ความเป็นพิษของอนุภาคนาโนชนิดซิลเวอร์ ซิลเวอร์คอปเปอร์ และซิลเวอร์ทิน
ในเซลล์เพาะเลี้ยงทีเอชพี-1 และเซลล์แมคโครฟาจที่เปลี่ยนแปลงมาจากทีเอชพี-1

นางสาวปฐมาภรณ์ มนต์ประสิทธิ์

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TOXICITY OF SILVER, SILVER-COPPER AND SILVER-TIN NANOPARTICLES IN THP-1 CELL LINE AND THP-1 DIFFERENTIATED MACROPHAGE CELLS

Miss Patamaporn Monprasit

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	TOXICITY OF SILVER, SILVER-COPPER AND SILVER-TIN NANOPARTICLES IN THP-1 CELL LINE AND THP-1 DIFFERENTIATED MACROPHAGE CELLS
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ปฐมาภรณ์ มนต์ประสิทธิ์ : ความเป็นพิษของอนุภาคนาโนชนิดซิลเวอร์ ซิลเวอร์คอปเปอร์ และซิลเวอร์ทินในเซลล์เพาะเลี้ยงทีเอชพี-1 และเซลล์แมคโครฟาจที่เปลี่ยนแปลงมาจากทีเอชพี-1 (TOXICITY OF SILVER, SILVER-COPPER AND SILVER-TIN NANOPARTICLES IN THP-1 CELL LINE AND THP-1 DIFFERENTIATED MACROPHAGE CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. พ.ต.ท. หญิง ดร. สมทรง ลาวัณย์ประเสริฐ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ภญ. ดร. รวิวรรณ มณีรัตนโชติ, 78 หน้า.

อนุภาคนาโนซิลเวอร์ (AgNPs) และอนุภาคนาโนในรูปแบบซิลเวอร์-โลหะผสม ได้แก่ ซิลเวอร์คอปเปอร์ (AgCuNPs) และซิลเวอร์ทิน (AgSnNPs) ได้ถูกนำมาใช้ในอุตสาหกรรมและในทางการแพทย์มากมาย เพื่อศึกษาผลของอนุภาคนาโนเหล่านี้ต่อระบบภูมิคุ้มกัน การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลพิษของ AgNPs, AgCuNPs และ AgSnNPs ต่อเซลล์โมโนไซต์ของมนุษย์ คือเซลล์ทีเอชพี-1 และเซลล์แมคโครฟาจที่เปลี่ยนแปลงมาจากทีเอชพี-1 การศึกษานี้ทำการศึกษาลักษณะของอนุภาคนาโน ความเป็นพิษต่อเซลล์ การเหนี่ยวนำให้เกิดอนุมูลอิสระภายในเซลล์ รวมทั้งการทำหน้าที่ของเซลล์ในการหลั่งไซโตไคน์ (TNF-α, IL-1β และ IL-8) เมื่อเซลล์สัมผัสกับอนุภาคนาโน ผลการศึกษาพบว่า อนุภาคนาโนทั้งสามชนิดไม่มีผลพิษต่อเซลล์ทีเอชพี-1 ที่ความเข้มข้นของอนุภาคนาโนระหว่าง 5-100 ไมโครกรัม/มิลลิลิตร AgCuNPs และ AgSnNPs ที่ความเข้มข้นสูง มีผลลดเปอร์เซ็นต์การมีชีวิตของเซลล์แมคโครฟาจอย่างมีนัยสำคัญทางสถิติ การเพิ่มการเหนี่ยวนำอนุมูลอิสระในเซลล์ ไม่ได้เป็นสาเหตุเดียวที่ทำให้เซลล์ตาย อนุภาคนาโนทั้งสามมีผลรบกวนการทำหน้าที่ของเซลล์ทีเอชพี-1 ในการหลั่ง TNF-α แต่มีผลกระตุ้นการหลั่ง IL-8 ในเซลล์แมคโครฟาจ ผลทางชีววิทยาของอนุภาคนาโนต่อระบบภูมิคุ้มกันยังคงอยู่ในความสนใจที่จะต้องมีการศึกษาต่อไป

ภาควิชา	เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต	.....
สาขาวิชา	เภสัชวิทยาและพิษวิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
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PATAMAPORN MONPRASIT: TOXICITY OF SILVER, SILVER-COPPER AND SILVER-TIN NANOPARTICLES IN THP-1 CELL LINE AND THP-1 DIFFERENTIATED MACROPHAGE CELLS. ADVISOR: ASSOC. PROF. POL. LT. COL. SOMSONG LAWANPRASERT, Ph.D., CO-ADVISOR: RAWIWAN MANIRATANACHOTE, Ph.D., 78 pp.

Silver nanoparticles (AgNPs) and nanoparticles in the form of silver-metal alloy, such as silver-copper (AgCuNPs) and silver-tin (AgSnNPs) have been applied in various industries and medical applications. To assess effects of these nanoparticles on human immune system, this study aimed to assess toxicity of AgNPs, AgCuNPs and AgSnNPs on human monocytes, THP-1 cells, and THP-1 differentiated macrophage cells. The nanoparticles were characterized and assessed for cytotoxicity and generation of intracellular ROS. Effects of the nanoparticles on the release of cytokines (TNF-α, IL-1β and IL-8) were also assessed. The results demonstrated no significant cytotoxicity of silver and silver-metal nanoparticles at the concentration range of 5-100 µg/mL in THP-1 cells. AgNPs and AgCuNPs decreased cell viability of macrophages only at high concentrations. Intracellular ROS generation was not the only cause of cell death. All three nanoparticles significantly impaired the release of TNF-α in THP-1 cells but caused significant increase of IL-8 release in THP-1 differentiated macrophage cells. Biological effects of metal-based nanoparticles are still a topic of interest for further investigations.

Department:	Pharmacology and Physiology	Student's Signature .....
		Advisor's Signature .....
Field of Study:	Pharmacology and Toxicology	Co-Advisor's Signature .....
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CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

%	percent
ºC	degree Celsius
ANOVA	analysis of variance
AgNPs	silver nanoparticles
AgCuNPs	silver copper nanoparticles
AgSnNPs	silver tin nanoparticles
CO_2	carbondioxide
DCFH-DA	2', 7'-dichlorofluorescein diacetate
DI	deionized water
DLS	dynamic light scattering
et al.	et alii, and other
FBS	fetal bovine serum
g	gram (s)
h	hour (s)
IgG	immunoglobulin G
µg	microgram (s)
µL	microliter (s)
µM	micromolar
mg	milligram (s)
min	minute (s)
mL	milliliter (s)
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

ng	nanogram (s)
nm	nanometer (s)
NPs	nanoparticles
PBS	phosphate buffer saline
ROS	reactive oxygen species
H_2O_2	hydrogenperoxide
S.D.	standard deviation
TEM	transmission electron microscope

CHAPTER I

INTRODUCTION

Nanoparticles are objects with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly dimension. Thus, they normally have high surface area to volume ratio. Nanoparticles have different atom or molecular arrangement leads them to unique properties [1]. Change of physical properties of nanoparticles cause them to possess chemical and biological characteristics differently from bulk materials [2]. Some of them are reactive or catalytic with other substance. Because of these special properties which are superior than the bulk materials, nanoparticles are incorporated in wide variety of industries, agricultural, medical and consumer products [3]. Most toxicities of nanoparticles depend on their size because small particles can distribute into blood stream and pass more easily through cell membrane of many tissues [4]. Metal nanomaterials widely used in industrial sectors are nano-gold, nano-silver, nano-copper, nano-tin, nano-aluminium, nano-nickel, nano-cobalts, etc [3].

Silver nanoparticles (AgNPs) are currently incorporated into many medical products and textiles because of their antibacterial properties [5]. In addition, nanoparticles in the form of silver-metal alloy, such as silver-copper nanoparticles (AgCuNPs) and silver-tin nanoparticles (AgSnNPs) are also used in medical and industrial

products such as medical devices and textile because both copper and tin also have antibacterial properties as silver [2, 6-8]. Metal alloy nanomaterials are incorporated in various electronic industrial products and replaced lead in the electronic connecting circuit [7]. AgNPs and AgCuNPs are usefully incorporated in the conductive ink, printed conductors for printed RFID (radio frequency identification) applications [8]. Nanoparticles entered into the body from various routes such as skin contact, inhalation and ingestion [9]. Toxicity of nanoparticles is depended on size, shape, chemical and physical properties as well as particle aggregation [10]. It is know that the most common adverse effect of silver is irreversible pigmentation of the skin or argyria [11]. Toxicity of AgNPs has been conducted both *in vivo* and *in vitro*. An *in vivo* study demonstrated that AgNPs affected motility rate of zebrafish embryo [12]. *In vitro* studies have been performed in various types of cell lines. It was shown that AgNPs caused toxicity differently depending on the size, surface coating, ion-release from the nanoparticles as well as the tested cell types [13, 14]. Generation of intracellular reactive oxygen species (ROS) is the predominant mechanism leading to nanoparticle-induced toxicity [3], in addition to ROS-independent (cell cycle arrest) toxicity [14]. Toxicities of AgNPs on immune cells were assessed *in vitro* using different sizes and coating materials of AgNPs in rat liver cells and macrophage cells [15-17] and cytokine release from macrophage cells were measured [16]. It was found that toxic effect of AgNPs was depend on size, surface and type of macrophage cells. Besides no effect on induction of pro-inflammatory cytokine release from macrophage cells, AgNPs were

shown to inhibit IL-6 release which may explain the anti-inflammatory effect of AgNPs [19]. Even though there are few studies regarding the toxicity of AgNPs in immune cells, there is no report regarding toxicity of silver-metal alloy such as AgCuNPs and AgSnNPs. To obtain more safety information, this study aimed to investigate effects of AgNPs, AgCuNPs and AgSnNPs on THP-1 and THP-1 differentiated macrophage cells, representing cells in the immune system.

Objectives

1. To investigate the toxicological effects of AgNPs, AgCuNPs and AgSnNPs on THP-1 cells and THP-1 differentiated macrophage cells.
2. To investigate the effect of AgNPs, AgCuNPs and AgSnNPs on intracellular ROS generation and cytokine release in THP-1 cells and THP-1 differentiated macrophage cells.

Hypothesis

AgNPs, AgCuNPs and AgSnNPs are cytotoxic to THP-1 and THP-1 differentiated macrophage cells. All three nanoparticles cause intracellular ROS generation and cytokine release from THP-1 cells and THP-1 differentiated macrophage cells.

Expected benefits

The information regarding toxic effects of AgNPs, AgCuNPs and AgSnNPs on THP-1 cells and THP-1 differentiated macrophage cells which are the cells in the immune system.

CHAPTER II

LITERATURE REVIEWS

1. Nanoparticles (NPs)

Nanoparticles are objects with all external dimensions in the nanoscale where the lengths of the longest and the shortest axes of the nano-object do not differ significantly dimension [1]. NPs have been used for several decades in various product categories, including agriculture, medicine, clothing & textile, cosmetics and food. In medical aspect, NPs are applied in biomedical devices for various purposes including treatment, diagnosis, drug delivery, medical device coating and personal health care [2].

