmful effects could be because LC aids in the transport of DHA into mitochondria for the incorporation of DHA into mitochondrial phospholipids to repair damaged phospholipids affected by AgNPs' attack.

As AuNPs are usually associated with low or no cytotoxicity (Connor et al., 2005, Bar-Ilan et al., 2009, Asharani et al., 2011), AuNPs were used as a positive control in the study. As expected, one hour incubation with AuNPs did not lead to significant cell death, indicating its relative non-toxicity. However, AuNPs may demonstrate other cellular damage. Incubation of SH-SY5Y cells with AuNPs for one hour led to increased ATP levels, no changes in ADP levels, and decreased ADP/ATP ratio as compared to vehicle control. This could possibly be due to depressed cellular processes following the one hour incubation with AuNPs, resulting in decreased consumption of ATP, and consequently, an accumulation of ATP. Lack of significant alterations in ADP levels following AuNPs exposure suggests that ATP is unlikely to be synthesized. Several studies have shown that exposure to AuNPs led to a depression of ATP-dependent cellular

processes such as cell proliferation, cell cycle progression, and motility (Pernodet et al., 2006, Balasubramanian et al., 2010, Liu et al., 2014). The reduction in ATP expenditure could contribute to the observed accumulation of ATP following one hour incubation with AuNPs.

CHAPTER 2

CALCIUM-INDEPENDENT PHOSPHOLIPASE A_2

AND ACUTE SILVER NANOTOXICITY

2.1. Introduction

In the previous chapter, it was established that acute exposure of AgNPs to SH-SY5Y cells resulted in mitochondrial dysfunction and subsequently decreased ATP production, without leading to cell death. The harmful effects exerted by AgNPs were rescued through the co-supplementation of DHA and LC. Apart from disrupting mitochondrial function, nanotoxicity is often associated with the triggering of inflammatory responses (Park and Park, 2009, Xue et al., 2012, Panas et al., 2013). AgNPs exposure in various cell lines led to the induction of inflammation (Romoser et al., 2012, Prasad et al., 2013). Additionally, mice (Cha et al., 2008) and rats (Kim et al., 2010) exposed to AgNPs showed changes in expression of genes and proteins that moderate ROS formation, inflammation, and apoptosis. On the contrary, AuNPs do not show overt toxicity, although it could be identified as foreign materials by immune cells, leading to acute inflammation and apoptosis (Cho et al., 2009).

PLA_2 enzymes play integral roles in brain and are crucial for signal transduction, production of eicosanoids and lipid mediators, inflammation, membrane remodeling, and lipid metabolism (Dennis, 1997, Farooqui et al., 2000, Ong et al., 2010, Sun et al., 2014). Under normal conditions, PLA_2 enzymes prevent membrane lipid peroxidation through the removal of oxidized fatty acids from phospholipids (Tan et al., 1984, Sevanian and Kim, 1985, McLean et al., 1993, Ong et al., 2015). In pathological conditions however, elevated activation of PLA_2

enzymes result in increased production of fatty acids and lysophospholipids that will be metabolized to form second messengers and metabolites that could lead to inflammation (Sun et al., 2014, Ong et al., 2015). $cPLA_2$ preferentially releases AA from glycerophospholipids (Murakami and Kudo, 2002, Sun et al., 2004, Ong et al., 2015), where AA becomes precursors for eicosanoids and prostanoids, which lead to inflammatory responses in cells (Calder, 2008, Sun et al., 2014). Elevated $cPLA_2$ activity is often associated with oxidative stress and neuroinflammation (Ong et al., 2003, Farooqui and Horrocks, 2006, Tanaka et al., 2012). On the other hand, $iPLA_2$ mediates release of DHA, which is further metabolized to docosanoids that antagonize effects of AA-derived pro-inflammatory eicosanoids thus exhibiting anti-inflammatory and anti-apoptotic characteristics (Mukherjee et al., 2004, Bazan, 2009, Ong et al., 2015).

In this chapter, the relationship between AgNPs and PLA_2 enzymes, in particular $cPLA_2$ and $iPLA_2$, was examined. The mechanism underlying AgNPs-induced inflammatory responses in SH-SY5Y cells – induction of $cPLA_2$, depression of $iPLA_2$ expression and function, or both – was also determined.

2.2. Materials and Methods

2.2.1. Cell Culture

SH-SY5Y cells were cultured in complete growth medium, and grown in 100 mm dishes. SH-SY5Y cells were incubated under standard conditions of 37 °C and 5 % CO_2, and regularly passaged with 0.25 % Trypsin-EDTA. At 70 % confluence, cells were used for treatments.

2.2.2. Synthesis, Purification, and Characterization of AgNPs and AuNPs

26 nm AgNPs and 20 nm AuNPs were synthesized, purified, and characterized as described in chapter 1 (pages 40 – 43).

2.2.3. Trypan Blue Exclusion Cell Viability Assay

SH-SY5Y cells were plated in a 6-well plate at 0.5 X 10^6 cells/well, and allowed to attach overnight. Three groups of cells were treated with the following reagents: (1) vehicle, water, (2) 50 µg/mL AgNPs, (3) 91.3 µg/mL AuNPs, and incubated for six hours. PBS was used to wash the cells. The assay was then carried out as described in chapter 1 (page 44).

2.2.4. Cell Treatment

2.2.4.1. Treatment with AgNPs

Two groups of SH-SY5Y cells were treated with the following reagents: (1) vehicle, water, (2) 50 μg/mL AgNPs, and were incubated for one and six hours. PBS was used to wash the cells. SH-SY5Y cells were then harvested for further real-time RT-PCR, immunocytochemistry, and lipidomic profiling analyses.

2.2.4.2. Treatment with AgNPs and AuNPs

Three groups of SH-SY5Y cells were treated with the following reagents: (1) vehicle, water, (2) 50 μg/mL AgNPs, (3) 91.3 μg/mL AuNPs, and incubated for one and six hours. PBS was used to wash the cells. SH-SY5Y cells were then harvested for further real-time RT-PCR analyses.

2.2.4.3. Treatment with AgNPs, DHA, and LC

Eight groups of SH-SY5Y cells were treated with the following reagents: (1) vehicle, ethanol and water, (2) 10 μM DHA, (3) 100 μM LC, (4) 10 μM DHA and 100 μM LC, (5) 50 μg/mL AgNPs, (6) 50 μg/mL AgNPs + 10 μM DHA, (7) 50 μg/mL AgNPs + 100 μM LC, (8) 50 μg/mL AgNPs + 10 μM DHA + 100 μM LC, and incubated for six hours. PBS was used to wash the cells. SH-SY5Y cells were then harvested for further real-time RT-PCR analysis.

2.2.5. Real-time RT-PCR

RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands) was used to extract and purify RNA of treated cells, which was then reverse transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems®, Life Technologies, Carlsbad, CA, USA) with the stated reaction parameters: 10 minutes at 25 °C, 120 minutes at 37 °C, and 5 minutes at 85 °C. Using a 7,500 Real-time PCR system, real-time PCR amplification was carried out with the following reagents: TaqMan® Universal PCR Master Mix (#4304437) and probes for human $cPLA_2$ (Hs00233352_m1), $iPLA_2$ (Hs00185926_m1), and ACTB (beta actin) (#4326315E) (Applied Biosystems®, Life Technologies, Carlsbad, CA, USA), and with the stated parameters: 2 minutes at 50 °C, 10 minutes at 95 °C, 40 15 seconds cycles at 95 °C, and 1 minute at 60 °C. Amplified transcripts were quantified using the comparative threshold cycle (CT) method (Livak and Schmittgen, 2001), where relative fold change is calculated with the following equation:

$$2^{-\Delta\Delta CT}$$

CT is the number of cycles where reporter fluorescence emission surpasses preset threshold level. CT value inversely correlates with target mRNA levels.

2.2.6. Immunocytochemistry

SH-SY5Y cells were plated on poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) coated coverslips in a 24-well plate at 2.0 X 10^5

cells/well, and allowed to attach overnight. Two groups of cells were treated with the following reagents: (1) vehicle, water, (2) 50 µg/mL AgNPs, and incubated for six hours. PBS was used to wash the cells. SH-SY5Y cells were then fixed with 2 % paraformaldehyde (PanReac AppliChem, Barcelona, Spain), washed with PBS, and reacted with 70 % formic acid (Sigma-Aldrich, St. Louis, MO, USA) for 20 minutes and 0.1 % PBS-Triton® X-100 (USB Corporation, Cleveland, OH, USA) for 15 minutes. Cells were incubated with 1 % bovine serum albumin (BSA) (Calbiochem®, Merck Millipore, Billerica, MA, USA) in PBS for one hour, and anti-iPLA$_2$ antibody (#160507, Cayman Chemical, Ann Arbor, MI, USA; diluted 1:50) in 1 % BSA overnight at 4 °C. PBS was used to wash the cells. SH-SY5Y cells were incubated with Alexa Fluor® 488 Donkey Anti-Rabbit lgG (H+L) Antibody (Applied Biosystems®, Life Technologies, Carlsbad, CA, USA; diluted 1:200) for one hour at room temperature, and subsequently mounted onto microscope slides using ProLong Gold anti-fade reagent with DAPI (Invitrogen™, Life Technologies, Carlsbad, CA, USA), which is also used for nuclear counterstaining. Cells were viewed using the Olympus FluoView FV1000 confocal microscope at 40 X magnification. Total cell fluorescence corrected for background was determined with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.2.7. Lipidomic Profiling

2.2.7.1. Lipid Extraction

Lipid extraction was conducted using a modified protocol from Bligh and Dyer (1959). SH-SY5Y cells were passed through 27 G needles and sonicated in 750 µL chloroform/methanol, 1:2 (v/v) and 5 µL of internal standards solution containing PC 14:0 14:0, LysoPC 20:0, PE 14:0 14:0, PS 14:0 14:0, SM 30:1, and Cer 17:0 (Avanti Polar Lipids, Alabaster, AL, USA) for 30 minutes at 4 °C. Samples were supplemented with 250 µL chloroform and 450 µL 0.88 % potassium chloride solution, and centrifuged for two minutes at 9,000 *g* at 4 °C. 250 µL chloroform was used to re-extract the aqueous phase and the organic fractions were combined, where the lipids were then isolated and vacuum-dried (Thermo Scientific™ Savant™ SpeedVac™, Life Technologies, Carlsbad, CA, USA).

2.2.7.2. Lipid Analysis

Quantification of individual polar lipids was conducted using an Agilent HPLC-Chip system connected with an Agilent Technologies 6460 Triple Quad LC/MS (Santa Clara, CA, USA), where the column used was Kinetex 2.6 µM HILIC 100 Å chip with a LC column of 150 X 2.10 mm size. Solvents used were 50 % acetonitrile + 50 % 25 µM ammonium formate buffer pH 4.6 (solvent A) and 95 % acetonitrile + 5 % 25 µM ammonium formate buffer pH 4.6 (solvent B). Analytes were eluted under the stated parameters: 0.1 % solvent A and 99.9 %

solvent B (0 to 6 minutes), 75 % solvent A and 25 % solvent B (6 to 7 minutes), 90 % solvent A and 10 % solvent B (7 to 7.1 minutes), and 0.1% solvent A and 99.9% solvent B (7.1 to 10.1 minutes) using a constant 0.5 mL/min flow rate. For the MS source parameters, gas temperature was set at 300 °C with a 5 L/min flow rate and nebulizer at 45 psi. Sheath gas temperature was 250 °C with a 11 L/min gas flow rate. Data was then extracted and analyzed using the Agilent MassHunter acquisition, Agilent MassHunter Qualitative and Agilent MassHunter Quantitative software (Santa Clara, CA, USA).

2.2.8. Statistical Analyses

Mean and standard error of values were determined for each experimental group, and possible significant differences among various groups were analyzed using two-tailed unpaired Student's t-test or one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $p < 0.05$ was deemed significant.

2.3. Results

2.3.1. Real-time RT-PCR Analyses

2.3.1.1. Effect of AgNPs Treatment on $cPLA_2$ and $iPLA_2$ mRNA Expression

2.3.1.1.1. 1 Hour

One hour incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 1.26-fold (p = 0.44) change in $cPLA_2$ mRNA expression compared to vehicle control (Fig. 3.2.1). 50 µg/mL AgNPs-treated SH-SY5Y cells exhibited a 0.74 fold (p = 0.004) statistically significant change in $iPLA_2$ mRNA expression compared to vehicle control.

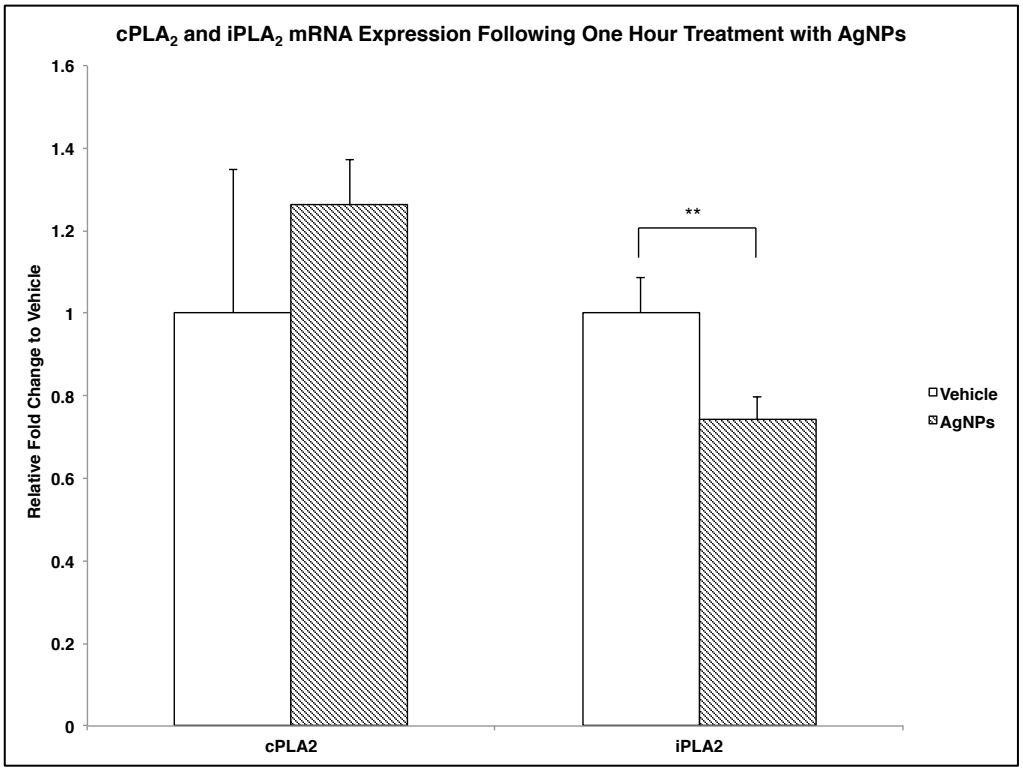


Fig. 3.2.1 Fold change in $cPLA_2$ and $iPLA_2$ mRNA expression in SH-SY5Y cells following one hour incubation with vehicle control and 50 µg/mL AgNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by two-tailed unpaired Student's t-test. ** $p < 0.01$.