Nanotechnology products can be classified into several categories, such as metals, metal oxides, carbon, silica and semiconductor nanomaterials. Metal nanoparticles such as nano-gold, nano-silver, nano-copper, nano-aluminum, nano-cobalt, and others have been studies for their toxicities. Metal nanoparticles are important industrial materials and widely used in cosmetics, pharmaceutics, etc. In this study, AgNPs and Ag-metal alloy such as AgCuNPs and AgSnNPs were investigated for their toxicity.

2. Toxicity of Nanoparticles

Toxicity of NPs has been studied in different systems both *in vitro* using cell lines and *in vivo* of different organisms such as human cell lines, rodent and zebrafish [18-

26]. Different NPs demonstrated different toxic potency, for exam, nano-CuO was shown to be most potent cytotoxic and cause DNA damage compared with metal-oxide and carbon nanotube [27]. Mechanisms of toxicity of NPs have been investigated.

An important mechanism of nanotoxicity is ROS generation resulting in the formation of oxidative stress inside the cells [18]. Generation of ROS also plays an important role in genotoxicity, thus, leading to mutagenesis, carcinogenesis and aging-related diseases. Because of their small size, high surface area and high surface reactivity, thus NPs may cause more production of high level of ROS and more cytotoxicity and genotoxicity than the bulk-size counterparts.

It has been reported that ROS formation from NPs is dependent on the physical and chemical properties of the NPs and the testing systems (cell type). Thus factors that influence the toxicity of NPs are the following:

1. Size and shape of NPs

The smaller particles, the greater tendency to enter the target tissues.

2. Particle surface, charges and surface containing groups

NPs with higher surface positive charges demonstrated greater cytotoxicity and ROS formation [26].

3. Solubility and particle dissolution

Intracellular solubility of NPs is a determinant affecting NPs induced cytotoxicity [28].

4. Metal ion released from metal NPs

For example, Ag^+ ion is the reactive species leading to AgNPs toxicity.

5. Light activation

Activation by light irradiation, some NPs are excited and reacted with oxygen to generate ROS and cytotoxicity.

6. Aggregation and mode of interaction with cells

Degree of aggregation affects the level of ROS and cytotoxicity.

7. Inflammation leading to ROS generation

Inflammation effect of NPs can generate ROS resulting in cell apoptosis and cell death [16].

8. pH of the system

Lower pH might facilitate Fenton reactions to generate hydroxyl radicals, resulting ROS formation.

3. Nanoparticles and immune system

NPs are engineered for drug delivery to either avoid recognition, improving drug stability, or to selectively interact with the immune system. Immunosuppression or immunostimulation of the NPs to the immune system can be either beneficial or undesirable. Immunosuppression of the NPs is desirable in term of the purpose for treatment of inflammatory disorder/autoimmune diseases but undesirable for the adverse effects of myelosuppression and lower body's response to infection and cancers. Immunostimulation of NPs is desirable in term of the vaccine efficacy and

antitumor effects but undesirable for the adverse events such as hypersensitivity reactions and inflammation [29].

The example of immunosuppression of NPs is demonstrated that inhalation of carbon nanotubes inhibit B cell formation and the production of TGF-β by alveolar macrophage in mice [30]. The example of immunostimulation of NPs is demonstrated by the finding that single-wall and multiwall carbon nanotubes increase allergenicity of egg albumin following administration to mice [31].

4. Silver nanoparticles (AgNPs)

4.1 Applications of AgNPs

AgNPs are among the most commercialized nanoparticles. They have gained considerable attention in the area of nanotechnology because of their attractive properties, particularly their ability to protect against a wide range of bacteria, virus, fungi as well as anti-inflammatory activity [32, 34-41]. AgNPs are incorporated in a large number of consumer and medical products. Many biomedical applications of AgNPs in human health care are shown in Figure 1.

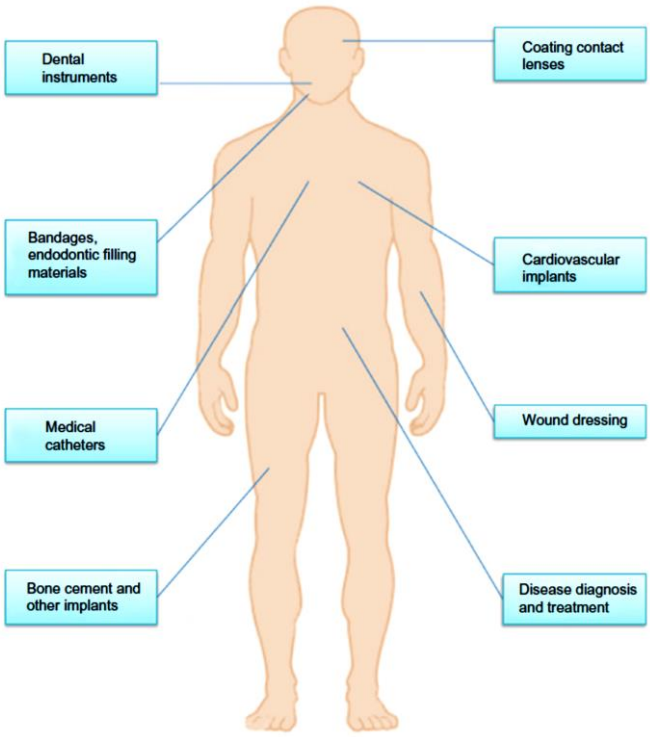

Figure 1 Biomedical applications of silver nanoparticles in human health care [2]

4.2 Synthesis of AgNPs [2]

AgNPs are synthesized by different methods to yield the nanoparticles with variable sizes, shapes, morphology and stability. The methods can be classified as physical, chemical and biological systhesis. Physical synthesis comprises evaporation/condensation and laser ablation techniques. Chemical synthesis by chemical reduction is the most common method used for AgNPs synthesis. This method requires silver salt (mostly $AgNO_3$), reductants and stabilizer or capping agents. The reductants are borohydride, citrate, ascorbate or hydrogen gas. Stabilizers including surfactants and ligands or polymers (such as polyvinylpyrrolidone, polyethylene glycol, etc) are used to prevent particle aggregation (Figure 2).

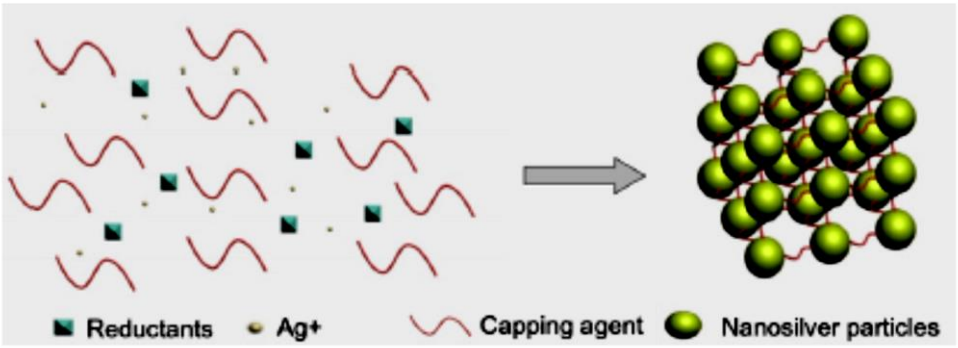

Figure 2 Chemical synthesis of AgNPs [2]

Biological or green synthesis uses eco-friendly reducing and capping agents such as protein, carbohydrate, various biological systems such as bacteria, fungi, yeast, algae and plants. Biological synthesis include enzymatic (using NADP reductase) and non enzymatic reaction using microorganisms or plants (Figure 3).

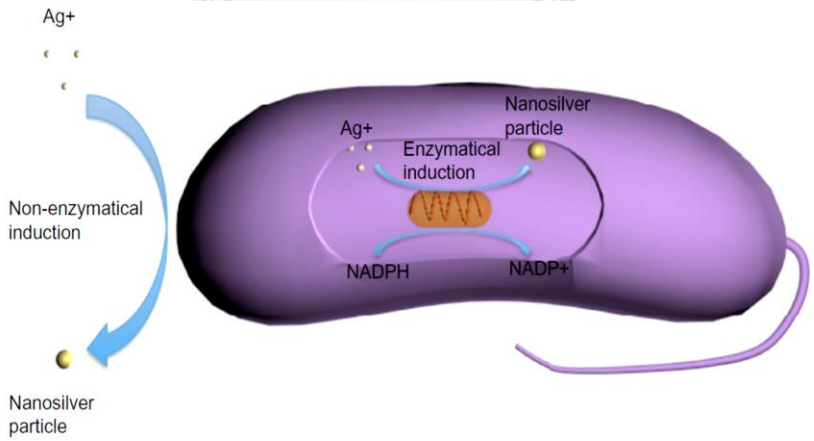

Figure 3 Biological (or green) synthesis of AgNPs [2]

4.3 Pharmacological effects of AgNPs

AgNPs possess beneficial pharmacological effects including

1. Antibacterial effects

AgNPs posses antibacterial effects on many gram-negative and gram-positive bacteria [34]. Its efficacy depends on size, concentration, and shape of the particles. It is proposed that AgNPs anchor to and penetrate bacterial cell wall, cause structure change of cell membrane (via free radical formation and subsequently free radical-induced membrane damage), increase cell permeability leading to cell death [34].

2. Antifungal effects

AgNPs possess antifungal effects against 44 strains of 6 fungal species [35]. It is proposed that AgNPs disrupt cellular membrane and inhibit fungal normal budding process [36, 37].

3. Antiviral effects

AgNPs possess antiviral effects against HIV-1 [38], hepatitis B virus [39], herpes simplex virus [40], etc. Inhibition of the initial stage of HIV-1 cycle and inhibition of CD4-dependent binding, fusion and infectivity are the propose mechanism of AgNPs against HIV-1 [41].

4. Anti- inflammatory effect

AgNPs alter the expression of pro-inflammatory cytokines such as TNF-α was decrease after treated with silver nanocrystalline [42], inhibited production of IL-4, IL-10 in mouse [43] explaining the anti-inflammatory effect of the nanoparticles.

4.4 Toxicity of AgNPs

***In vitro* study**

Toxicity of AgNPs have been investigated in various cell types. AgNPs affected human mesenchymal stem cells by decrease cell viability after incubated with 0.1 µg/mL of albumin-capped AgNPs [44]. In normal human lung fibroblast cells, or human glioblastoma cells, AgNPs showed genotoxicity at 50 µg/mL and above [45], while AgNPs capped with albumin demonstrated genotoxic effect at around 2 µg/mL in mouse peritoneal macrophage cell line [16]. AgNPs capped with polysaccharides showed toxic effect at 50 µg/mL in mouse embryonic stem cells and fibroblasts [46]. In addition, AgNPs were studied in epithelial cells such as Human Chang Liver (HeLa) and A549 cells showing that after treatment with AgNPs the morphology and cell growth were changed [47, 48]. Lung epithelial cells treated with AgNPs showed reduction of cell viability and leakage of lactate dehydrogenase (LDH), alteration of cell cycle distribution, upregulation of apoptotic gene expression and down regulation of anti-apoptotic genes [48]. AgNPs induced ROS formation in normal human lung bronchial epithelial cell line (BEAS-2B) [49]. Moreover, AgNPs showed toxic effect on endothelial cells. In this regard, Kalishwaralal *et al* [50] found that AgNPs caused inhibition of cell survival, VEGF-induced cell viability, cell proliferation, and migration through the activity of caspase-3 and suppression of Akt phosphorylation [50, 51]. AgNPs showed neurotoxic effect in dopaminergic neuronal cell line, PC12 by interfering

enzymatic function resulting in dopamine depletion [52]. Besides, AgNPs significantly induce cytotoxicity through activation of caspase-3, oxidative stress, depletion of anti-oxidant molecules and reduced intracellular calcium levels on cerebellum granule cells (CGCs) [53].

***In vivo* study**

AgNPs had no effect on zebrafish embryo at low concentration (25 μg/mL) but showed genotoxic effect when increased the concentration [12]. AgNPs caused abnormal sperm heads after injection to in mice [54]. Moreover, AgNPs showed pulmonary toxicity by increasing pro-inflammatory cytokines and ROS generation in lung [55-57]. In addition, there have been interested reports on oral exposure that AgNPs or Ag ion were translocated from the gut into the blood, systemically inducing liver damage [57].

4.4.1 Toxicity of AgNPs in immune cells

AgNPs showed significant toxic effects on the immune cells. For example, murine peritoneal macrophages exhibited decreased cell viability and nitric oxide (NO) production after treatment with AgNPs [59]. In addition, U937 cells which are the monocytic cell lines showed reduction of cell viability, increased ROS generaion and greater IL-8 production depending on size of AgNPs [60]

4.4.2 Mechanism of AgNP toxicity

The major toxic mechanism of AgNPs is induction of intracellular ROS. The overproduction of intracellular ROS can lead to oxidative stress, resulting in cellular dysfunction [61, 62]. ROS may also induce lipid peroxidation [63-65], DNA-strand break, modification of nucleic acids [66], modulation of gene expression through activation of redox-sensitive transcription factors [58, 59], and modulation of inflammatory responses through signal transcription [67], leading to cell death and genotoxic effects [65]. Intracellular ROS is associated with biological mechanisms involving mutagenesis, carcinogenesis and other ageing-diseases in human [3]. On the other hand, ROS-independent partway is also involved in AgNPs toxicity. Chairuangkitti et al.[14] reported that AgNPs caused cell cycle arrest in A549 cells. AgNPs increased the proportion of cells in the sub G-1 phase, caused S phase arrest and down regulation of the cell cycle associated with the proliferating cell nuclear antigen (PCNA) protein [14]. Moreover, AgNPs can induce pro-inflammatory cytokine release, such as IL-1, IL-6 and TNF-α as well as IL-1β by activate caspase-1, resulting in inflammation and cytotoxicity [73].

5. Silver copper nanoparticles (AgCuNPs)

5.1 Applications of AgCuNPs

Because copper is cheaper than silver or gold, but has very high conductivity, AgCuNPs are interested as a replacement metal used in inkjet printing industry [74].

5.2 Toxicity of AgCuNPs

Copper nanoparticles are very toxic to biological system, CuNPs can induce kidney, liver, spleen and renal toxicity [75]. However, no information regarding toxicity of AgCuNPs available so far.

6. Silver tin nanoparticles (AgSnNPs)

6.1 Applications of AgSnNPs

SnNPs have been used as an electrical interconnect material in the electronic packaging instead of lead in the solder because the vapour of lead are very toxic [7].

6.2 Toxicity of AgSnNPs

SnNPs showed no effect on dapthnia magma [78] but report on AgSnNPs toxicity is limited.

7. Cytokines

Cytokines are small proteins (~5–20 kDa) that are important in cell signaling. They are released by cells and affect behavior of other cells. They are produced by a broad range of cells, including immune cells like monocytes, macrophages, B

lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells [80].

7.1 Proinflammatory cytokines, Anti-inflammatory cytokines and chemokines

Cytokine have an important role in pathogenesis of the diseases and immunity response to infection. The induction of proinflammatory and anti-inflammatory cytokines is important to determine whether the immune system is successful in providing protection against specific pathogenic organism.

Pro-inflammatory cytokines are cytokines that are important in cell signaling and promoting systemic inflammation. They are produced predominantly by activated macrophages and are involved in the upregulation of inflammatory reactions. The examples of pro-inflammatory cytokines are TNF-α, IL-1α and IL-1β (Table 1).

Anti-inflammatory cytokine are series of immunoregulatory molecules that control the proinflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. The examples of anti-inflammatory cytokines are TGF-β and IL-10.

Chemokines are a family of small cytokines, or signaling proteins secreted by cells. Their names are derived from their ability to induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines. The examples of chemokines are IL-8 (Table 1), MCP-1 and MCP-2 [80]

Table 1 TNF-α, IL-1β and IL-8 source and activity [80]

Cytokine. MW. Synonyms	Sources	Activity
Tumor necrosis factor alpha (TNF-α). 52 kDa. Cachectin, TNF ligand superfamily member 2 (TNFSF2).	Monocytes, macrophages, and other cell types, including activated T cells, NK cells, neutrophils, and fibroblasts.	Strong mediator of inflammatory and immune functions. Regulates growth and differentiation of a wide variety of cell types. Cytotoxic for many types of transformed and some normal cells. Promotes angiogenesis, bone resorption, and thrombotic processs. Suppresses lipogenic metabolism.
Interleukin 1 (IL-1). IL-1α 17.5 kDa, IL-1β 17.3 kDa. Lymphocyte-activating factor (LAF); mononuclear cell factor (MCF); endogenous pyrogen (EP).	Many cell types, including monocytes, macrophages, dendritic cells, NK cells, and non-immune system cells such as epithelial and endothelial cells, fibroblasts, adipocytes, astrocytes, and some smooth muscle cells.	Displays a wide variety of biological activities on many different cell types, including T cells, B cells, monocytes, eosinophils and dendritic cells, as well as fibroblasts, liver cells, vascular endothelial cells, and some cells of the nervous system. The in vivo effects of IL-1 include induction of

		local inflammation and systemic effects such as fever, the acute phase response, and stimulation of neutrophil production.
Interleukin 8 (IL-8). 6-8 kDa. Neutrophil attractant/activating protein (NAP-1); neutrophil-activating factor (NAF); granulocyte chemotactic protein 1(GCP-1); CXCL8 chemokine.	Many cell types, including monocytes, macrophages, lymphocytes, granulocytes, and nonimmune system cells such as fibroblasts, endothelial and epithelial cells, and hepatocytes.	Chemokine that functions primary as a chemoattractant and activator of neutrophils; also attracts basophils and some subpopulations of lymphocytes; has angiogenic activity.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Nanoparticles

In this study, AgNPs (Cat. No. : 576832, Formular: Ag) coated with polyvinylpyrrolidone (PVP) have an average size of < 100 nm, containing 99.5% metals basis. AgCuNPs (Cat. No. : 576824, Formular: $CuAg_{25}$) have an average size of < 100 nm, containing 90-100% silver and 1-2.5% copper. AgSnNPs (Cat. No. : 677434, Formular: $AgSn_{25}$) have an average size of < 150 nm, containing 1-10% silver and 90-100% tin. All nanoparticles and $AgNO_3$ (powder) were purchased from Sigma-Aldrich (MO, USA).

3.1.2 Cells line and Chemicals

Human acute monocytic leukemia (THP-1, TIB-202™) cells were obtained from American Type Culture Collection (Virginia, USA). Roswell Park Memorial Institute-1640 (RPMI 1640) medium, fetal bovine serum (FBS), glutamine and were from Gibco (NY, USA). Dimethyl sulfoxide, 0.25% trypsin-EDTA solution, 2', 7'-dichlorofluorescein diacetate, phorbol 12-myristate 13-acetate (PMA), hydrogen peroxide solution (H_2O_2), and HBSS buffer were from Sigma-Aldrich (MO, USA). Tetrazolium compound [3-(4, 5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H tetrazolium (MTS) was purchased from Promega (WI, USA). Ethanol was obtained from Merck (Darmstadt, Germany). All other chemicals were of the highest grade available.

3.1.3 Instruments

Theese following instruments were used in this study

1. 10 mm pertri dish, 6-well plate, 96-well plate, and black 96-well plate (Corning Costar, NY, USA)
2. Cell culture flask 25 and 75 cm^2 (Corning Costar, NY, USA)
3. Centrifuge tube 15, 50 ml (Corning Costar, NY, USA)
4. Compact inverted microscope (CKX41, Olympus, Tokyo, Japan)
5. High speed refrigerated micro centrifuge: MX-305 (Tomy, Fremont, CA, USA)
6. Centrifuge Bechman Allegra® X-15R (Beckman Coulter, CA, USA)
7. Electron microscopy grids size 100 mesh × 250 μm pitch, copper (Sigma-Aldrich, St. Louis, MO, Germany)
8. Elmasonic S30H sonicator (Elma, SG, Germany)
9. Flow cytometer (FACSAria^{TM}II): BD Biosciences, San Jose, CA, USA
10. Microcentrifuge tube size 1.5-2.0 milliliter (Eppendorf, Hamburg, Germany)
11. Micro pipette size 20, 200 and 1,000 microliter (Eppendorf, Hamburg, Germany)
12. Multi-mode microplate reader: SpectraMax M5, Molecular Devices (Sunnyvale, CA, USA)

13. Pipette tips size 0.1-10 microliter and 100-1,000 microliter (Eppendorf, Hamburg, Germany)
14. Spectra/Por® 6 dialysis membrane (Merck, Darmstadt, Germany)
15. Thermomixer (TEM; JEM-2010, Jeol, MA, USA)
16. Zetasizer Nano ZS (Mavern Instrument Ltd, Malvern, UK)

3.2 Methods

The experimental design of this study is demonstrated in Figure 4.