2.3.1.1.2. 6 Hours

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 1.12-fold (p = 0.69) change in $cPLA_2$ mRNA expression compared to vehicle control (Fig. 3.2.2). 50 µg/mL AgNPs-treated SH-SY5Y cells exhibited a 0.48 fold ($p < 0.001$) statistically significant change in $iPLA_2$ mRNA expression compared to vehicle control.

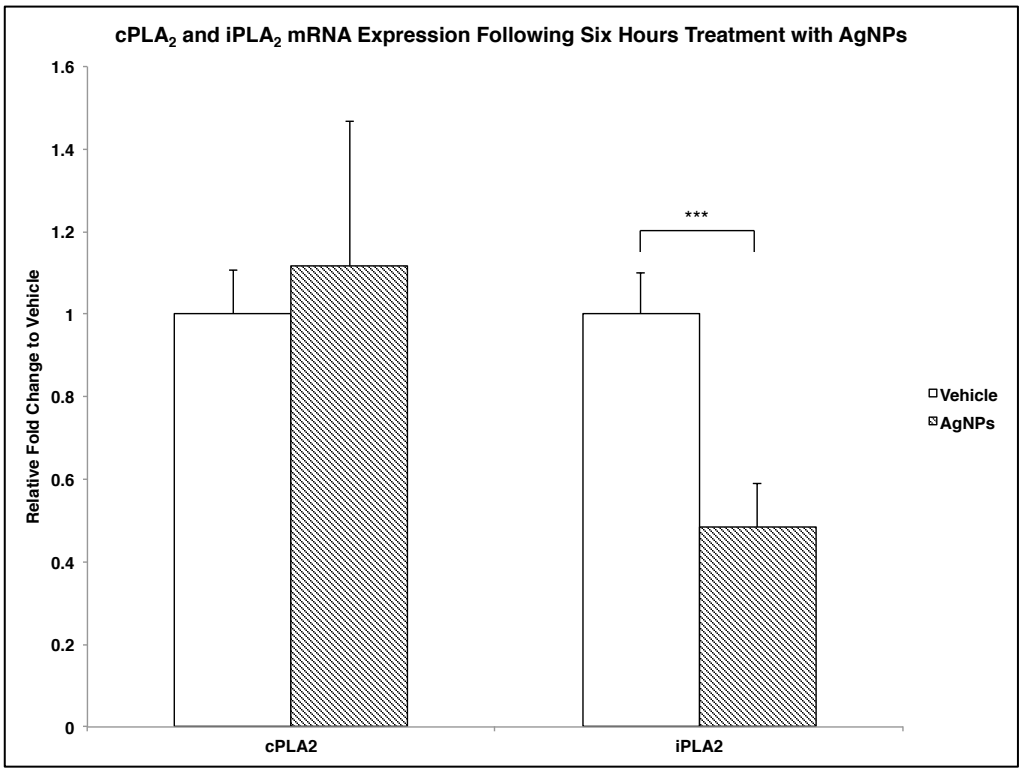


Fig. 3.2.2 Fold change in $cPLA_2$ and $iPLA_2$ mRNA expression in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by two-tailed unpaired Student's t-test. *** $p < 0.001$.

2.3.1.2. Effect of AgNPs and AuNPs Treatment on iPLA$_2$ mRNA Expression

2.3.1.2.1. 1 Hour

One hour incubation of SH-SY5Y cells with 91.3 µg/mL AuNPs resulted in a 0.48-fold ($p < 0.001$) statistically significant change in iPLA$_2$ mRNA expression compared to vehicle control (Fig. 3.2.3). Similar to previous findings, 50 µg/mL AgNPs-treated SH-SY5Y cells led to a 0.74-fold ($p = 0.006$) statistically significant change in iPLA$_2$ mRNA expression compared to vehicle control.

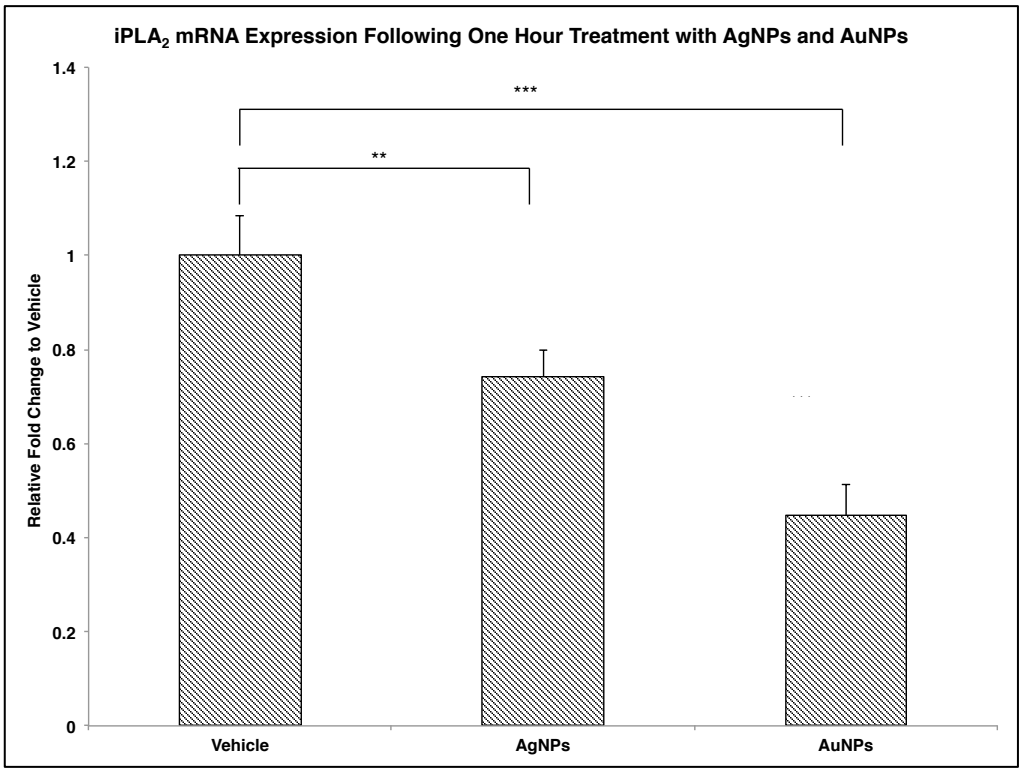


Fig. 3.2.3 Fold change in iPLA$_2$ mRNA expression in SH-SY5Y cells following one hour incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 91.3 µg/mL AuNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$.

2.3.1.2.2. 6 Hours

Six hours incubation of SH-SY5Y cells with 91.3 µg/mL AuNPs resulted in a 0.97-fold (p = 1.000) change in $iPLA_2$ mRNA expression compared to vehicle control (Fig. 3.2.4). Similar to previous findings, 50 µg/mL AgNPs-treated SH-SY5Y cells led to a 0.48-fold (p = 0.009) statistically significant change in $iPLA_2$ mRNA expression compared to vehicle control.

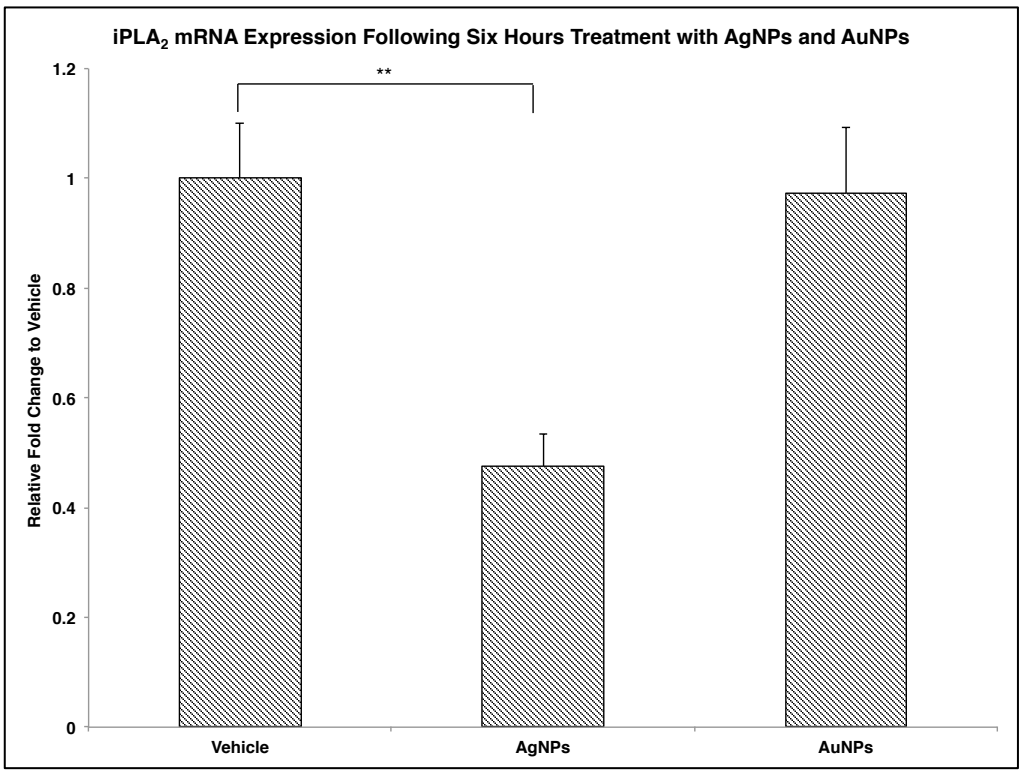


Fig. 3.2.4 Fold change in $iPLA_2$ mRNA expression in SH-SY5Y cells following six hours incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 91.3 µg/mL AuNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. ** $p < 0.01$.

2.3.1.3. Effect of AgNPs, DHA, and LC Treatment on $iPLA_2$ mRNA Expression

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 0.29-fold ($p < 0.001$) statistically significant change in $iPLA_2$ mRNA expression compared to vehicle control (Fig. 3.2.5). 10 µM DHA + 100 µM LC-treated cells exhibited a 0.51-fold ($p = 0.016$) significant change in $iPLA_2$ mRNA expression compared to vehicle control. There was a 0.30-fold ($p < 0.001$), 0.34-fold ($p < 0.001$), and 0.28-fold ($p < 0.001$) change in $iPLA_2$ mRNA expression in cells treated with the various rescue efforts – 50 µg/mL AgNPs + 10 µM DHA, 50 µg/mL AgNPs + 100 µM LC, and 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC – compared to vehicle control. Compared to 50 µg/mL AgNPs-treated SH-SY5Y cells, no significant changes in $iPLA_2$ mRNA expression were observed. Statistically significant reductions in $iPLA_2$ mRNA expression were also observed in cells treated with 10 µM DHA compared to 50 µg/mL AgNPs + 10 µM DHA ($p < 0.001$), and 100 µM LC compared to 50 µg/mL AgNPs + 100 µM LC ($p = 0.002$).

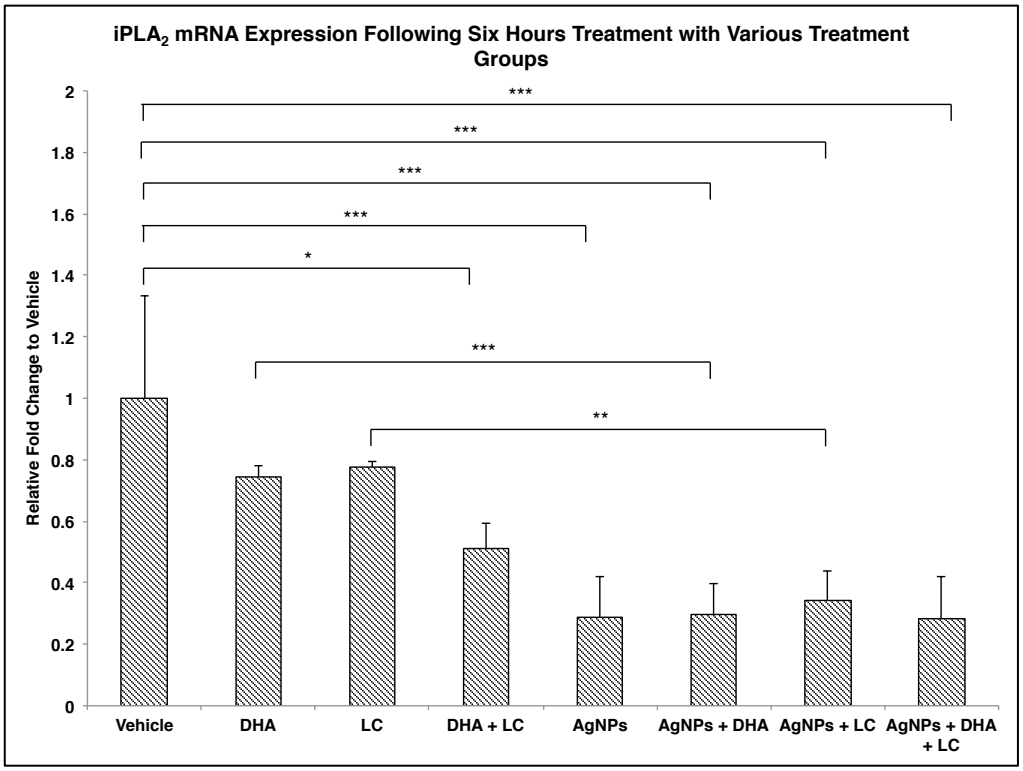


Fig. 3.2.5 Fold change in $iPLA_2$ mRNA expression in SH-SY5Y cells following six hours incubation with various treatment groups – vehicle control; 10 µM DHA; 100 µM LC; 10 µM DHA + 100 µM LC; 50 µg/mL AgNPs; 50 µg/mL AgNPs + 10 µM DHA; 50 µg/mL AgNPs + 100 µM LC; 50 µg/mL AgNPs + 10 µM DHA + 100 µM LC (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3.2. Immunocytochemistry Analysis

$iPLA_2$ immunofluorescence labeling showed localization of $iPLA_2$ protein in the cytoplasm of SH-SY5Y cells (Fig. 3.2.6). Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 0.77-fold ($p < 0.001$) statistically significant change in $iPLA_2$ fluorescence intensity compared to vehicle control (Fig. 3.2.7).

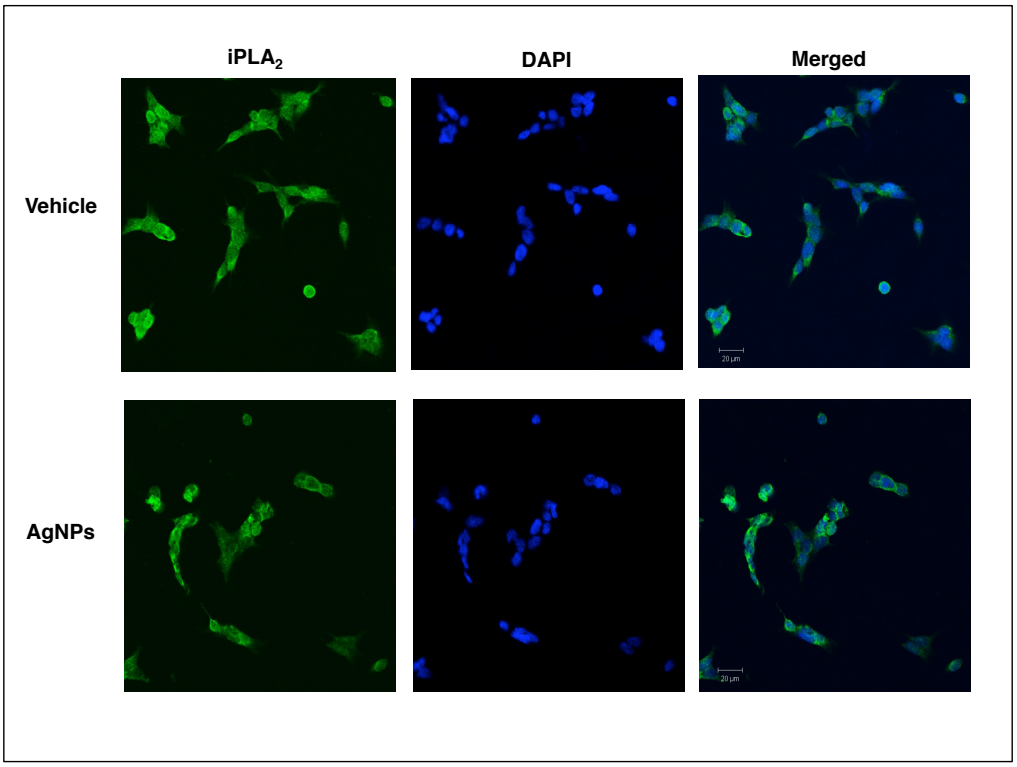


Fig. 3.2.6 Immunocytochemistry photos of $iPLA_2$ protein expression in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Scale: 20 µm.

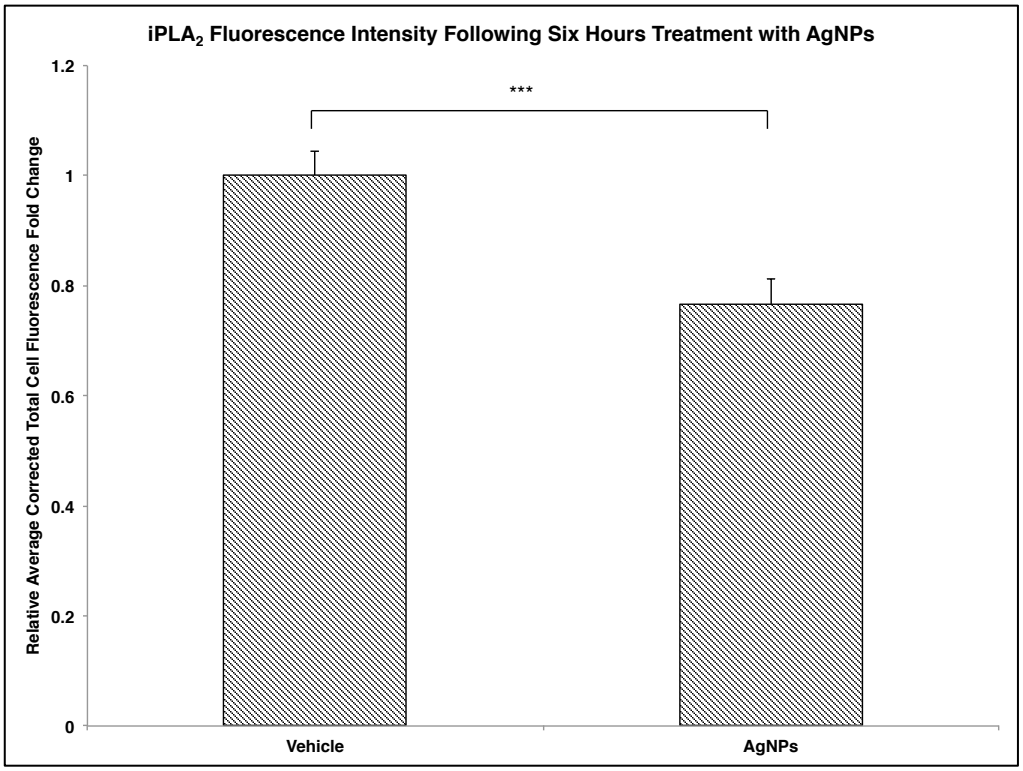


Fig. 3.2.7 Fold change in $iPLA_2$ fluorescence intensity in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by two-tailed unpaired Student's t-test. *** $p < 0.001$.

2.3.3. Trypan Blue Exclusion Cell Viability Assay Analysis

Six hours incubation of SH-SY5Y cells resulted in cell viabilities of 98.3 %, 94.0 %, and 98.2 % for vehicle control, 50 µg/mL AgNPs-, and 91.3 µg/mL AuNPs-treated cells respectively (Fig. 3.2.8). No significant differences were observed between vehicle control and 50 µg/mL AgNPs- and 91.3 µg/mL AuNPs-treated cells.

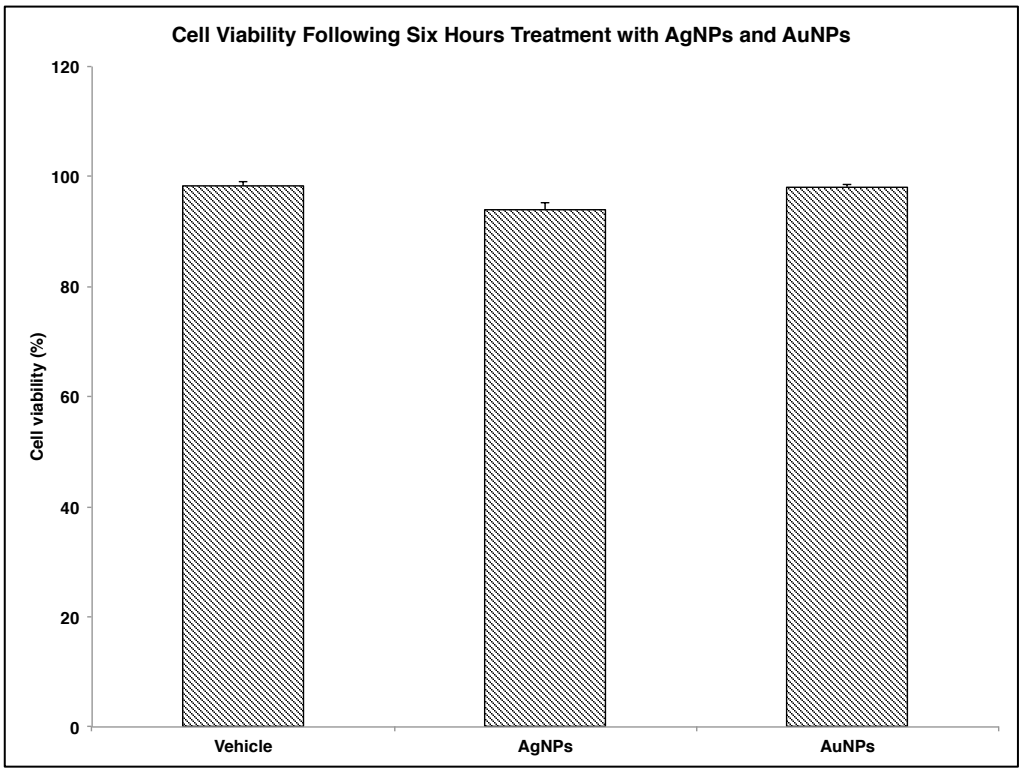


Fig. 3.2.8 Cell viability of SH-SY5Y cells following six hours incubation with various treatment groups – vehicle control; 50 µg/mL AgNPs; 91.3 µg/mL AuNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM.

2.3.4. Lipidomic Profiling Analysis

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in significant differences in relative abundance of various phospholipid and lysophospholipid species compared to vehicle control (Table 3.2.1) – phosphatidylcholine (PC) (Fig. 3.2.9A & 3.2.9B), lysophosphatidylcholine (LysoPC) (Fig. 3.2.10A & 3.2.10B), phosphatidylethanolamine (PE) (Fig. 3.2.11A & 3.2.11B), and phosphatidylserine (PS) (Fig. 3.2.13). Significant increases in relative abundance of PC 30:0p, PC 30:0, PC 32:1e, PC 32:0e, PC 32:0p, PC 32:2, PC 34:2e, PC 34:1e, PC 34:1p, PC 34:5, PC 34:4, PC 34:3, PC 34:2, PC 36:2e, PC 36:6, PC 36:5, PC 36:3, PC 36:1, PC 36:0, PC 38:4e, PC 38:4p, PC 38:7, PC 18:2/20:4, PC 38:2, PC 40:7e, PC 40:5e, PC 40:6p, PC 40:8, PC 40:7, PC 40:6, PC 40:4, PE 34:1e, PE 34:3, PE 34:2, PE 34:1, PE 35:2, PE 36:5e, PE 36:3, PE 36:2, PE 36:1, PE 36:0, PE 16:0p/20:4, PE 18:0p/18:1, PE 38:4, PE 38:3, PE 16:0p/22:6, PE 16:0p/22:5, PE 40:7, PE 40:6, PE 40:5, PE 18:0p/22:6, PS 36:2, PS 36:1, PS 38:4, PS 38:3, PS 40:6, PS 40:5, LysoPC 18:0, LysoPC 24:0e, and LysoPC 26:0 lipid species were observed in 50 µg/mL AgNPs-treated SH-SY5Y cells as compared to vehicle control. In contrast, PC 32:3 and PE 32:0 lipid species showed a significant reduction in relative abundance in 50 µg/mL AgNPs-treated cells as compared to vehicle control. No significant differences were observed in lysophosphatidylethanolamine (LysoPE) lipid species (Fig. 3.2.12).

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs also resulted in significant differences in relative abundance of sphingomyelin (SM) (Fig. 3.2.14) and ceramide (Cer) (Fig. 3.2.15) lipid species (Table 3.2.1). Significant increases in relative abundance of SM 31:1, SM 32:2, SM 32:1, SM 32:0, SM 33:1, SM 34:2, SM 34:1, SM 34:0, SM 36:2, SM 36:1, SM 38:1, SM 39:1, SM 41:2, SM 34:1, SM 42:1, Cer 16:0, Cer 18:0, Cer 20:0, Cer 22:0, Cer 24:1, and Cer 24:0 lipid species were observed in 50 µg/mL AgNPs-treated SH-SY5Y cells as compared to vehicle control.

Table 3.2.1 Lipid species with significant changes in relative abundance following six hours incubation with vehicle control and 50 µg/mL AgNPs.

	Vehicle		AgNPs		
Lipid Species	**Mean (/10^6 cells)**	**S.E. (/10^6 cells)**	**Mean (/10^6 cells)**	**S.E. (/10^6 cells)**	***p*-Value**
PC 30:0p	2.18E-03	2.47E-04	2.46E-03	2.01E-04	0.047
PC 30:0	2.79E-01	3.44E-03	2.90E-01	1.81E-02	0.039
PC 32:1e	1.96E-02	1.28E-03	3.19E-02	1.87E-03	0.019
PC 32:0e	4.41E-02	3.83E-03	5.57E-02	3.71E-03	0.014
PC 32:0p	4.81E-03	2.56E-04	6.29E-03	7.80E-04	0.023
PC 32:3	1.21E-02	1.48E-03	1.66E-02	1.19E-03	0.046
PC 32:2	8.67E-02	9.66E-03	1.21E-01	8.80E-03	0.041
PC 34:2e	1.12E-02	1.95E-04	1.80E-02	8.54E-04	0.032
PC 34:1e	1.39E-01	9.13E-03	1.93E-01	1.07E-02	0.006
PC 34:1p	1.07E-02	6.35E-04	1.65E-02	1.15E-03	0.002
PC 34:5	3.18E-04	4.57E-05	4.08E-04	2.28E-05	0.009
PC 34:4	9.45E-03	9.07E-04	1.17E-02	8.21E-04	0.035
PC 34:3	5.79E-02	4.99E-03	7.75E-02	4.68E-03	0.019
PC 34:2	2.89E-01	3.52E-03	3.23E-01	2.01E-02	0.023
PC 36:2e	8.41E-03	7.85E-04	1.26E-02	7.03E-04	0.029
PC 36:6	3.82E-03	3.32E-04	5.14E-03	2.78E-04	0.005
PC 36:5	2.52E-02	5.82E-04	2.52E-02	1.41E-03	0.008
PC 36:3	6.90E-02	4.34E-03	8.21E-02	4.43E-03	0.032
PC 36:1	1.64E-01	3.66E-03	1.78E-01	1.03E-02	0.015