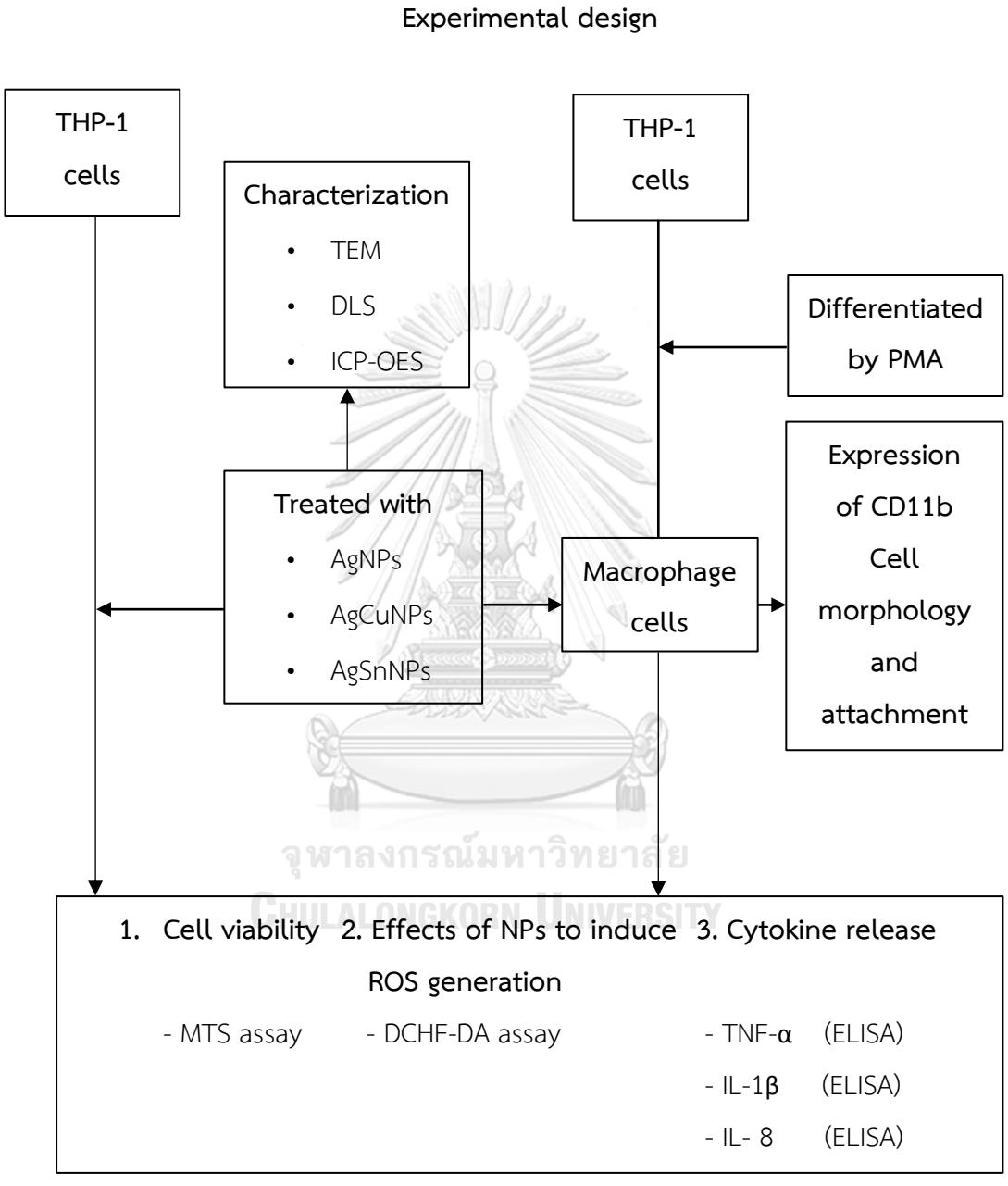

Figure 4 The experimental design of the study of toxicity of AgNPs, AgCuNPs and AgSnNPs in THP-1 cell lines and THP-1 differentiated macrophage cells.

3.2.1 Characterization of AgNPs, AgCuNPs and AgSnNPs (Modified from McNeil, 2011 [91]; Chairuangkitti et al., 2013 [14])

According to the product information, AgNPs and AgCuNPs have particle size of less than 100 nm, while AgSnNPs have particle size of less than 150 nm. To confirm and further clarify the morphology of those nanoparticles, AgNPs, AgCuNPs and AgSnNPs were dispersed in ethanol at 2 mg/mL, dropped on copper grids and allowed the solvent to be evaporated at room temperature for 24 h. Then the morphology of the nanoparticles wasa measured by a TEM. The primary sizes of nanoparticles were calculated from the average of 100 particles scanned from left to right in 10 fields of view.

3.2.2 Stability of AgNPs, AgCuNPs and AgSnNPs (Modified from McNeil, 2011 [91]; Chairuangkitti et al., 2013 [14])

To determine the stability of AgNPs, AgCuNP and AgSnNPs, the nanoparticle were dispersed at the concentration of 2 mg/ml in deionized water using various sonication times (0, 1, 2, 5, and 10 min), standed at room temperature for 0, 6 or 24 h. The zeta potential and dynamic light scattering (DLS) were measured using Zetasizer.

3.2.3 Ion release from AgNPs, AgCuNPs and AgSnNPs in deionized water (Modified from Kittler et al., 2010 [92])

To determine the ions released from AgNPs, AgCuNP and AgSnNPs, 10 mL of 2 mg/mL of each nanoparticle in deionized water was put into the dialysis bag with pore size of 1 kilo Dalton (kD) and the bag was left in the beaker containing 990 mL of deionized water for 24 h. The solution outside the dialysis bag was brought to measured for Ag^{+}, Cu^{2+} and Sn^{2+} ion by an inductively coupled plasma optical emission spectrometer (ICP-OES) (Figure 5).

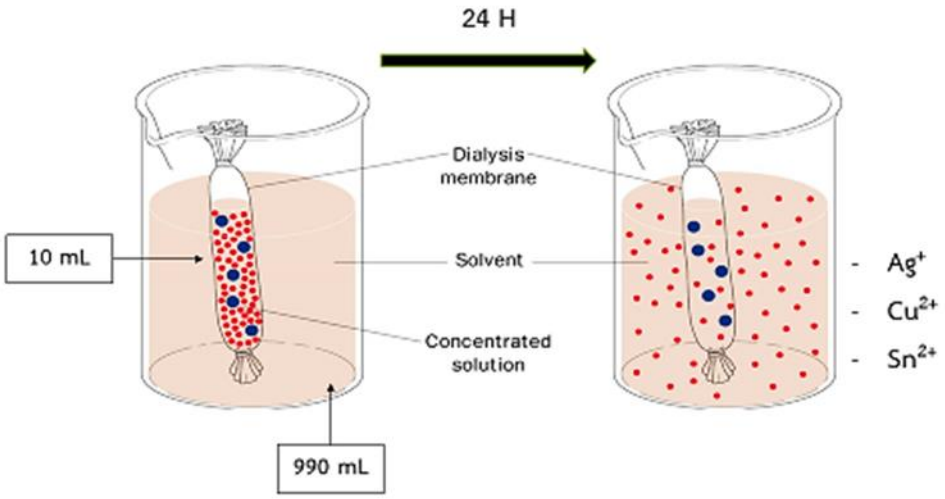

Figure 5 Metal ions released from AgNPs, AgCuNPs and AgSnNPs into deionized water

3.2.4 Cytotoxicity of AgNPs, AgCuNPs and AgSnNPs on THP-1 cells

Cytotoxicity of the nanoparticles was assessed using MTS assay, a mitochondrial-based cell viability assay according to the method of O'Toole et al (2003) [93]. THP-1 cells ($5x10^4$ cells/well) were seeded in 96-well plates. After 24 h, the cell were incubated with AgNPs, AgCuNPs or AgSnNPs at various concentrations (5, 10, 25, 50 and 100 µg/mL) for 24 h. At the end of the treatment period, 10 µL of MTS solution was added to the cells and further incubated for 3 h. The absorbance was measured at 490 nm using a multifunctional microplate reader (SpectraMax M5) with respective to the corresponding background of each nanoparticle. The percent cell viability was calculated from the ratio of the absorbance obtained from each treatment with respect to the corresponding control without nanoparticles treatment. Each experiment was performed independently in triplicate.

3.2.5 Intracellular ROS generation by AgNPs, AgCuNPs and AgSnNPs in THP-1 cells

Intracellular ROS were measured by DCF assay according to the method of Aranda et al (2013) [94]. THP-1 cells ($5x10^4$ cells/well) were seeded in black 96-well plates. Cells were incubated with 50 µM DCFH-DA in HBSS buffer for 40 mins, washed twice with PBS followed by incubation with AgNPs, AgCuNPs and AgSnNPs at various concentrations (5, 10, 25, 50 and 100 µg/mL) for 3 h. H_2O_2 at the concentration of 500 µM in sterile HBSS buffer was used as a positive control [97]. The fluorescent

compound in form DCF was detected using a SpectraMax M5 microplate fluorometer at an excitation and emission wavelength of 485 and 528 nm, respectively. Fluorescence intensity of the treatment groups was compared to those of the control. Each experiment was performed independently in triplicate.

3.2.6 Induction of THP-1 cells to macrophage cells using phorbol 12-myristate 13-acetate (PMA)

THP-1 cells were differentiated into macrophages using the method of Daigneault et al (2010) [95], with some modifications. The cells were treated with PMA at various concentrations (10, 25, 50, 100 and 200 ng/mL) for 72 h. The specific protein surface marker of macrophage, CD11b expression was detected by flow cytometry and morphology of the cells was determined by microscopy. The THP-1 differentiated macrophage cells were trypsinized and resuspended with completed RPMI media, then centrifuged at 300 g for 5 min, washed twice with cold PBS, followed by Fc block and incubated at room temperature for 15 min, then washed twice with cold PBS. The cells were resuspended with cold PBS and stained with CD11b antibody conjugated with FITC (Cat.No.4225554, BD Pharmingen®) for 30 minutes at room temperature. Cold PBS of 1 ml was added to the cells, then centrifuged for 5 min and resuspended with 500 µl of cold PBS. Expression of CD-11b was measured using flow cytometry. Fold increase or change of fluorescence intensity are calculated from the following equation:

$$\text{Fold increase or change of fluorescence intensity} = \frac{\text{Av. PMA-CD11b - Av.PMA-IgG1}}{\text{Av.THP-1-CD11b - Av.THP-1-IgG1}}$$

3.2.7 Cytotoxicity of AgNPs, AgCuNPs and AgSnNPs on THP-1 differentiated macrophage cells

To determine cytotoxicity of AgNPs, AgCuNPs and AgSnNPs in THP-1 differentiated macrophage cells, the macrophage cells ($5x10^4$ cells/well) were incubated with AgNPs, AgCuNPs and AgSnNPs at various concentrations (5, 10, 25, 50 and 100 µg/mL) for 24 h and the experiments were performed as described in 3.2.4.

3.2.8 Intracellular ROS generation by AgNPs, AgCuNPs and AgSnNPs in THP-1 differentiated macrophage cells

Intracellular ROS levels were measured by the DCF assay. THP-1 differentiated macrophage cells ($5x10^4$ cells/well) were seeded in black 96-well plates and the experiments were performed as described in 3.2.5.

3.2.9 Detection of TNF-α, IL-1β and IL-8 cytokine release in THP-1 cells and THP-1 differentiated macrophage cells after treatment with AgNPs, AgCuNPs and AgSnNPs (Modified from the method of Lee et al., 1993 [96])

Cytokine release were detected by collecting supernatant of THP-1 cells or THP-1 differentiated macrophage cell after treatment with various concentrations (10, 50 and 100 µg/mL) of AgNPs, AgCuNPs and AgSnNPs for 24 h. To eliminate trace amount of nanoparticles, the supernatants were centrifuged at 1300 rpm, 25°c, for 5 min. Each cytokine in the supernatant was detected by using enzyme-linked immunosorbent assay (ELISA) kits. The Lipopolysaccharide (LPS) 1 ng/mL [98] was used as positive control.

3.2.10 Statistic analysis

All data were presented as mean ± standard deviation (SD). Three independent experiments were conducted to confirm the reproducibility of the experiments. Difference among groups was determined by one way analysis of variance (ANOVA) follow by Dunnett's test for multiple comparisons using SPSS program version 22.0 (Network license purchased by Chulalongkorn university). The value of $p<0.05$ was considered as statistically significant.