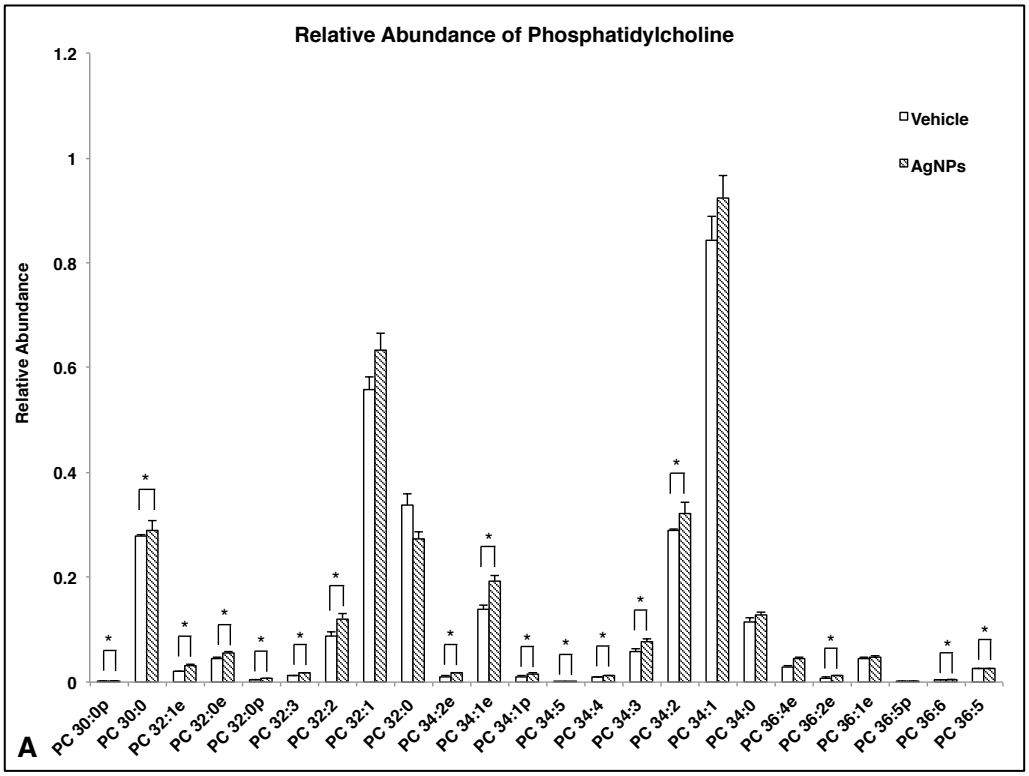
Lipid Species	Vehicle Mean (/10^6 cells)	Vehicle S.E. (/10^6 cells)	AgNPs Mean (/10^6 cells)	AgNPs S.E. (/10^6 cells)	*p*-Value
PC 36:0	2.44E-02	1.39E-03	2.82E-02	1.46E-03	0.022
PC 38:4e	1.08E-02	9.65E-04	1.82E-02	1.82E-03	0.010
PC 38:4p	1.38E-02	6.64E-04	1.90E-02	1.44E-03	0.010
PC 38:7	6.47E-03	7.14E-04	8.55E-03	6.08E-04	0.001
PC 18:2/20:4	4.71E-02	6.05E-04	5.28E-02	3.33E-03	0.003
PC 38:2	9.36E-03	7.63E-04	1.19E-02	8.52E-04	0.020
PC 40:7e	9.27E-03	7.26E-04	1.70E-02	1.17E-03	0.004
PC 40:5e	6.34E-03	4.16E-04	8.83E-03	7.73E-04	0.016
PC 40:6p	3.10E-03	3.57E-04	6.11E-03	7.06E-04	0.002
PC 40:8	4.80E-03	3.17E-04	6.08E-03	3.60E-04	0.046
PC 40:7	3.89E-02	2.11E-03	4.55E-02	2.48E-03	0.006
PC 40:6	1.08E-02	6.44E-04	1.13E-02	5.61E-04	0.021
PC 40:4	1.18E-02	1.25E-04	1.36E-02	8.89E-04	0.010
PE 32:0	2.49E-02	1.16E-03	2.29E-02	6.33E-04	0.036
PE 34:1e	1.11E-02	1.24E-03	2.89E-02	1.52E-03	0.014
PE 34:3	3.27E-02	3.63E-03	4.88E-02	4.33E-03	0.029
PE 34:2	1.40E-01	1.64E-02	1.89E-01	1.36E-02	0.027
PE 34:1	3.71E-01	2.33E-02	4.50E-01	2.59E-02	0.028
PE 35:2	2.28E-02	1.63E-03	2.98E-02	1.18E-03	0.020
PE 36:5e	1.02E-02	5.41E-04	1.46E-02	1.50E-03	0.040
PE 36:3	9.67E-02	9.62E-04	1.22E-01	4.93E-03	0.009

Lipid Species	Vehicle Mean (/10^6 cells)	Vehicle S.E. (/10^6 cells)	AgNPs Mean (/10^6 cells)	AgNPs S.E. (/10^6 cells)	*p*-Value
PE 36:2	4.47E-01	1.23E-02	5.36E-01	3.64E-02	0.002
PE 36:1	6.99E-01	5.81E-02	7.97E-01	4.80E-02	0.027
PE 36:0	8.64E-02	1.67E-03	9.50E-02	7.82E-03	0.014
PE 16:0p/20:4	1.03E-02	5.17E-04	1.46E-02	1.50E-03	0.041
PE 18:0p/18:1	3.60E-03	2.10E-04	4.35E-03	8.09E-04	0.009
PE 38:4	1.10	1.42E-02	1.22	8.18E-02	0.003
PE 38:3	4.76E-02	2.33E-03	6.45E-02	3.59E-03	0.003
PE 16:0p/22:6	1.82E-02	3.26E-03	2.90E-02	3.06E-03	0.009
PE 16:0p/22:5	5.31E-02	3.60E-03	9.69E-02	5.51E-03	0.011
PE 40:7	1.34E-01	1.57E-02	1.76E-01	1.31E-02	0.017
PE 40:6	2.60E-01	2.07E-02	3.44E-01	1.79E-02	0.019
PE 40:5	3.24E-01	5.13E-03	3.47E-01	2.85E-02	0.004
PE 18:0p/22:6	3.32E-02	4.34E-03	4.87E-02	3.32E-03	0.008
PS 36:2	3.87	4.16E-01	5.67	4.55E-01	0.005
PS 36:1	3.31E01	5.11E-01	4.54E01	3.35	0.017
PS 38:4	2.42	1.10E-01	2.81	1.91E-01	0.010
PS 38:3	3.08	2.14E-01	4.35	2.79E-01	0.006
PS 40:6	9.84	7.14E-01	1.44E01	1.42	0.004
PS 40:5	1.14E01	7.29E-01	1.44E01	1.21	0.009

Lipid Species	Vehicle Mean (/10^6 cells)	Vehicle S.E. (/10^6 cells)	AgNPs Mean (/10^6 cells)	AgNPs S.E. (/10^6 cells)	*p*-Value
LysoPC 18:0	3.31E-01	1.85E-02	4.82E-01	5.60E-02	0.030
LysoPC 24:0e	1.62E-02	1.97E-03	2.63E-02	1.78E-03	0.012
LysoPC 26:0	8.56E-02	4.52E-03	1.32E-01	1.88E-02	0.034
SM 31:1	9.22E-03	3.28E-04	1.16E-02	1.05E-03	0.002
SM 32:2	1.05E-02	9.72E-04	1.20E-02	1.03E-03	0.018
SM 32:1	3.82E-01	3.40E-02	4.68E-01	3.38E-02	0.017
SM 32:0	5.01E-02	3.87E-03	6.10E-02	2.83E-03	0.033
SM 33:1	7.49E-01	9.25E-02	9.27E-01	8.43E-02	0.018
SM 34:2	8.57E-01	7.28E-02	1.02	1.03E-03	0.021
SM 34:1	9.17	7.51E-01	1.17E01	8.68E-01	0.018
SM 34:0	4.91E-01	4.41E-02	5.95E-01	5.00E-02	0.014
SM 36:2	4.10E-01	2.01E-02	5.05E-01	3.26E-02	0.024
SM 36:1	6.68E-01	2.88E-02	8.25E-01	4.93E-02	0.003
SM 38:1	2.18E-01	5.06E-03	2.41E-01	1.93E-02	0.008
SM 39:1	4.69E-01	3.99E-02	7.25E-01	5.47E-02	0.009
SM 41:2	4.14E-01	4.88E-02	6.95E-01	4.83E-02	0.011
SM 41:1	1.39E-01	1.29E-02	2.02E-01	1.89E-02	0.006
SM 42:1	1.33E-01	3.36E-04	1.34E-01	1.36E-02	0.001
Cer 16:0	3.77E-01	2.55E-02	5.96E-01	2.70E-02	0.014
Cer 18:0	9.36E-02	6.62E-03	1.40E-01	1.31E-03	0.013
Cer 20:0	1.06E-02	1.65E-03	1.33E-02	7.52E-04	0.043

Lipid Species	Vehicle		AgNPs		*p*-Value
	Mean (/10^6 cells)	S.E. (/10^6 cells)	Mean (/10^6 cells)	S.E. (/10^6 cells)	
Cer 22:0	8.38E-02	2.19E-03	9.09E-02	5.08E-03	0.005
Cer 24:1	3.76E-01	5.78E-03	4.11E-01	2.65E-02	0.002
Cer 24:0	1.07E-01	3.23E-03	1.24E-01	8.74E-03	0.005

2.3.4.1. Phosphatidylcholine and Lysophosphatidylcholine



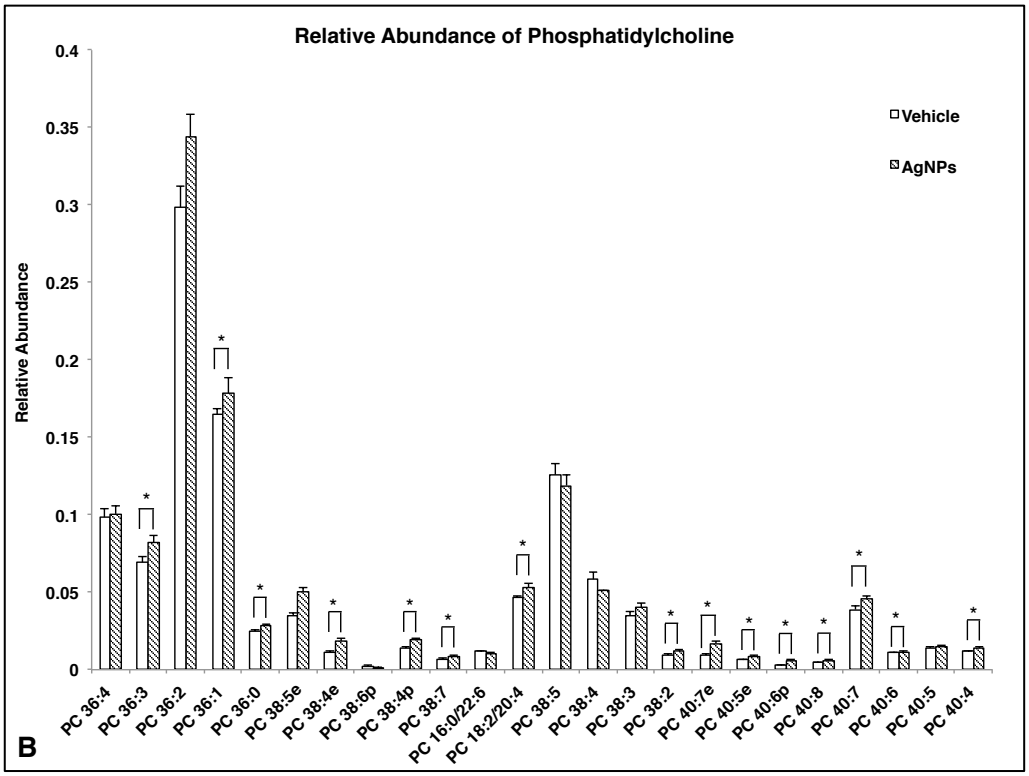
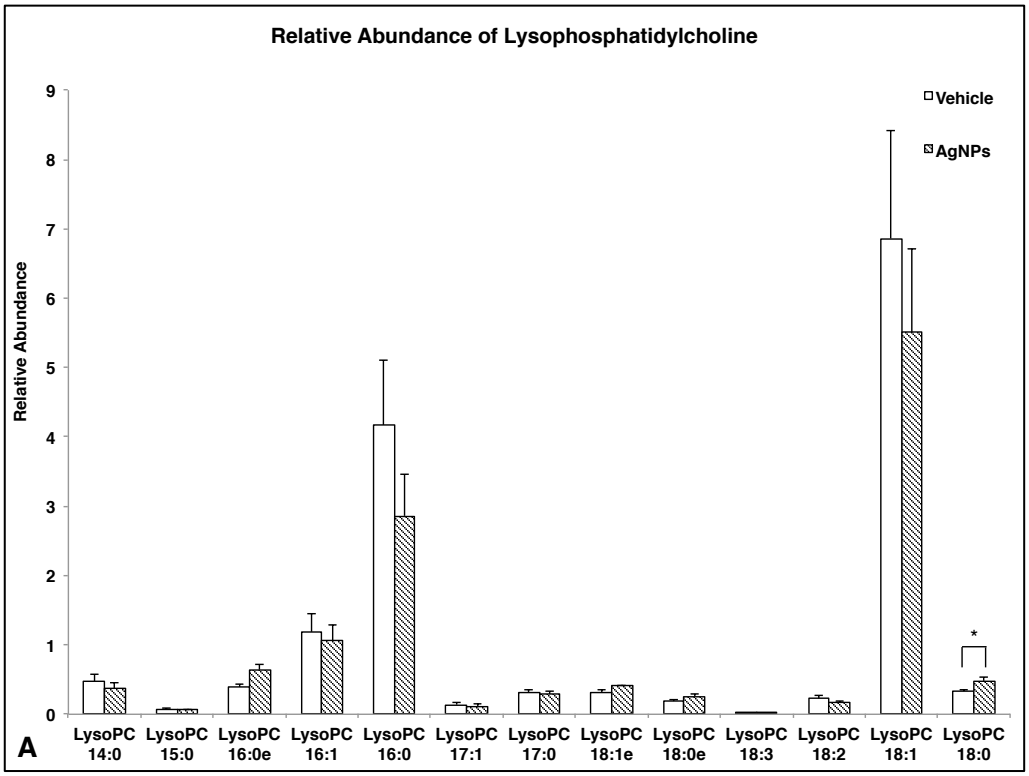


Fig. 3.2.9 Relative abundance of phosphatidylcholine in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in relative abundance by two-tailed unpaired Student's t-test. * $p < 0.05$. PC – phosphatidylcholine.



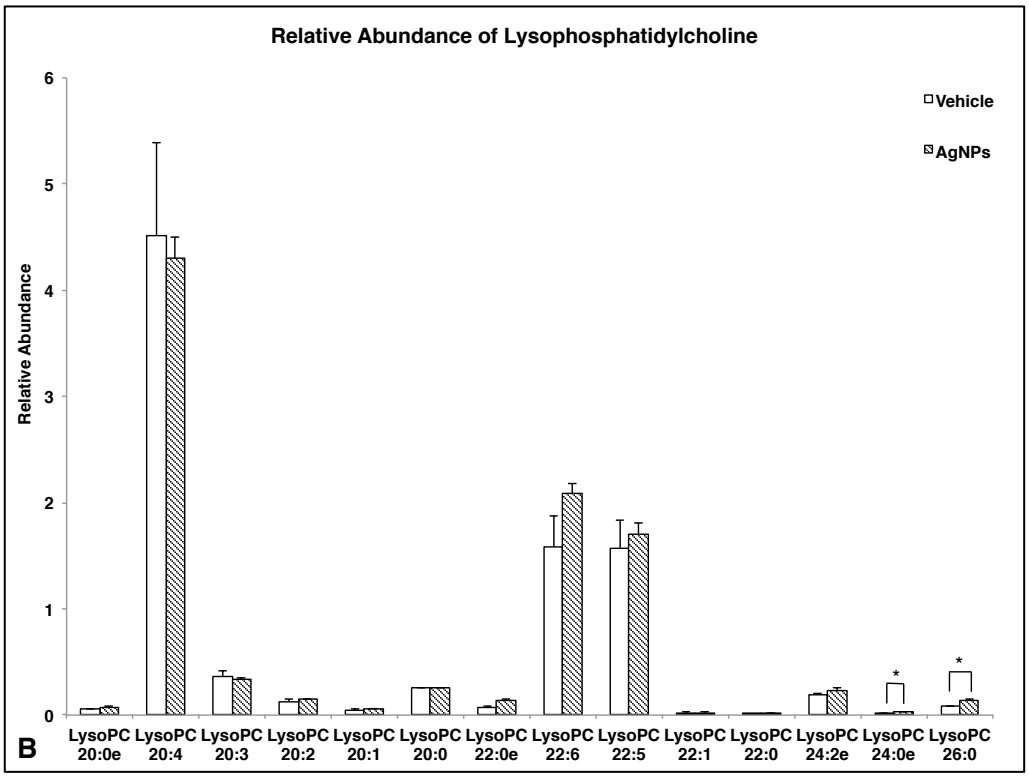
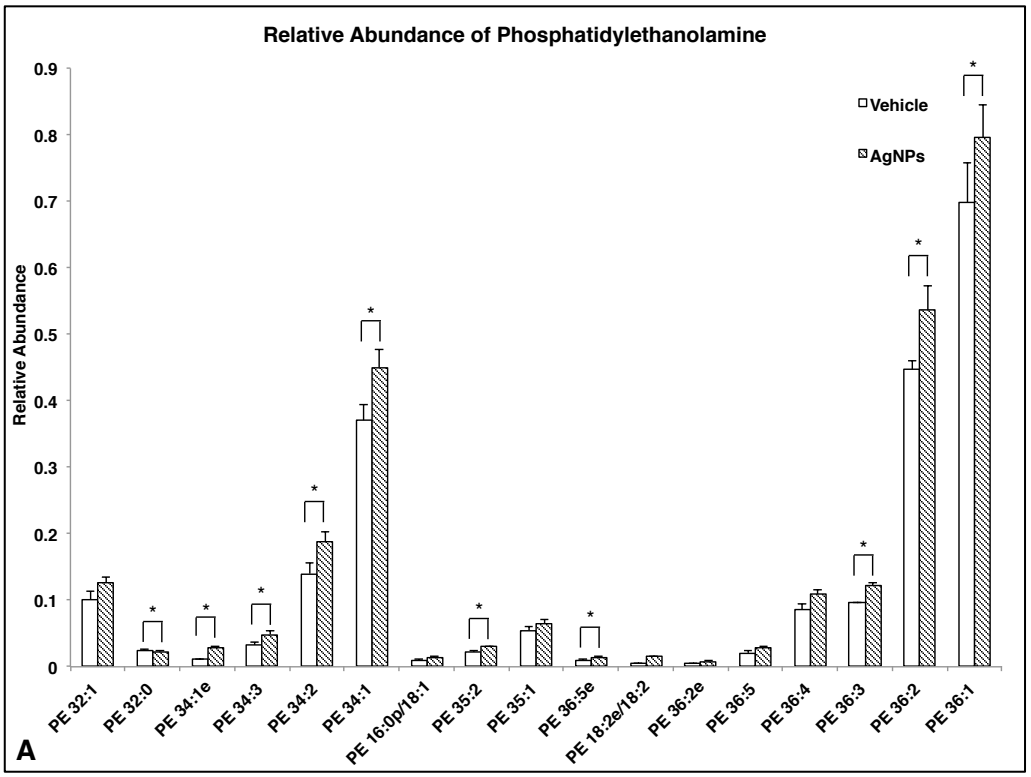


Fig. 3.2.10 Relative abundance of lysophosphatidylcholine in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in relative abundance by two-tailed unpaired Student's t-test. * $p < 0.05$. LysoPC – lysophosphatidylcholine.

2.3.4.2. Phosphatidylethanolamine and Lysophosphatidylethanolamine



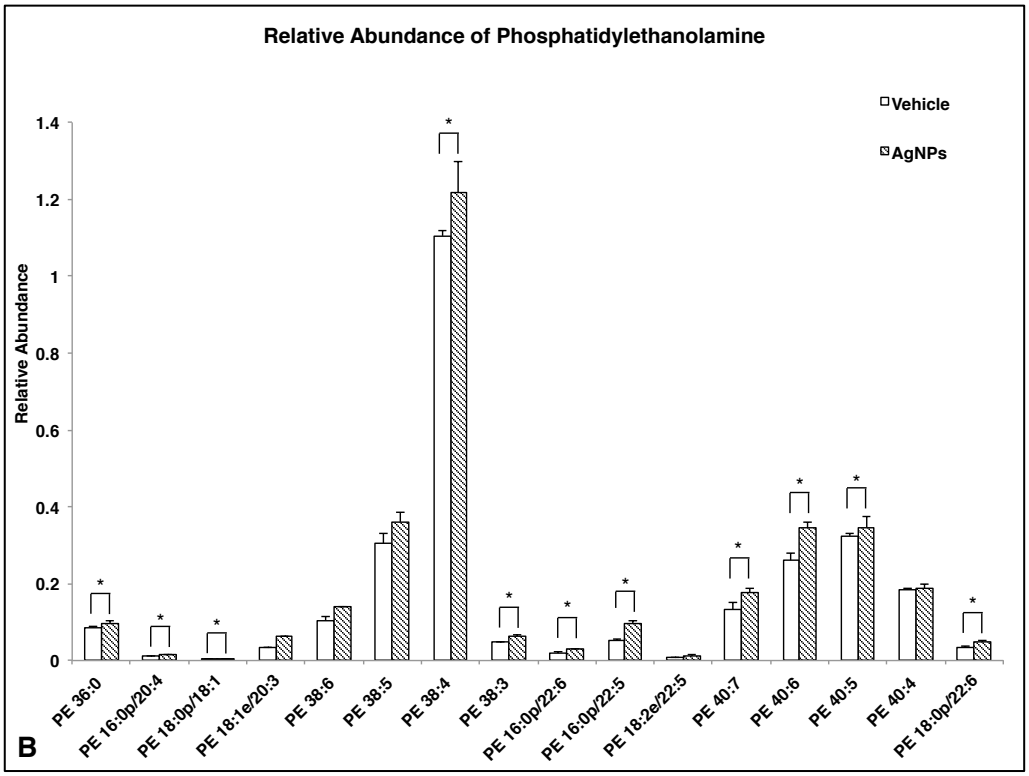


Fig. 3.2.11 Relative abundance of phosphatidylethanolamine in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in relative abundance by two-tailed unpaired Student's t-test. * $p < 0.05$. PE – phosphatidylethanolamine.

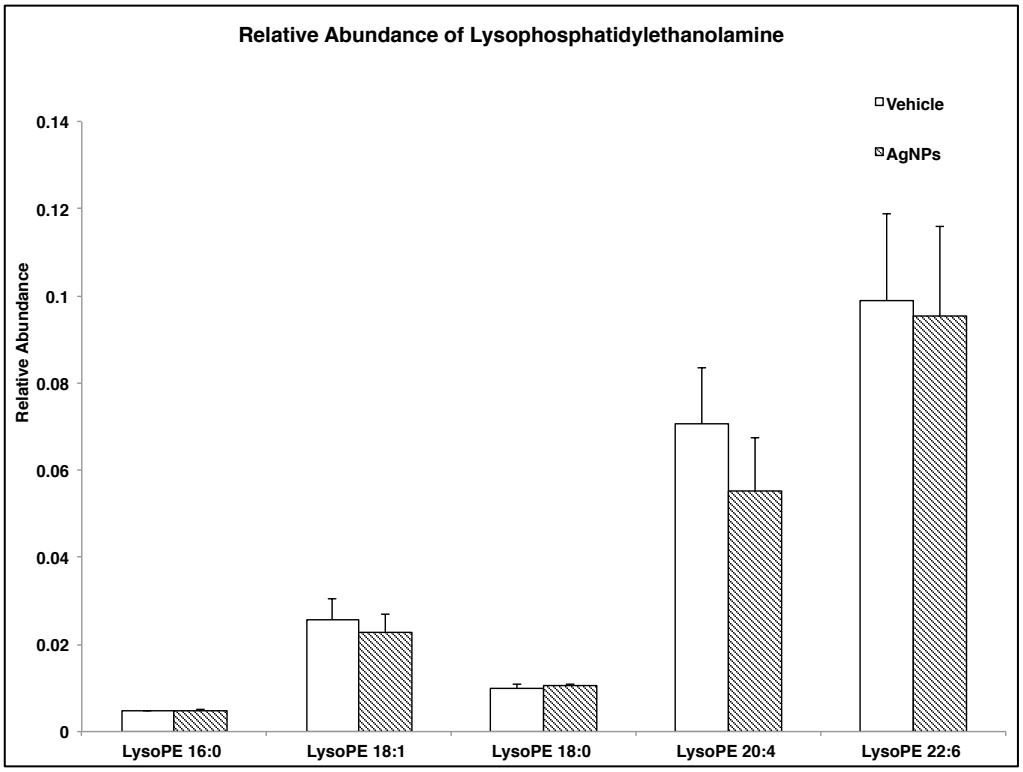


Fig. 3.2.12 Relative abundance of lysophosphatidylethanolamine in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Each bar in the figure denotes mean + SEM. LysoPE – lysophosphatidylethanolamine.

2.3.4.3. Phosphatidylserine

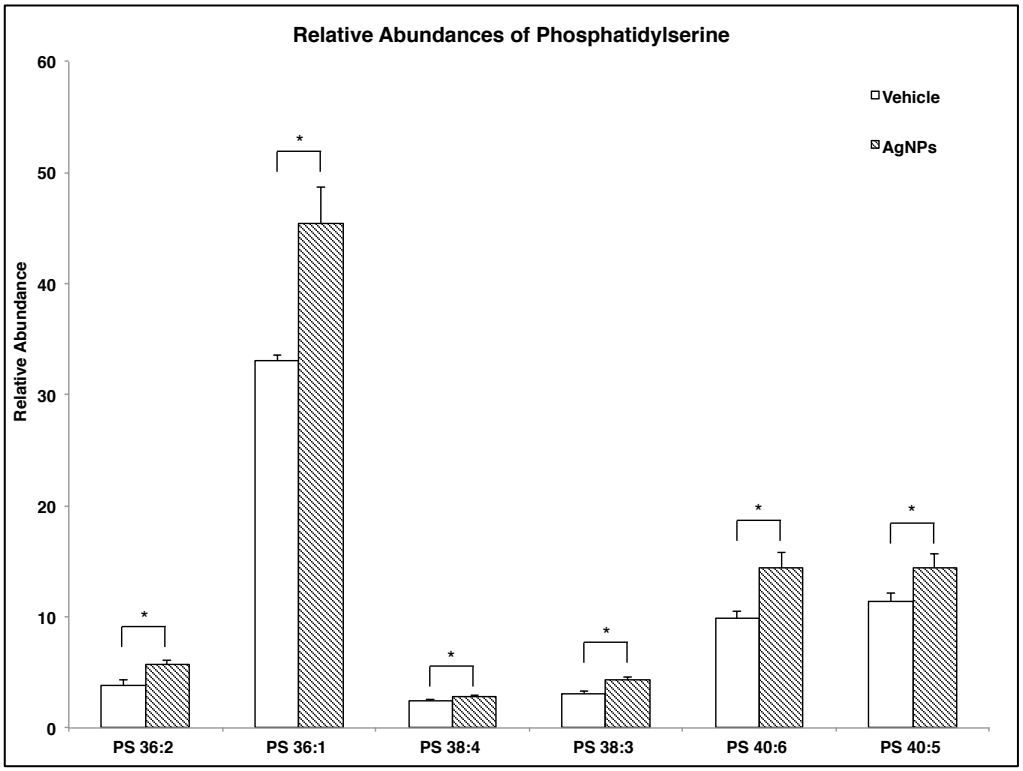


Fig. 3.2.13 Relative abundance of phosphatidylserine in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in relative abundance by two-tailed unpaired Student's t-test. * $p < 0.05$. PS – phosphatidylserine.