CHAPTER IV

RESULTS

4.1 Characterization of AgNPs, AgCuNPs and AgSnNPs

The morphology and primary size of the nanoparticles were characterized by TEM. The results demonstrated that all NPs were spherical and primary size (mean ±SEM) of AgNPs were 19.77±1.17 nm (Figure 6, Table A1), AgCuNPs were 50.72±3.72 nm (Figure 7, Table A2) and AgSnNPs were 82.58±3.85 nm (Figure 8, Table A3), respectively.

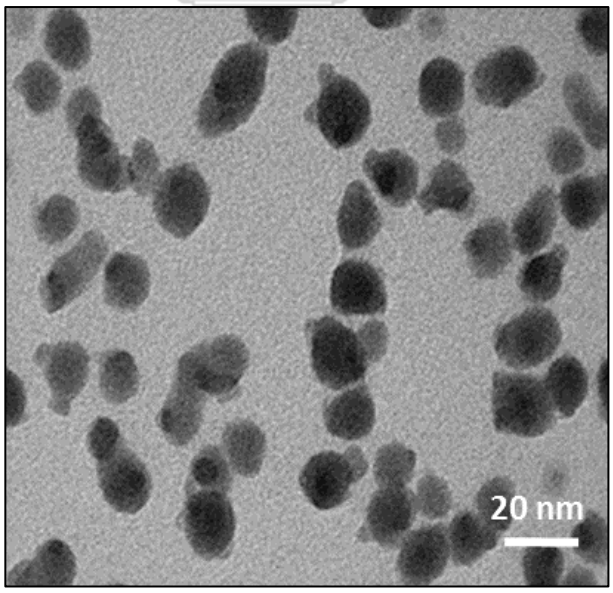

Figure 6 TEM image of AgNPs

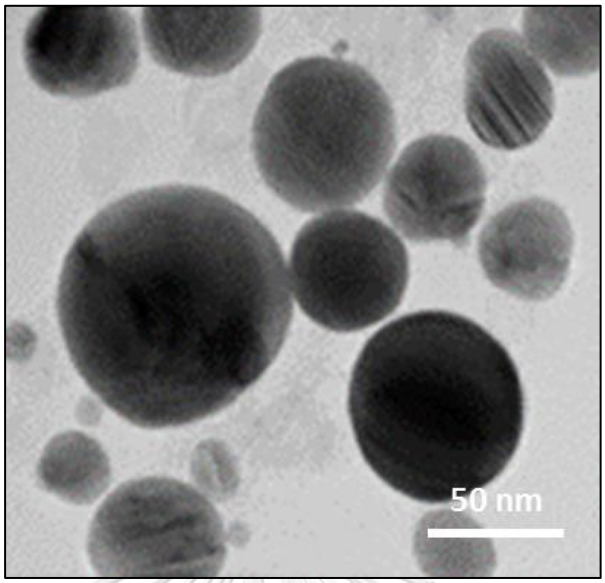

Figure 7 TEM image of AgCuNPs

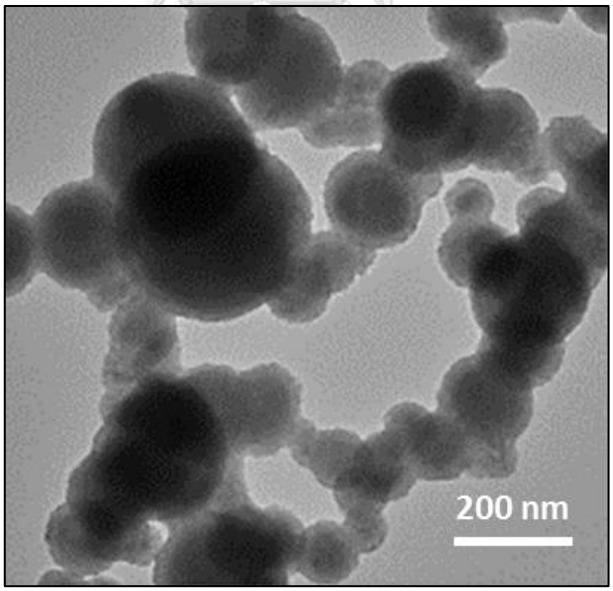

Figure 8 TEM image of AgSnNPs

4.2 Stability of AgNPs, AgCuNPs and AgSnNPs

The results from DLS showed that hydrodynamic size of AgNPs and AgCuNPs were approximately 177 nm and 116 nm, respectively. While AgSnNPs was about 371 nm. (Table 2, Table A4)

Sonication time selected from NPs 2 mg/ml dispersed in complete RPMI-1640 sonicated in various time (0, 1, 2, 5 and 10 min) observed at 0, 6 and 24 h at room temperature.

AgNPs before sonication were not well dispersed, they agglomerated on the solution surface. After sonicated for various times (1, 2, 5 and 10 min), the dispersion of AgNPs was improved. The suspended nanoparticles were then observed at 0, 6 and 24 h thereafter. The results showed AgNPs at sonication time 5 min were the best condition according to hydrodynamic size and their appearance after 24 h. For AgCuNPs, the best condition is 1 min sonication. On the other hand, AgSnNPs showed a good dispersion without sonication (Table2, A4).

Table 2. Hydrodynamic size and sonication times of AgNPs, AgCuNPs and AgSnNPs in DI water

Title	**AgNPs**	**AgCuNPs**	**AgSnNPs**
Hydrodynamic size (nm) mean ± SD (n=3)	176.67±7.81	115.73±2.45	370.93±1.46
Optimized Sonication time (min)	5	1	0

Table 3. Nanoparticle suspension after sonication and standing at room temperature for various times.

Time (h)	AgNPs	AgCuNPs	AgSnNPs
0			
6			
24			

Sonication Time (min) 0 1 2 **5** 10 0 **1** 2 5 10 **0** 1 2 5 10

4.3 Metal ions released from AgNPs, AgCuNPs and AgSnNPs into deionized water

Metal ions are among the factors affected biological responses and toxicity. In this study, all nanoparticles were investigated their release of metal ions into DI water over 24 h of dialysis. The results demonstrated very low amount of silver ion released from AgNPs (<0.035% w/w) and AgCuNPs (0.285% w/w) (Table 4). Neither silver nor tin

ions were detectable from AgSnNPs, whereas dissolution of $AgNO_3$ in DI water was 96.06% w/w. A moderate amount (9.05% w/w) of copper ion was found to be dissolved from AgCuNPs (Table 4).

Table 4 Ion release from AgNPs, AgCuNPs and AgSnNPs in deionized water

No.	Particles	Formula	Dissolved silver ion		Dissolved copper ion		Dissolved tin ion	
			(mg/L)	%	(mg/L)	%	(mg/L)	%
1	$AgNO_3$ (MW=169.87)	$AgNO_3$	12.2	96.06	-	-	-	-
2	AgNPs	Ag	< 0.007	<0.035	-	-	-	-
3	AgCuNPs	$CuAg_{25}$	0.057	0.285	1.81	9.05	-	-
4	AgSnNPs	$AgSn_{25}$	N.D.	N.D.	-	-	N.D.	N.D.
5	H_2O	H_2O	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

N.D.; not detectable

4.4 Cytotoxicity of AgNPs, AgCuNPs and AgSnNPs on THP-1 cells

Effects of AgNPs, AgCuNPs and AgSnNPs on cell viability were assessed using MTS assay. The results showed that all nanoparticles had no significant effects on THP-1 cells, at all concentration used in this study (5, 10, 25, 50 and 100 µg/mL) (Figure 9; Table A5, A6, A7)

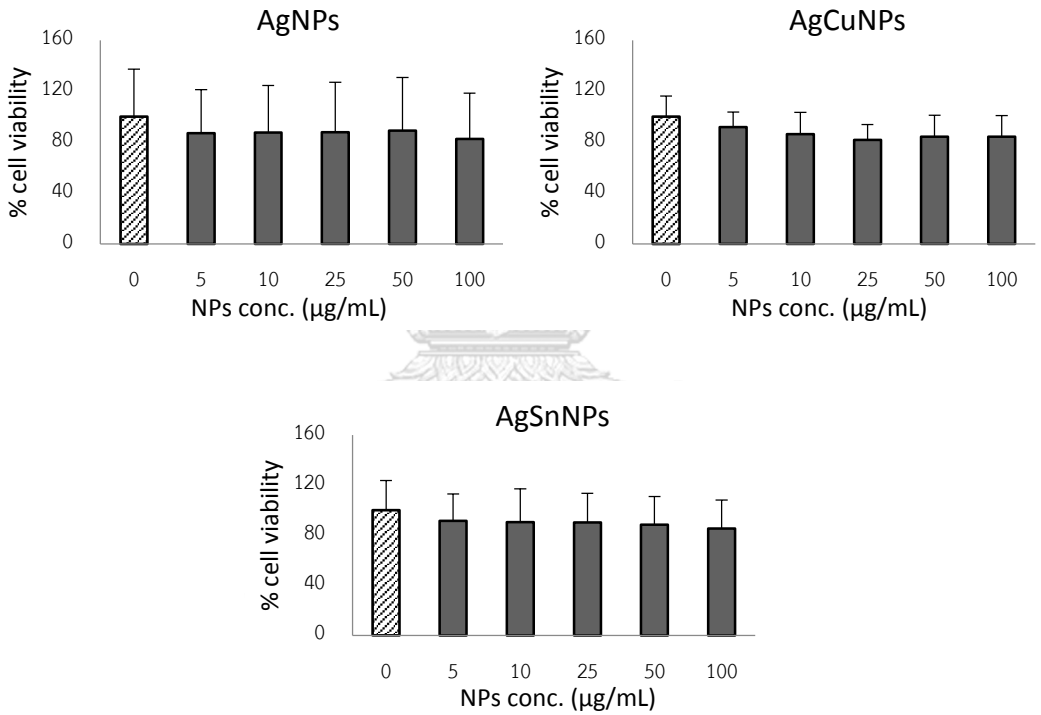

Figure 9 Cytotoxicity of AgNPs, AgCuNPs and AgSnNPs on THP-1 cells assessed by MTS assay

4.5 Intracellular ROS generation by AgNPs, AgCuNPs and AgSnNPs in THP-1 cells

Intracellular ROS was detected by DCF assay. The results showed that AgNPs and AgSnNPs had no effected on THP-1 cells, except for AgCuNPs that generated intracellular ROS in THP-1 cells at the high concentrations (50, 100 µg/mL). H_2O_2 solution which was used as positive control caused significant increase of intracellular ROS as compared to the non-treated control (Figure 10; Table A8, A9, A10)