2.3.4.4. Sphingomyelin

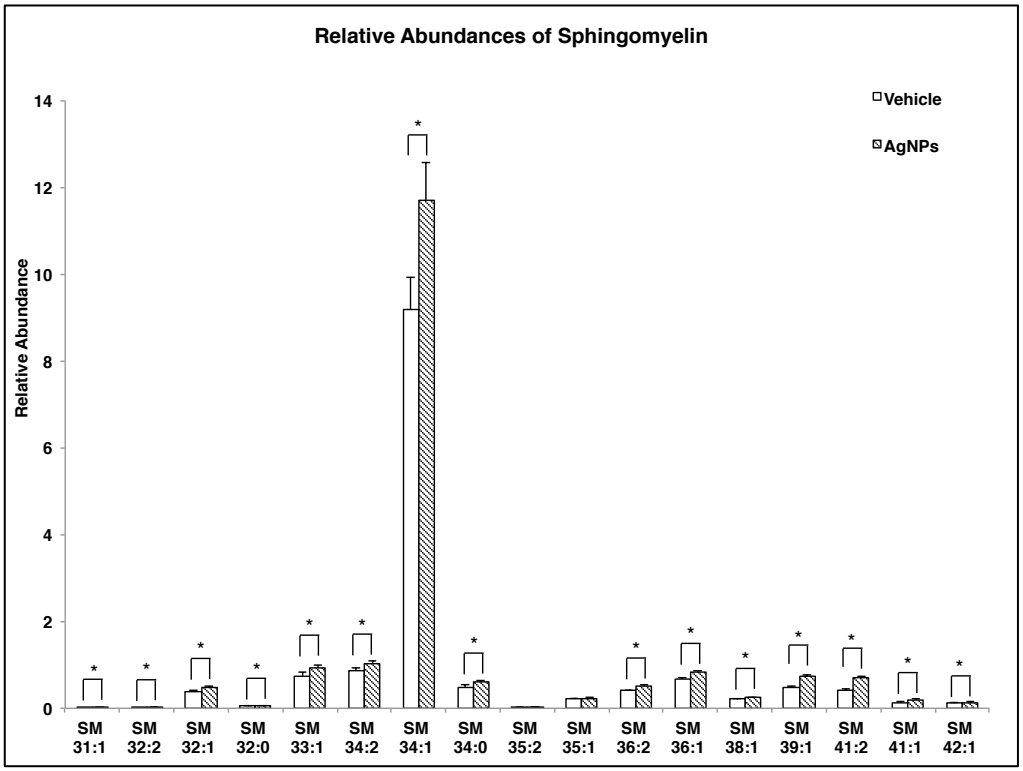


Fig. 3.2.14 Relative abundance of sphingomyelin in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in relative abundance by two-tailed unpaired Student's t-test. * $p < 0.05$. SM – sphingomyelin.

2.3.4.5. Ceramide

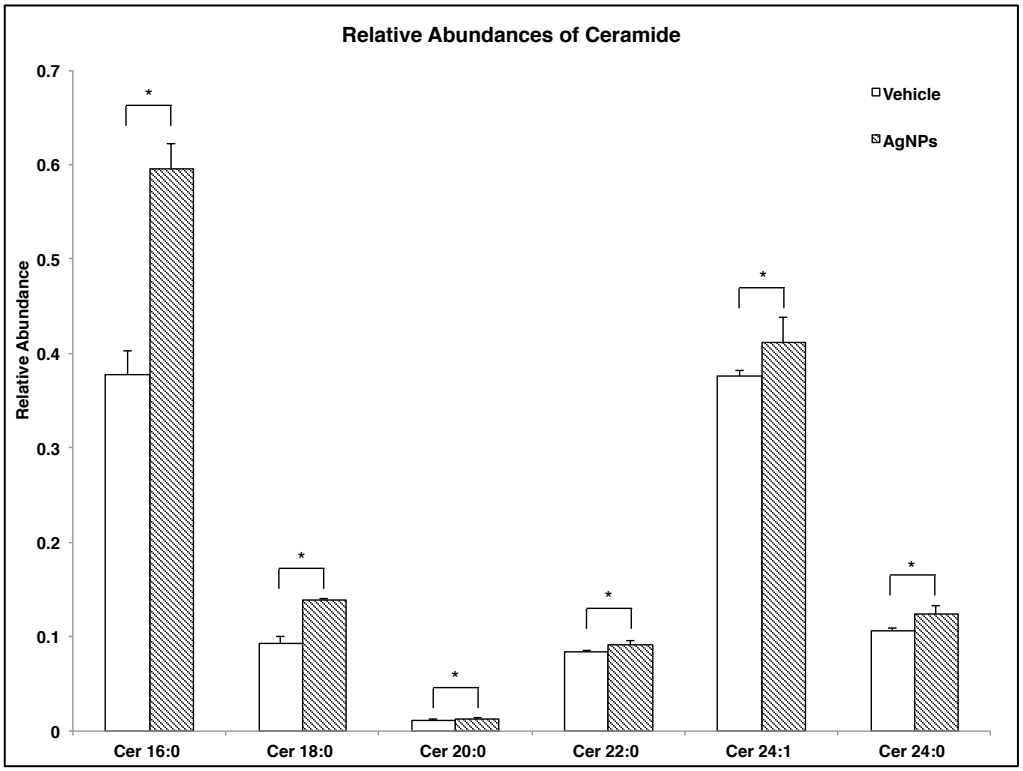


Fig. 3.2.15 Relative abundance of ceramide in SH-SY5Y cells following six hours incubation with vehicle control and 50 µg/mL AgNPs. Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in relative abundance by two-tailed unpaired Student's t-test. * $p < 0.05$. Cer – ceramide.

2.4. Discussion

This chapter was conducted to examine the relationship between AgNPs and PLA_2 enzymes, in particular $cPLA_2$ and $iPLA_2$, in SH-SY5Y human neuroblastoma cells. Since AgNPs have demonstrated inflammatory properties (Carlson et al., 2008, Cha et al., 2008, Kim et al., 2010), I wanted to determine if AgNPs-induced toxicity is due to an induction of $cPLA_2$, depression of $iPLA_2$ expression and function, or both. $sPLA_2$ activity has been heavily implicated in inflammatory conditions, however, it lacks specificity for the cleavage of fatty acids at the sn-2 position of glycerophospholipids (Burke and Dennis, 2009, Ong et al., 2015). As such, the present study focuses on $cPLA_2$, which preferentially releases AA, a potent pro-inflammatory mediator (Sun et al., 2004, Dennis et al., 2011), and $iPLA_2$, which preferentially releases DHA, a potent anti-inflammatory mediator (Green et al., 2008, Basselin et al., 2010).

Interestingly, no significant changes in $cPLA_2$ gene expression following incubation with AgNPs for up to six hours, while a significant reduction in $iPLA_2$ gene expression from one to six hours was observed. This indicates that $iPLA_2$ is more susceptible to AgNPs' toxicity as compared to $cPLA_2$, suggesting that AgNPs exerts its harmful effects through the inhibition of anti-inflammatory mechanisms instead of elevating pro-inflammatory pathways in SH-SY5Y cells. Moreover, $iPLA_2$ protein expression decreased significantly after six hours exposure to AgNPs, as reflected by the significant drop in

fluorescence intensity using immunocytochemistry analysis. AgNPs exposure has also been reported to cause cell death in various cell lines (Braydich-Stolle et al., 2005, Carlson et al., 2008, Eom and Choi, 2010). The observed decrease in $iPLA_2$ expression could thus be potentially due to the loss of cells after exposure to AgNPs. However, six hours incubation of SH-SY5Y cells with AgNPs did not result in significant cell death. This implies that AgNPs exert its toxicity in SH-SY5Y cells, in part by affecting $iPLA_2$ expression. On the contrary, AuNPs showed a greater reduction in $iPLA_2$ mRNA expression at one hour as compared to AgNPs-treated cells, although $iPLA_2$ expression returned to similar to that of vehicle control after six hours exposure. The observed recovery suggests that SH-SY5Y cells are able to overcome the inhibitory effects of AuNPs on $iPLA_2$ gene expression. This corroborates with other findings that AuNPs are generally safe, exhibiting lesser toxic effects as compared to AgNPs (Bar-Ilan et al., 2009, Asharani et al., 2011).

Additionally, it was shown in the previous chapter that acute exposure of SH-SY5Y cells to AgNPs resulted in mitochondrial dysfunction. The use of DHA and LC was able to rescue the harmful effects exerted by AgNPs in SH-SY5Y cells. However, co-treatment of DHA and/or LC with AgNPs did not result in significant changes in $iPLA_2$ mRNA expression of SH-SY5Y cells, suggesting that supplementation of DHA and/or LC was unable to overcome changes observed in $iPLA_2$ expression despite their ability to rescue harmful

effects exerted by AgNPs on mitochondria. As an integral housekeeping gene, $iPLA_2$ plays many crucial roles in cells, including maintenance of mitochondrial integrity and its functions (Gadd et al., 2006, Seleznev et al., 2006, Beck et al., 2011). This suggests that AgNPs affect $iPLA_2$ expression upstream that of its effects exerted on human neuronal mitochondria, explaining for the inability of DHA and/or LC to alter $iPLA_2$ expression.

Alterations in activity of the $iPLA_2$ enzyme following AgNPs treatment were investigated via lipidomic profiling. Since $iPLA_2$ preferentially releases DHA from phospholipids (Strokin et al., 2003, Green et al., 2008, Ong et al., 2015), it was postulated that AgNPs exposure to SH-SY5Y cells would result in lowered activity of the enzyme, i.e., less hydrolysis of glycerophospholipids to produce DHA and 2-lysophospholipids. Based on the molecular weights of PC, LysoPC, PE, and LysoPE lipid species, relative amount of released DHA by the action of $iPLA_2$ enzyme can be determined. For example, PC 36:6 is hydrolyzed to produce LysoPC 14:0 and DHA, i.e. PC 36:6 – LysoPC 14:0 = 22:6 (DHA). Similarly, PE 40:7 releases LysoPE 18:1 and DHA. Lipidomic analysis revealed significant increases in PC and PE species and little to no significant changes in LysoPC and LysoPE species that are involved in the release of DHA, indicating decreased $iPLA_2$ activity after six hours incubation with AgNPs. Decreased levels of PC and PE species such as PC 32:0, PC 36:5, and PE 32:0 were also detected. However, these are non-DHA-containing PC and PE

species as they have five or lesser double bonds in their fatty acid chains. Mitochondrial phospholipids are mainly made up of PC, PE, and cardiolipin lipid species (Zinser et al., 1991). Hence, alterations in these lipid species would have an effect on mitochondrial function, for instance, mitochondrial membrane integrity, permeability, and intracellular trafficking (Yorio and Frazier, 1990, Stenger et al., 2009, Sivaprakasam and Nachiappan, 2015). Taken together, the results suggest that AgNPs could exert its pro-inflammatory properties in SH-SY5Y cells by modulating $iPLA_2$ expression and function, in turn resulting in mitochondrial dysfunction.

Lipidomic analysis also showed a significant increase in Cer lipid species following six hours incubation with AgNPs. Cer are lipid messengers that are involved with suppression of cell growth and induction of apoptosis (Obeid and Hannun, 1995). They can also work on mitochondria, causing the opening of the MPTP and release of cytochrome C, leading to mitochondrial dysfunction (Cutler et al., 2004). $iPLA_2$ is involved in the induction of ER stress that causes an increase in Cer levels (Lei et al., 2007). Additionally, the presence of ROS from AgNPs exposure could result in an increase in Cer levels (Cutler et al., 2004, Alessenko et al., 2005, Ichi et al., 2009). However, the resultant increase in Cer species is often due to the activation of sphingomyelinase, an enzyme that cleaves SM to produce Cer and phosphocholine (Alessenko et al., 2005, Lei et al., 2007). This contradicts the present study's results as SM levels were found to

increase significantly following AgNPs treatment. Intriguingly, the addition of tumor necrosis factor, an inflammatory cytokine known to generate ROS in cells, increased both SM and Cer levels (Pekary and Hershman, 1998), indicating that ROS could affect phospholipid species in various ways. Additionally, it is possible that incubation with AgNPs resulted in increased SM levels so as to produce more Cer as SM are precursors of Cer. Six hours incubation with AgNPs also saw a significant increase in PS lipid species levels. Under apoptotic conditions, the translocation of PS from the inner to outer membrane of the plasma membrane act as a phagocytic “eat-me” signal (Verhoven et al., 1995). The increased PS lipid species could possibly be due to the preparation of apoptosis, in response to AgNPs exposure.

CHAPTER 3

ROLE OF OXIDATIVE STRESS IN SILVER NANOPARTICLES-INDUCED CALCIUM-INDEPENDENT PHOSPHOLIPASE A_2 CHANGES

3.1. Introduction

In the previous chapters, it was established that acute exposure of AgNPs affects $iPLA_2$ expression and function, in turn leading to mitochondrial dysfunction. AgNPs have been reported to cause cytotoxicity in cells due to generation of ROS, which react with cellular components like DNA and lipids, leading to mitochondrial dysfunction, DNA damage, and ultimately cell death (AshaRani et al., 2009, Costa et al., 2010, Hwang et al., 2012, Kang et al., 2012, Mukherjee et al., 2012). $iPLA_2$ has been reported to protect cells against oxidant-induced lipid peroxidation (Cummings et al., 2002, Kinsey et al., 2008, Eady et al., 2012), and the inhibition of $iPLA_2$ activity increases oxidant-induced cell death (Peterson et al., 2007). While $iPLA_2$ plays a protective role against oxidative stress, the enzyme can also be inactivated by ROS due to the oxidation of $iPLA_2$ sulfhydryl groups (Cummings et al., 2004, Song et al., 2006). Decreased $iPLA_2$ activity may thus lead to reduced cleavage, reacylation, and reinsertion of the peroxidized phospholipids into membranes, resulting in increased lipid peroxidation, and ultimately leading to cell death (Cummings et al., 2002, Cummings et al., 2004, Peterson et al., 2007).

$iPLA_2$ expression is potentially modulated by several regulatory factors at the promoter including Sp1 transcription factor (SP1), urocortin, and sterol regulatory element-binding proteins (SREBPs). $iPLA_2$ contains several potential SP1 binding sites on its

promoter (Larsson Forsell et al., 1999), hence it could possibly regulate alterations in $iPLA_2$ gene expression following exposure to AgNPs. However, SP1 is a general transcription factor that binds to GC-rich elements in DNA, and has been shown to affect multiple genes including $cPLA_2$ (Tsou et al., 2008, Zhang et al., 2008). Similarly, urocortin regulates both $cPLA_2$ and $iPLA_2$ expression (Zhu et al., 2014). The results in chapter 2 indicate that AgNPs exposure in SH-SY5Y cells affects $iPLA_2$ but not $cPLA_2$ expression, suggesting that SP1 and urocortin are possibly not involved in the AgNPs-induced alterations of $iPLA_2$ expression.

On the other hand, $iPLA_2$ promoter contains a sterol regulatory element (SRE) binding site for SREBPs that is not found on $cPLA_2$ (Seashols et al., 2004, Chew and Ong, 2014). SREBPs are transcription factors that play an integral role in lipid homeostasis. In mammals, there are three isoforms, SREBP-1a, SREBP-1c, and SREBP-2 (Horton et al., 2003). SREBP-1a and SREBP-1c are alternatively spliced isoforms of the sterol regulatory element binding transcription factor 1 (SREBF1) gene found on human chromosome 17p11.2, while SREBP-2 gene (SREBF2) is found on human chromosome 22q13 (Hua et al., 1995, Brown and Goldstein, 1997, Horton et al., 2002). SREBP-1a enhances transcription of all SREBP-related genes, including genes involved in cholesterol and fatty acid synthesis, while SREBP-1c activates genes involved in fatty acid synthesis and SREBP-2 regulates expression of genes that play a role

in cholesterol synthesis and uptake. (Brown and Goldstein, 1997, Krycer and Brown, 2013).

In this chapter, the role of oxidative stress in mediating AgNPs-induced decrease in $iPLA_2$ expression and function was examined using antioxidants such as N-acetyl L-cysteine (NAC) and N-tert-Butyl-α-phenylnitrone (PBN). The potential mechanism underlying the down-regulation of $iPLA_2$ gene expression following acute exposure to AgNPs was also elucidated.

3.2. Materials and Methods

3.2.1. Cell Culture

SH-SY5Y cells were cultured in complete growth medium, and grown in 100 mm dishes. SH-SY5Y cells were incubated under standard conditions of 37 °C and 5 % CO_2, and regularly passaged with 0.25 % Trypsin-EDTA. At 70 % confluence, cells were used for treatments.

3.2.2. Synthesis, Purification, and Characterization of AgNPs

26 nm AgNPs were synthesized, purified, and characterized as described in chapter 1 (pages 40 – 43).

3.2.3. Cell Treatment

3.2.3.1. Treatment with NAC and AgNPs

Four groups of SH-SY5Y cells were treated with the following reagents: (1) vehicle, water, (2) 1mM NAC (Sigma-Aldrich, St. Louis, MO, USA), (3) 50 µg/mL AgNPs, (4) 1 mM NAC and 50 µg/mL AgNPs. Cells were pre-treated with 1 mM NAC for one hour prior to addition of AgNPs for another six hours. PBS was used to wash the cells. SH-SY5Y cells were then harvested for further real-time RT-PCR analysis.

3.2.3.2. Treatment with PBN and AgNPs

Four groups of SH-SY5Y cells were treated with the following reagents: (1) vehicle, ethanol, (2) 10 µM PBN (Sigma-Aldrich, St. Louis, MO, USA), (3) 50 µg/mL AgNPs, (4) 10 µM PBN and 50 µg/mL AgNPs. Cells were pre-treated with 10 µM PBN for one hour prior to addition of AgNPs for another six hours. PBS was used to wash the cells. SH-SY5Y cells were then harvested for further real-time RT-PCR analyses.

3.2.4. Real-time RT-PCR

RNA of treated cells was extracted and real-time RT-PCR analyses were carried out as described in chapter 2 (page 72), using probes for human $iPLA_2$ (Hs00185926_m1), SREBP-1 (Hs01088691_m1), SREBP-2 (Hs01081784_m1), and ACTB (beta actin) (#4326315E) (Applied Biosystems®, Life Technologies, Carlsbad, CA, USA).

3.2.5. Statistical analyses

Mean and standard error of values were determined for each experimental group, and possible significant differences among various groups were analyzed using one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. $p < 0.05$ was deemed significant.

3.3. Results

3.3.1. Real-time RT-PCR Analyses

3.3.1.1. Effect of NAC and AgNPs Treatment on $iPLA_2$ mRNA Expression

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 0.43-fold ($p < 0.001$) statistically significant change in $iPLA_2$ mRNA expression compared to vehicle control (Fig. 3.3.1). 1 mM NAC + 50 µg/mL AgNPs-treated cells also exhibited a 0.65-fold ($p = 0.005$) statistically significant change in $iPLA_2$ mRNA expression compared to vehicle control. Compared to 50 µg/mL AgNPs-treated SH-SY5Y cells, significant increase in $iPLA_2$ mRNA expression was observed in cells treated with 1 mM NAC + 50 µg/mL AgNPs ($p = 0.023$). Statistically significant reduction in $iPLA_2$ mRNA expression was also observed in cells treated with 1 mM NAC compared to 1 mM NAC + 50 µg/mL AgNPs ($p = 0.047$).

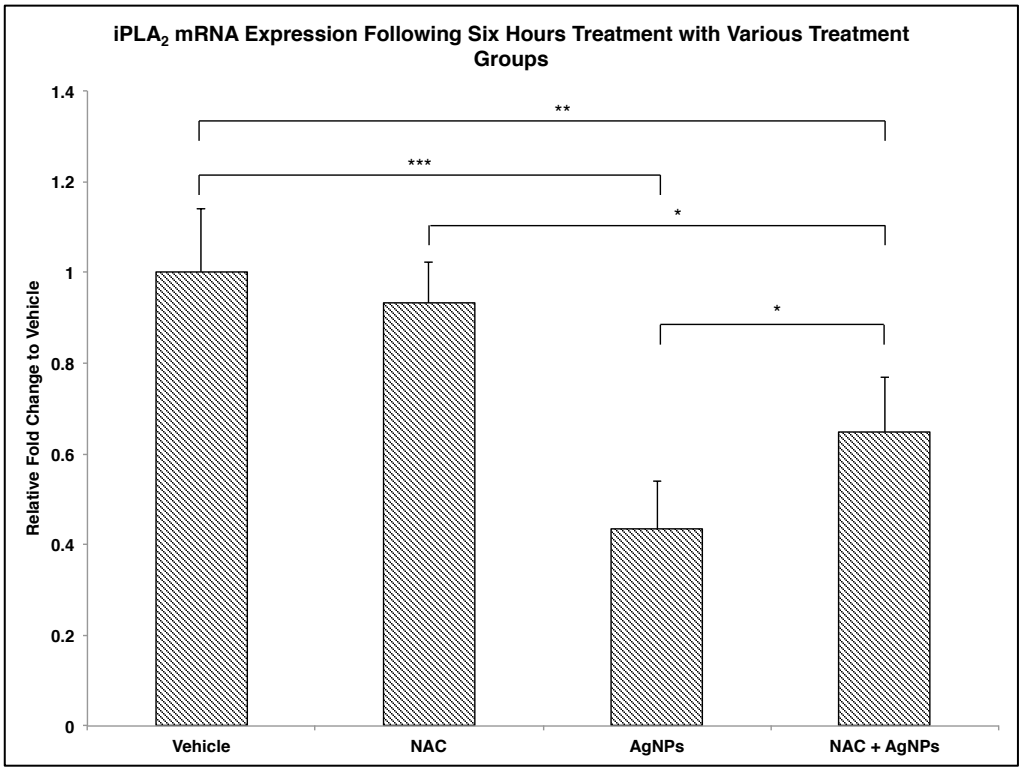


Fig. 3.3.1 Fold change in $iPLA_2$ mRNA expression in SH-SY5Y cells following six hours incubation with various treatment groups – vehicle control; 1 mM NAC; 50 µg/mL AgNPs; 1 mM NAC + 50 µg/mL AgNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.1.2. Effect of PBN and AgNPs Treatment on $iPLA_2$ mRNA Expression

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 0.48-fold (p = 0.001) statistically significant change in $iPLA_2$ mRNA expression compared to vehicle control (Fig. 3.3.2). No significant difference was observed between 10 µM PBN + 50 µg/mL AgNPs-treated cells and vehicle control. Compared to 50 µg/mL AgNPs-treated SH-SY5Y cells, significant increase in $iPLA_2$ mRNA expression was observed in cells treated with 10 µM PBN + 50 µg/mL AgNPs ($p < 0.001$).

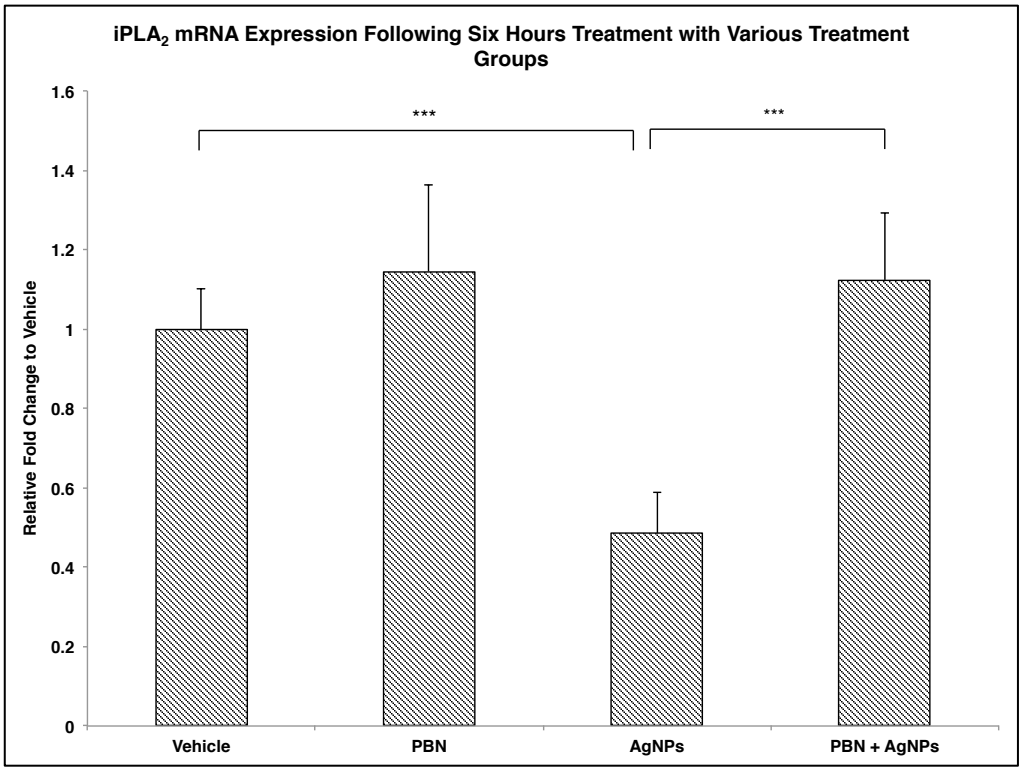


Fig. 3.3.2 Fold change in $iPLA_2$ mRNA expression in SH-SY5Y cells following six hours incubation with various treatment groups – vehicle control; 10 µM PBN; 50 µg/mL AgNPs; 10 µM PBN + 50 µg/mL AgNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. *** $p < 0.001$.

3.3.1.3. Effect of PBN and AgNPs Treatment on SREBP-1 mRNA Expression

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 0.32-fold ($p < 0.001$) statistically significant change in SREBP-1 mRNA expression compared to vehicle control (Fig. 3.3.3). 10 µM PBN-treated cells exhibited a 2.10-fold ($p < 0.001$) statistically significant increase in SREBP-1 mRNA expression compared to vehicle control. 10 µM PBN + 50 µg/mL AgNPs-treated cells exhibited a 0.58-fold ($p < 0.001$) statistically significant reduction in SREBP-1 mRNA expression compared to vehicle control. Compared to 50 µg/mL AgNPs-treated SH-SY5Y cells, significant increase in SREBP-1 mRNA expression was observed in cells treated with 10 µM PBN + 50 µg/mL AgNPs ($p < 0.001$). Statistically significant reduction in SREBP-1 mRNA expression was also observed in cells treated with 10 µM PBN compared to 10 µM PBN + 50 µg/mL AgNPs ($p < 0.001$).

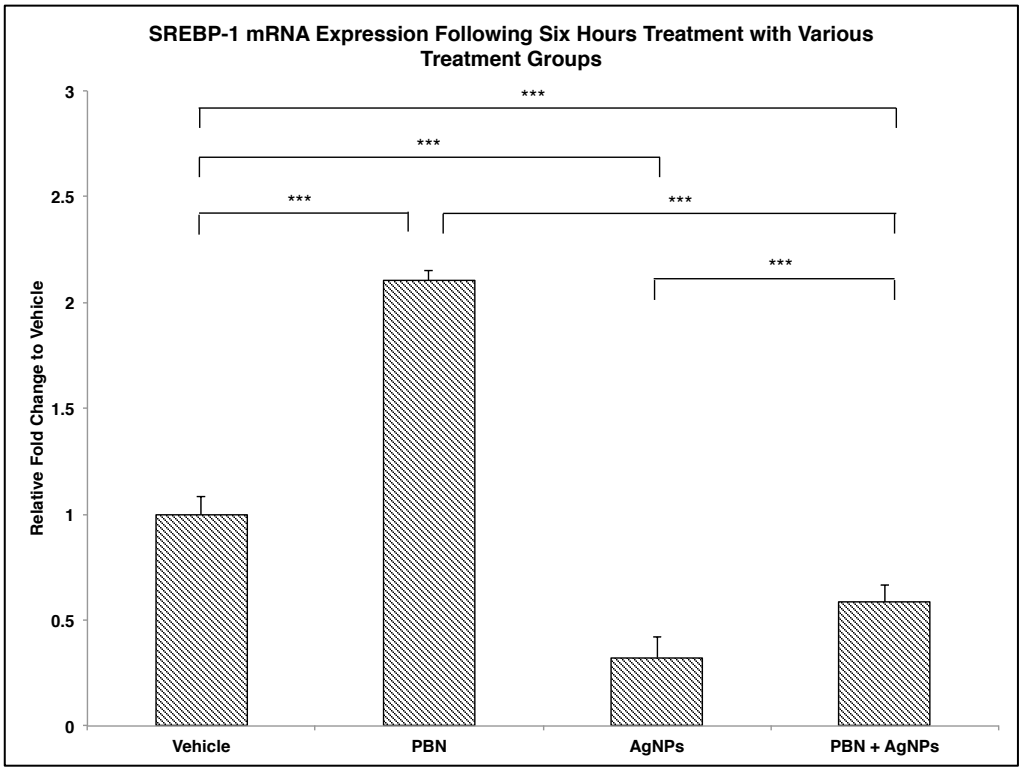


Fig. 3.3.3 Fold change in SREBP-1 mRNA expression in SH-SY5Y cells following six hours incubation with various treatment groups – vehicle control; 10 µM PBN; 50 µg/mL AgNPs; 10 µM PBN + 50 µg/mL AgNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. *** $p < 0.001$.