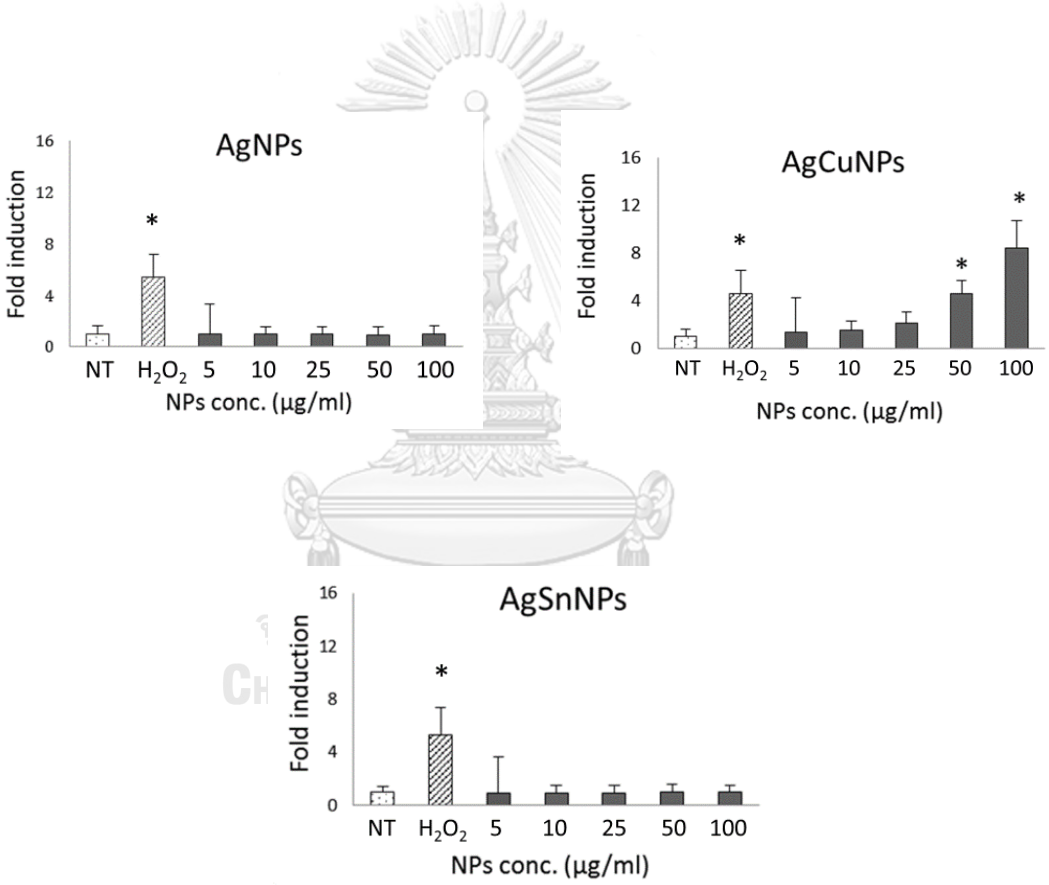

Figure 10 Intracellular ROS generation by AgNPs, AgCuNPs and AgSnNPs in THP-1 cells

* $P < 0.05$ compared with the non-treated control (NT).

4.6 Induction of THP-1 cells to macrophage cells using phorbol 12-myristate 13-acetate (PMA)

In this study PMA was used to differentiated THP-1 cells into THP-1 differentiated macrophage cells by varying the concentration of PMA (10, 25, 50, 100 and 200 ng/mL) for 72 h. The representation figures of cell morphology before and after treatment with PMA at 10, 25, 50, 100 and 200 ng/mL were shown in Figure 11, 12, 13, 14, 15 and 16, respectively. The results showed that at 100 ng/mL of PMA, the cells demonstrated good morphology and attached onto the well plate more than 80% of total cell population, indicating one of the macrophage characteristics (Figure 15).

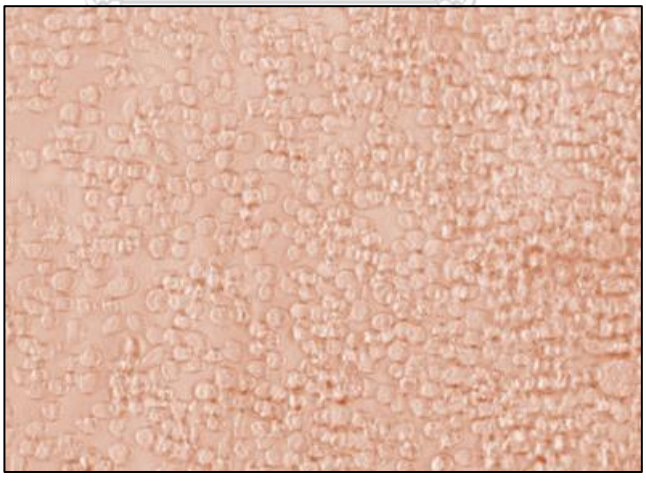

Figure 11 THP-1 cells before treatment with PMA

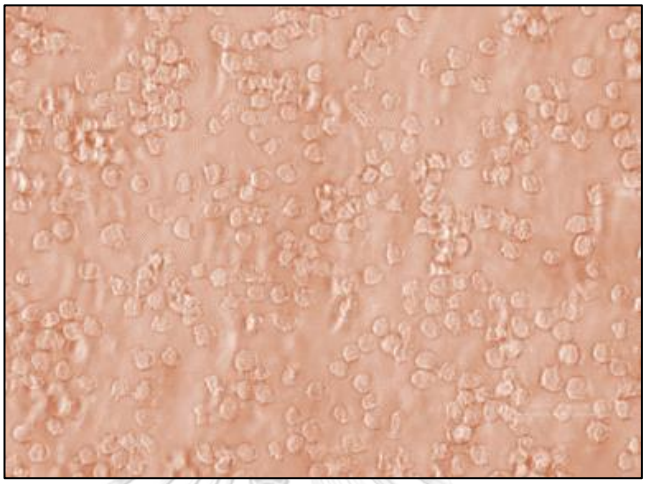

Figure 12 THP-1 cells treated with PMA at 10 ng/mL for 72 h

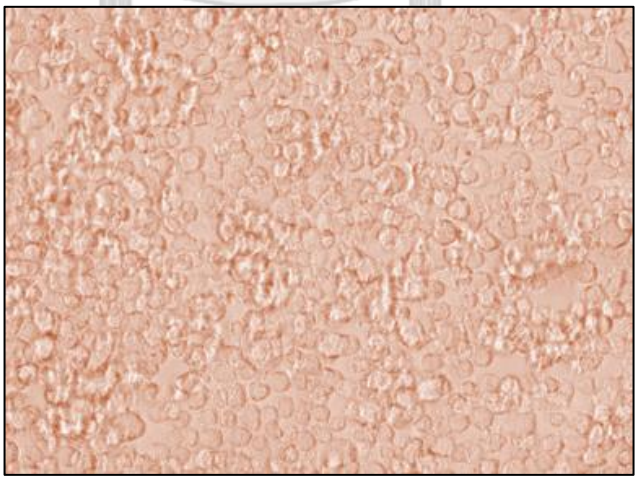

Figure 13 THP-1 cells treated with PMA at 25 ng/mL for 72 h

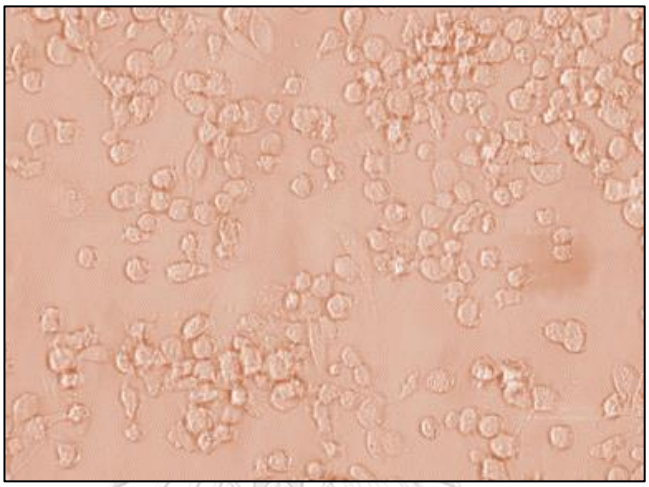

Figure 14 THP-1 cells treated with PMA at 50 ng/mL for 72 h

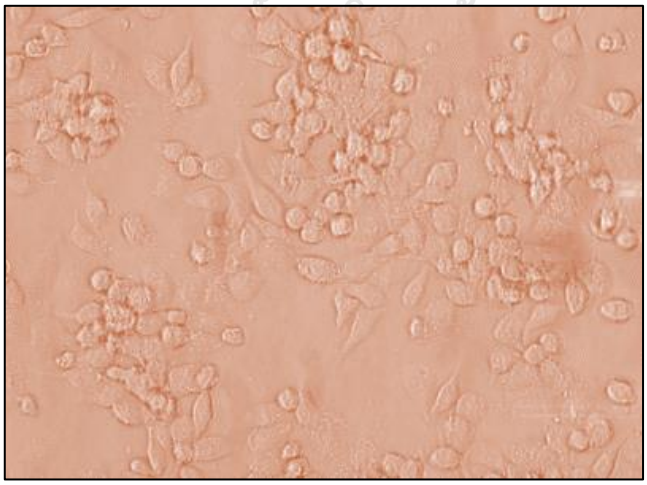

Figure 15 THP-1 cells treated with PMA at 100 ng/mL for 72 h

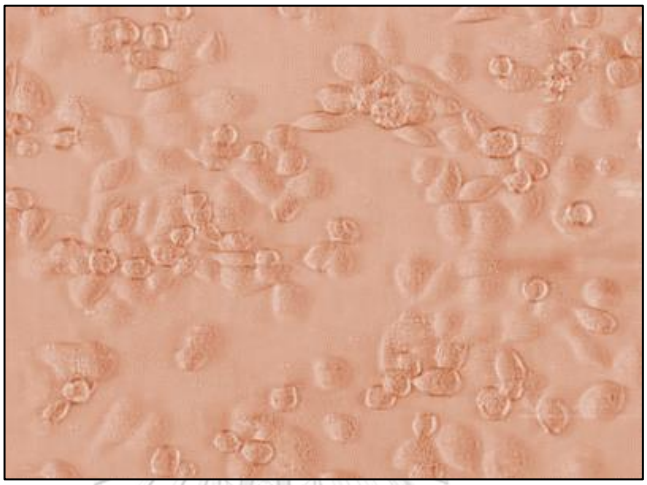

Figure 16 THP-1 cells treated with PMA at 200 ng/mL for 72 h

4.7 Verification of macrophage cells from CD11b expression

To confirm the macrophage characteristics, expression of CD11b, a specific surface marker of macrophage, was detected using flow cytometry. The results showed an increase of CD11b after THP-1 cells were treated with PMA at 100 ng/mL for 72 h (Figure 17).

No	Samples	% average
1	Av. PMA CD11b	13.25
2	Av.PMA-IgG1	0.15
3	Av.THP-1-CD11b	6.50
4	Av.THP-1-IgG1	0.75
Fold increase Intensity		= 2.28

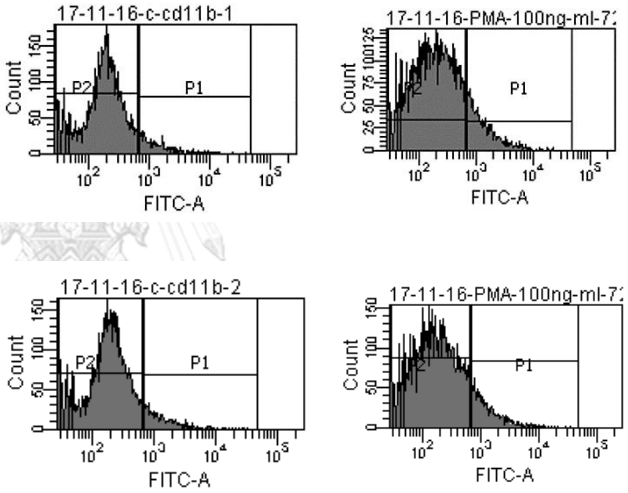

Figure 17 The expression of CD11b after THP-1 cells were treated with PMA at 100 ng/mL for 72 h

Fold increase or change of fluorescence intensity are calculated by

$$\frac{13.25 - 0.15}{6.50 - 0.75} = \frac{13.10}{5.75} = 2.28$$

4.8 Cytotoxicity of AgNPs, AgCuNPs and AgSnNPs on THP-1 differentiated macrophage cells

The results showed that AgNPs (100 µg/mL) and AgCuNPs (25, 50 and 100 µg/mL) caused significant decrease of cell viability while AgSnNPs at all concentrations used did not significantly affect cell viability (Figure 18; Table A11, A12, A13).