3.3.1.4. Effect of PBN and AgNPs Treatment on SREBP-2 mRNA Expression

Six hours incubation of SH-SY5Y cells with 50 µg/mL AgNPs resulted in a 0.68-fold ($p < 0.001$) statistically significant change in SREBP-2 mRNA expression compared to vehicle control (Fig. 3.3.4). 10 µM PBN-treated cells exhibited a 1.49-fold ($p < 0.001$) statistically significant increase in SREBP-2 mRNA expression compared to vehicle control. No significant difference was observed between 10 µM PBN + 50 µg/mL AgNPs-treated cells and vehicle control. Compared to 50 µg/mL AgNPs-treated SH-SY5Y cells, significant increase in SREBP-2 mRNA expression was observed in cells treated with 10 µM PBN + 50 µg/mL AgNPs ($p = 0.009$). Statistically significant reduction in SREBP-2 mRNA expression was also observed in cells treated with 10 µM PBN compared to 10 µM PBN + 50 µg/mL AgNPs ($p < 0.001$).

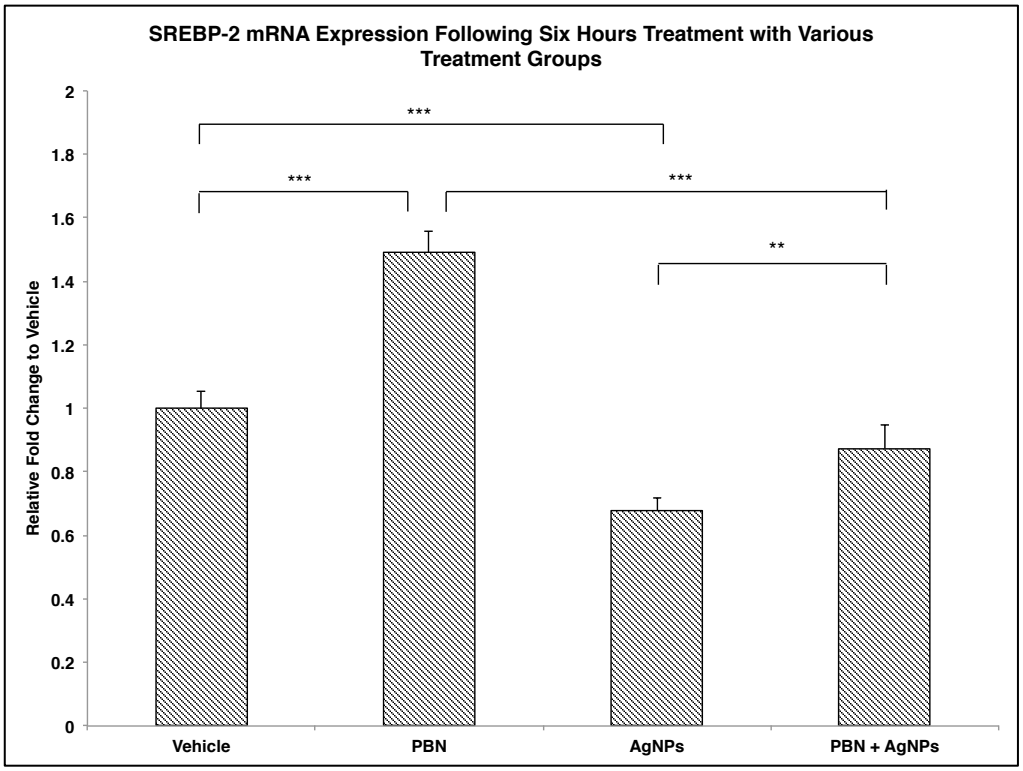


Fig. 3.3.4 Fold change in SREBP-2 mRNA expression in SH-SY5Y cells following six hours incubation with various treatment groups – vehicle control; 10 µM PBN; 50 µg/mL AgNPs; 10 µM PBN + 50 µg/mL AgNPs (n = 4 in each group). Each bar in the figure denotes mean + SEM. Asterisks (*) indicate statistically significant differences in fold change compared with vehicle control, by one-way ANOVA with Bonferroni's multiple comparison *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$.

3.4. Discussion

To determine if ROS and oxidative stress affects iPLA$_2$ expression, SH-SY5Y cells were pre-treated with the antioxidants NAC and PBN to observe for possible alterations in iPLA$_2$ gene expression. NAC is a commonly used antioxidant with the ability to reduce oxidative stress and the downstream effects associated with oxidative stress (Kerksick and Willoughby, 2005). NAC works as an antioxidant in two ways. Firstly, it is a precursor of cysteine, which is the rate-limiting enzyme in the synthesis of glutathione, a crucial endogenous antioxidant. Under oxidative stress, glutathione levels are depleted and this can be reversed via NAC supplementation (Dodd et al., 2008). NAC is also an active scavenger for free radicals (Kerksick and Willoughby, 2005, Dodd et al., 2008). Similar to NAC, PBN scavenges for a wide range of free radicals (Sack et al., 1996). PBN is commonly known as a spin-trapping compound. It detoxifies free radicals by adding them to its carbon-nitrogen double bond resulting in a stable nitroxide product, which is subsequently metabolized and excreted in the urine (Sack et al., 1996). Antioxidant pre-treatment prevented the observed decrease in iPLA$_2$ gene expression following AgNPs exposure, indicating that the AgNPs-induced down-regulation of iPLA$_2$ gene involves ROS. This corroborates with previous findings that acute exposure of AgNPs results in formation of ROS (Haase et al., 2012). Additionally, AgNPs-induced ROS affected iPLA$_2$ gene expression through an unknown mechanism.

Oxidative stress can alter gene expression of various genes in multiple ways. Different ROS could produce different effects on various genes' expression. Mackerness et al. (2001) found that H_2O_2 derived from $O_2^{\bullet-}$ caused an up-regulation of PR-1 gene and down-regulation of Lhcb gene, while $O_2^{\bullet-}$ resulted in an up-regulation of PDF1.2 gene. ROS could also alter gene expression by affecting cell signaling upstream of the gene. Wartenberg et al. (2001) determined that ROS down-regulated expression of Pgp gene via up-regulation of Erk1 and 2, and JNK. Additionally, ROS could affect transcription factors that have binding sites on the gene-of-interest's promoter (McCullough et al., 2001). It would be interesting to determine the mechanism underlying the down-regulation of $iPLA_2$ gene expression following six hours treatment with AgNPs.

Since the $iPLA_2$ promoter contains a SRE binding site for SREBPs that is not found on $cPLA_2$ (Seashols et al., 2004, Lei et al., 2010, Chew and Ong, 2014), it was decided to study the effects of oxidative stress on SREBP gene expression. As PBN pre-treatment resulted in a more significant change in $iPLA_2$ expression, PBN pre-treatment was used for subsequent investigations. AgNPs-treated cells showed significant reductions in both SREBP-1 and SREBP-2 expression as compared to vehicle control, indicating that acute exposure of SH-SY5Y cells to AgNPs causes dysregulation of SREBP gene expression. Although it is not known how AgNPs result in aberrant modulation of SREBP expression, transcriptional activity of

SREBPs have been reported to be regulated in a variety of ways including proteolytic processing and recruitment of transcriptional cofactors (Xiaoping and Fajun, 2012). Furthermore, cells treated with PBN and AgNPs showed a significant increase in both SREBP-1 and SREBP-2 expression as compared to cells treated with only AgNPs, suggesting that SREBPs are affected by AgNPs-induced ROS formation. Reduction in SREBP expression could potentially limit amount of SREBP transcription factors binding to the SRE binding site on the $iPLA_2$ promoter leading to lowered $iPLA_2$ expression, as observed in the present study.

Taken together, the present study's results suggest that SREBPs are highly involved in AgNPs-induced down-regulation of $iPLA_2$. Besides SREBPs, there may be other potential pathways involved. One possible mechanism is the AMPK signaling pathway, which has been shown to be activated by ROS in cultured cells (Hardie et al., 2012, Auciello et al., 2014). AMPK has also been demonstrated to inhibit SREBP expression and activity (Li et al., 2011, Liu et al., 2015). Thus, it is plausible that AgNPs-induced ROS production could first activate AMPK, which suppresses SREBPs, in turn leading to the down-regulation of $iPLA_2$. Nonetheless, additional work has to be done to validate these mechanisms.

SECTION IV

CONCLUSION

In chapter 1, the acute effects of AgNPs in SH-SY5Y mitochondria were investigated. Mitochondrial membrane potential assay analysis showed that acute exposure of AgNPs to SH-SY5Y cells led to a drop in 590/535 ratio suggesting mitochondrial membrane damage. Additionally, ATP levels decreased in AgNPs-treated cells indicating possible damage to mitochondrial respiratory chain. As ATP levels decline, a corresponding rise in ADP levels is expected. Conversely, a decrease in ADP levels was detected. This could possibly be due to the action of AK decreasing the adenine nucleotide source and diminishing ADP and ATP stores. No significant cell death was detected after acute exposure to AgNPs. DHA and LC were then used to rescue AgNPs-induced mitochondrial dysfunction. Taken together, acute exposure of AgNPs to SH-SY5Y cells resulted in mitochondrial dysfunction and subsequently decreased ATP production without leading to cell death, which could be rescued by co-supplementation with DHA and LC.

In chapter 2, the relationship between AgNPs and PLA_2 enzymes, in particular $cPLA_2$ and $iPLA_2$, were investigated in SH-SY5Y cells. No significant changes in $cPLA_2$ gene expression following incubation with AgNPs for up to six hours, while $iPLA_2$ gene expression was significantly reduced from one to six hours. Immunocytochemistry analysis also revealed a significant decrease in $iPLA_2$ protein expression following acute exposure to AgNPs. This observed reduction could possibly be due to loss of cells after AgNPs exposure,

however, no significant cell death was observed. Supplementation of DHA and/or LC was unable to overcome the $iPLA_2$ expression changes despite their ability to rescue harmful effects exerted by AgNPs on mitochondria, indicating that AgNPs affect $iPLA_2$ expression upstream that of its effects exerted on mitochondria. Alterations in $iPLA_2$ activity following AgNPs treatment were investigated via lipidomic profiling. Lipidomic analysis revealed significant increases in PC and PE species and little to no significant changes in LysoPC and LysoPE species that are involved in DHA release, indicating decreased $iPLA_2$ activity after acute exposure to AgNPs. Significant changes in Cer, SM, and PS lipid species were also detected. Taken together, AgNPs exert its inflammatory properties in SH-SY5Y cells by modulating $iPLA_2$ expression and function.

In chapter 3, the effects of AgNPs-induced ROS and oxidative stress on $iPLA_2$ expression were investigated, and the mechanism underlying changes observed was elucidated. Antioxidant pre-treatment prevented the observed decrease in $iPLA_2$ gene expression following AgNPs exposure. Since the $iPLA_2$ promoter contains a SRE binding site for SREBPs that is not found on $cPLA_2$, effects of oxidative stress on SREBP expression were investigated. Cells treated with PBN and AgNPs showed a significant increase in both SREBP-1 and SREBP-2 expressions as compared to cells treated with only AgNPs. Taken together, the observed down-regulation of $iPLA_2$ gene involves ROS production via acute exposure to AgNPs,

and the AgNPs-induced ROS formation affects SREBP expression. This could potentially reduce the amount of SREBP transcription factors binding to $iPLA_2$ promoter, leading to reduced $iPLA_2$ expression.

In conclusion, the present study examined the acute effects of AgNPs in SH-SY5Y human neuroblastoma cells. Despite a short incubation period of up to six hours, AgNPs exposure led to ROS production in SH-SY5Y cells, causing reduced SREBP and $iPLA_2$ expression and function. This subsequently leads to mitochondrial dysfunction, which could be rescued through DHA and LC co-supplementation (Fig. 4.1.1). The mechanism underlying AgNPs-induced mitochondrial dysfunction in SH-SY5Y cells was thus elucidated in the present study. Besides SREBPs, there may be other potential pathways involved. One possible mechanism is the AMPK signaling pathway. Nonetheless, additional work has to be done to validate these mechanisms. Establishment of such mechanisms could result in additional insights with respect to AgNPs' toxicity in human neuronal cells.

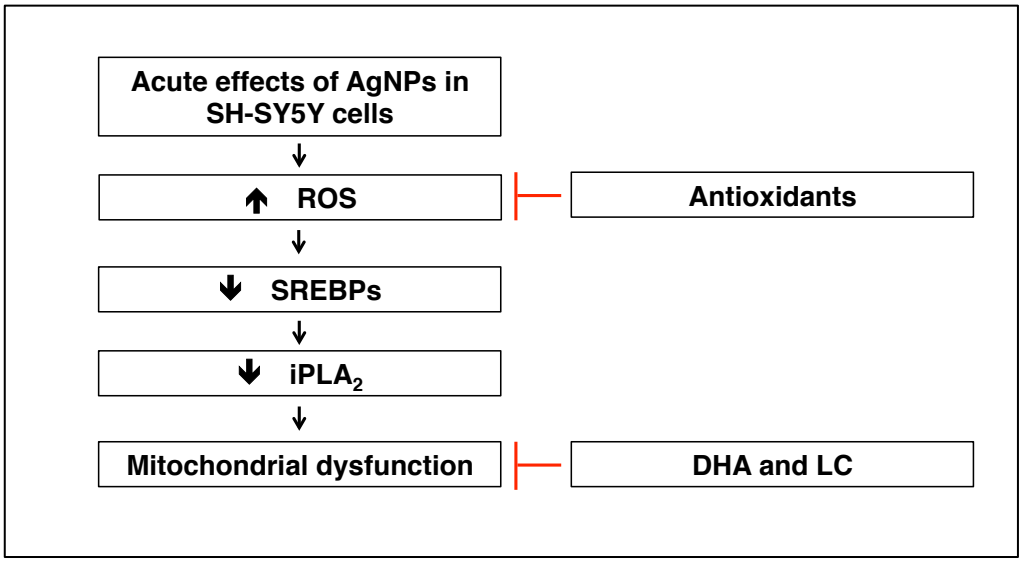


Fig. 4.1.1 Schematic flowchart of potential pathway and mechanism underlying acute effects of AgNPs in SH-SY5Y cells.

SECTION V

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