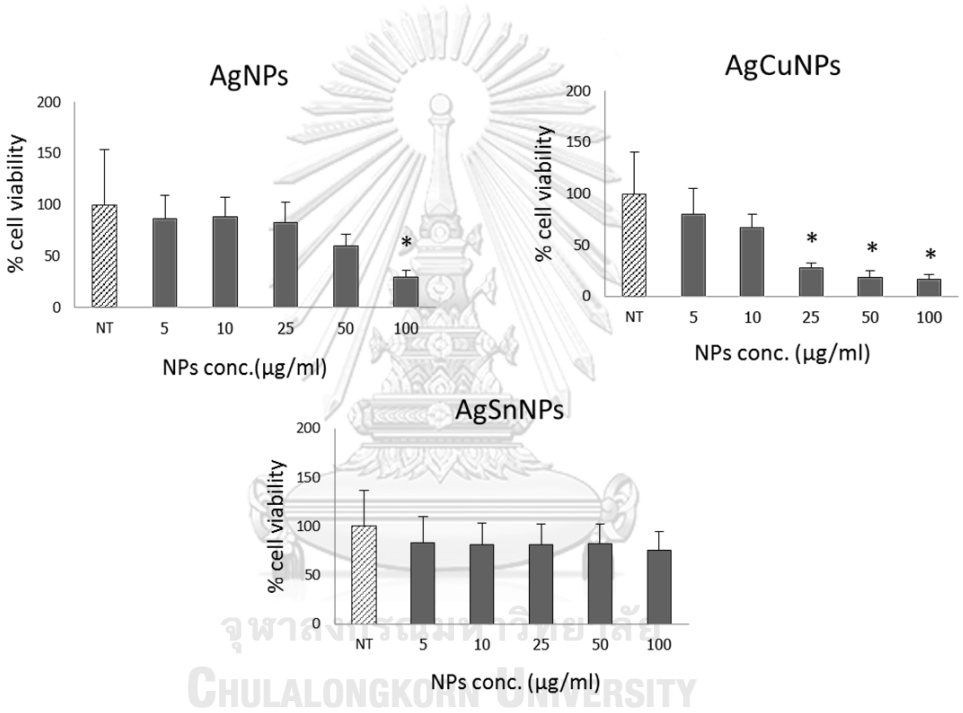

Figure 18 Cytotoxicity of AgNPs, AgCuNPs and AgSnNPs in THP-1 differentiated macrophage cells assessed by MTS assay. * $P < 0.05$ compared with the non-treated control (NT).

4.9 Intracellular ROS generation by AgNPs, AgCuNPs and AgSnNPs in THP-1 differentiated macrophage cells

Similar to the results in THP-1 cells, AgNPs and AgSnNPs did not cause intracellular ROS generation in THP-1 differentiated macrophages while AgCuNPs significantly increase intracellular ROS at the highest concentration used in this study (100 µg/mL) (Figure 19; Table A14, A15, A16).

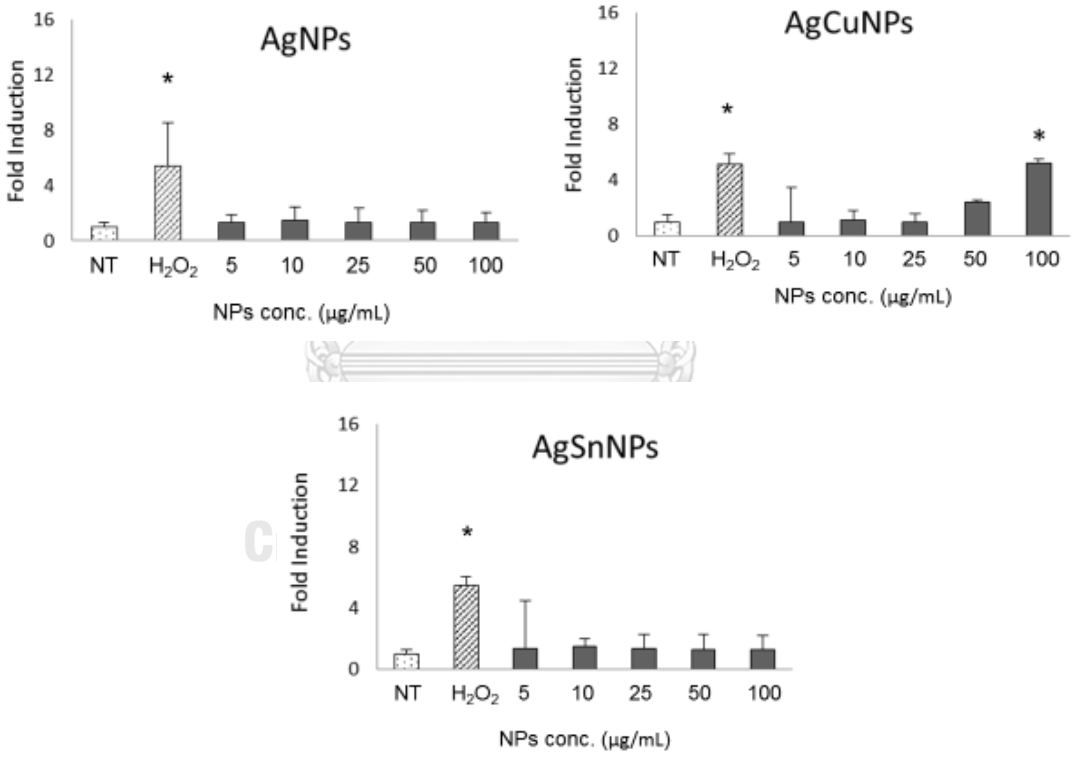

Figure 19 Intracellular ROS generation by AgNPs, AgCuNPs and AgSnNPs in THP-1 differentiated macrophage cells. * $P < 0.05$ compared with the non-treated control (NT).

4.10 Detection of TNF-α, IL-1β and IL-8 cytokine release after treatment with AgNPs, AgCuNPs and AgSnNPs in THP-1 cells and THP-1 differentiated macrophage cells

4.10.1 TNF-α release

The results showed that all nanoparticles at all concentrations used in this study (10, 50 and 100 μg/ml) caused significant decrease of TNF-α release from the THP-1 cells. LPS which was used as a positive control caused significant increase of TNF-α release from THP-1 cells (Figure 20).

TNF-α release from the macrophages was much less than that from THP-1 cells, despite without treatment with nanoparticles (in the non-treated control). Thus, the effects of nanoparticles on TNF-α release from macrophage cells were not seen, except for the AgCuNPs at 50 and 100 μg/mL that demonstrated the significant decrease of TNF-α release as compared to the non-treated control (Figure 21).

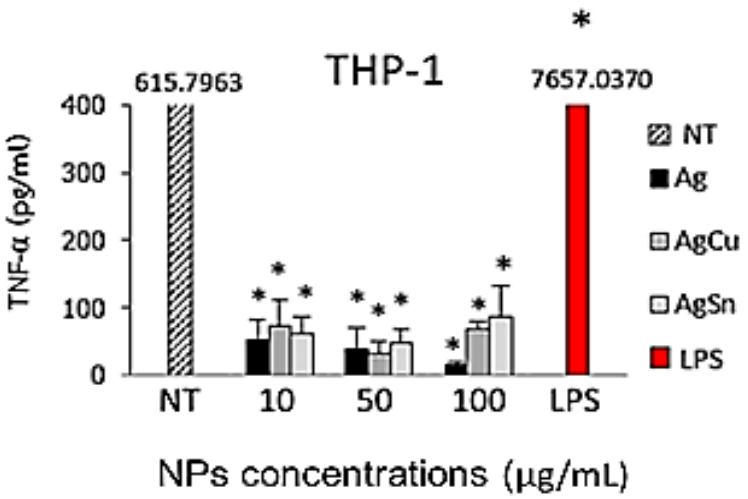

Figure 20 The release of TNF-α from THP-1 cells following treatment with AgNPs, AgCuNPs and AgSnNPs for 24 h. * $P < 0.05$ compared with the non-treated control (NT).

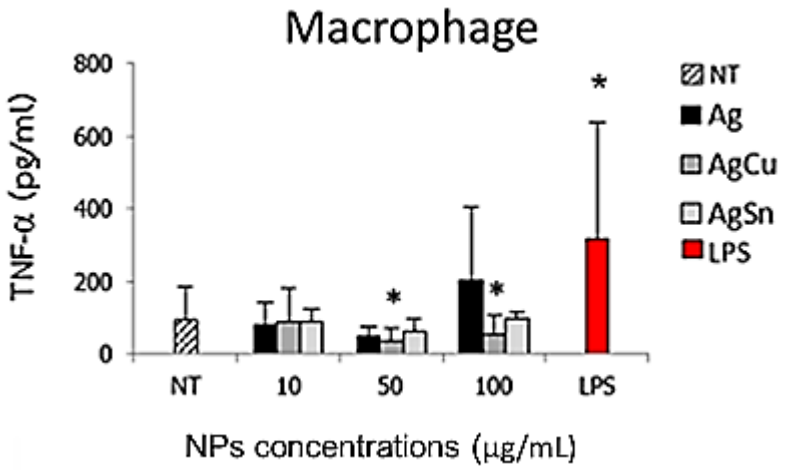

Figure 21 The release of TNF-α from THP-1 differentiated macrophage cells following treatment the cells with AgNPs, AgCuNPs and AgSnNPs for 24 h. * $P < 0.05$ compared with the non-treated control (NT).

4.10.2 IL-1β release

All nanoparticles used in this study seem not to affect the release of IL-1β from both THP-1 (Figure 22) and THP-1 differentiated macrophages (Figure 23). The exception was shown only for AgSnNPs at 10 and 50 μg/ml that demonstrated significant decrease of IL-1β release from THP-1 cells (Figure 22).

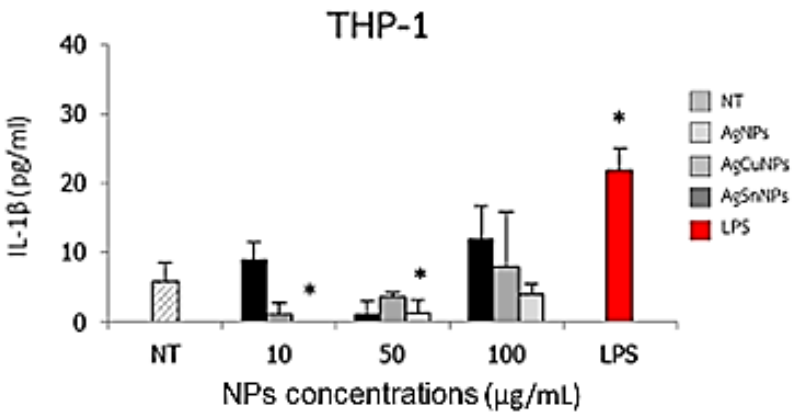

Figure 22 The release of IL-1β from THP-1 cells following treatment with AgNPs, AgCuNPs and AgSnNPs for 24 h. * $P < 0.05$ compared with the non-treated control (NT).

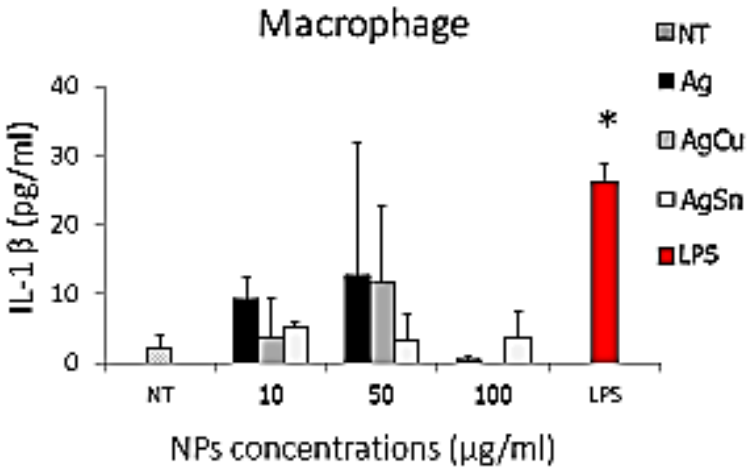

Figure 23 The release of IL-1β from THP-1 differentiated macrophage cells following treatment cells with AgNPs, AgCuNPs and AgSnNPs for 24 h. * $P < 0.05$ compared with the non-treated control (NT).

4.10.3 IL-8 release

AgNPs did not affect IL-8 release from THP-1 cells while AgCuNPs and AgSnNPs at 10 and 50 µg/ml caused significant increase of IL-8 release but not at the highest concentration (100 µg/ml) (Figure 24). In THP-1 differentiated macrophage cells, all nanoparticles at all concentrations used in this study caused significant increase of IL-8 release (Figure 25). LPS which was used as a positive control caused significant increase of IL-8 release in both THP-1 and macrophage cells (Figure 24, 25).

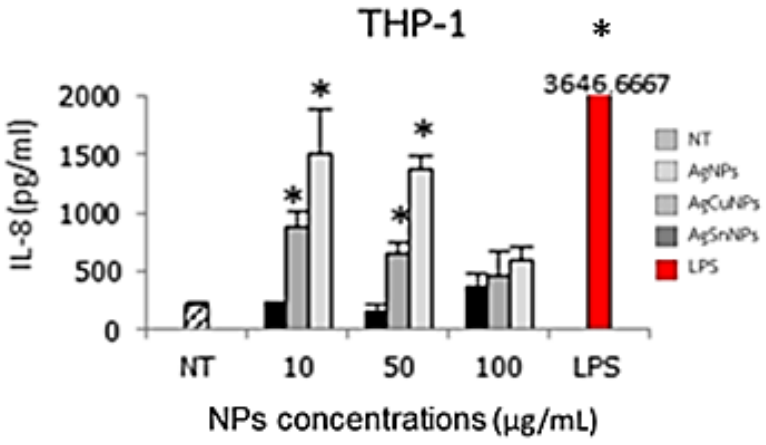

Figure 24 The release of IL-8 from THP-1 cells following treatment with AgNPs, AgCuNPs and AgSnNPs for 24 h. * $P < 0.05$ compared with the non-treated control (NT).

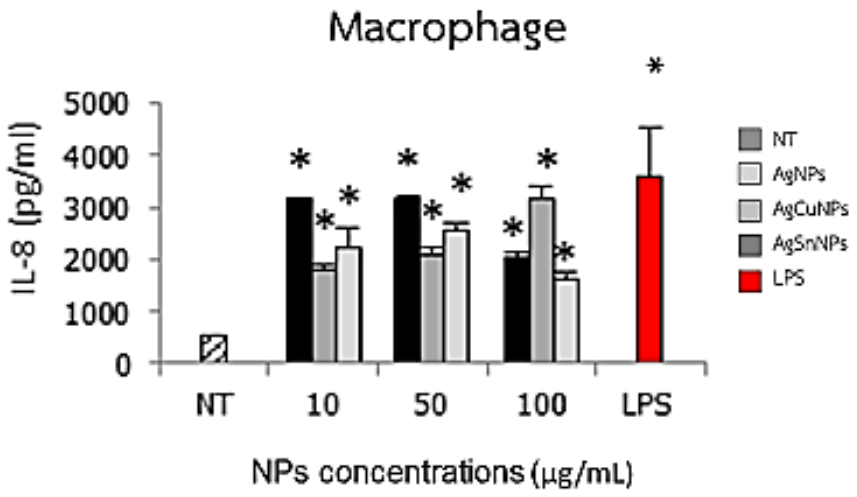

Figure 25 The release of IL-8 from THP-1 differentiated macrophage cells following treatment the cells with AgNPs, AgCuNPs and AgSnNPs for 24 h.

* $P < 0.05$ compared with the non-treated control (NT).

CHAPTER V

DISCUSSION AND CONCLUSION

This study aim to assess the toxic effects of AgNPs, AgCuNPs and AgSnNPs on THP-1 and THP-1 differentiated macrophage cells. THP-1 is the cell lines of human monocytes, which are one of the innate immune cells in the circulation and are normally differentiated to be tissue macrophages that play on important role in innate immune response. Beside the phagocytic function, macrophages also play a role of cytokine release and act as one of the antigen presenting cells leading to further induce the adaptive immune response. Thus, toxic effects of these three nanoparticles (particularly AgNPs which are used in many aspects of biomedical applications) on the immune cells are needed to be assessed.

Regarding THP-1 cells, the results demonstrated that all nanoparticles did not cause significant cytotoxic effect in THP-1 cells over the ranges of concentrations used in this study. However, intracellular ROS generation was increased in a concentration-dependent manner in AgCuNPs, but not for AgNPs or AgSnNPs. Elevation of ROS induced by AgCuNPs within 3 h was likely overcome by cellular defense mechanisms, as they did not cause cytotoxicity after 24 h of incubation [88]. The difference of oxidative response among silver and silver-metal nanoparticles is supported by a

previous study that copper oxide nanoparticles are highly associated with oxidative stress and up regulation of some heat shock proteins in THP-1 cells [9]. Since AgCuNPs used in this study contain only small amount of copper (1-2.5%), therefore the induction of intracellular ROS did not leading to cell death. ROS generation has been reported as a predominant mechanism of nanoparticle toxicity, and intracellular ROS is normally used as a crucial indicator to assess various toxic effects from nanoparticles [9, 14]. ROS independent pathway such as an interference of cell cycle is also involved [14].

Regarding THP-1 differentiated macrophages, the results demonstrated that AgNPs and AgCuNPs demonstrated cytotoxic effect at high concentrations. Significant decrease of cell viability were shown at the concentrations of 100 µg/mL of AgNPs; 25, 50 and 100 µg/mL of AgCuNPs. In contrast, AgSnNPs did not cause any significant toxic effect at all concentrations used. Significant increase of intracellular ROS generation was shown only at the concentration of 100 µg/mL of AgCuNPs. Thus, following treatment with AgNPs and AgCuNPs, the decrease of cell viability were not associated to the induction of intracellular ROS by the nanoparticles. Again, ROS-independent pathway may explain these results. Among these three nanoparticles, AgSnNPs did not show any cytotoxic effect in the range of concentrations used in this study while AgCuNPs were most toxic. This is because AgSnNPs contain only 1-10% of Ag and 90-100% of tin which is less toxic to cells [78] while AgCuNPs contain 90-100% of Ag and

1-2.5% of Cu which is more toxic to cells [27] adding more toxic effect to cell as compared to exposure to AgNPs.

This study also investigate the influence of AgNPs, AgCuNPs and AgSnNPs on the release 3 cytokines, TNF-α, IL-1β and IL-8 from THP-1 cells and THP-1 differentiated macrophages. The results demonstrated that in THP-1 cells, all 3 nanoparticles significantly decreased the release of TNF-α but only AgSnNPs significantly decreased the release of IL-1β (at 10 and 50 μg/ml) and both AgCuNPs and AgSnNPs significantly increased IL-8 (at 10 and 50 μg/ml). Regarding macrophage cells, only AgCuNPs decreased TNF-α release (at 50 and 100 μg/ml) and all three nanoparticles induced IL-8 release from these cells. Even through the results are somewhat different among type of cells as well as the type of metal nanoparticles, these nanoparticles tended to impair the secretion of pro-inflammatory cytokines such as TNF-α and IL-1β but increased the release of the chemokine, IL-8. Some previous studies have reported the interaction of AgNPs with the immune cells or others. Effect of AgNPs on cytokine release were varied among studies depending upon the different coating types and cell tested. Castillo et al (2008) [58] demonstrated that tiopronin coated AgNPs inhibited IL-6 release from RAW246.7 macrophages by TLR2, TLR2/6, TLR3 or TLR9 stimulation whereas Yen et al (2009) [89] found that AgNPs did not affect the release of IL-1, IL-6 and TNF-α from murine macrophage cell lines. Parnsamut and Brimson (2015) [87] investigated the effect of AgNPs on Jurkat and U937 cells, the results

showed that AgNPs decreased TNF-α production by suppressed ERK signaling, that is known to induce cell proliferation and cell survival. Shin et al (2007) [90] reported that AgNPs inhibited the release of IL-5, INF-γ and TNF-α from peripheral blood mononuclear cells (PBMCs) while Greulich et al (2009) [101] found that AgNPs inhibited the release of IL-6 and IL-11 but increase the release of IL-8 from human mesenchymal stem cell (hMSCs). The findings that all three nanoparticles affected the release of cytokines even though viability of the cells was not affected indicating that at the range of particle concentrations used, these nanoparticles only affected the cell functions without causing cell death in monocytes except at the high concentrations of AgNPs and AgCuNPs in macrophages. The only IL-8 that seemed to be increased following nanoparticles exposure could be because IL-8, which was a chemokine that immune cells particularly macrophages firstly released immediately following foreign compounds exposure to recruit other cells to the site of exposure before they caused disruption to other cytokines. The influence of AgNPs to impair the release of inflammatory cytokines from the immune cells was beneficial in term of safety form the allergic reactions from exposure to the nanoparticles but was disadvantageous from the impairment of body immune response to the microbial infections.

In this study, AgNPs, AgCuNPs and AgSnNPs were characterized for their size, stability and the ion release from the nanoparticles. It was demonstrated that the size of all three nanoparticles were less than 100 nm verifying that their sizes were in the

definition range of nano-sized particles (at least one dimension should be less than 100 nm). Stability of the nanoparticles was verified by zeta potential and hydrodynamic size. When they were suspended in the solution, after sonicated for various times, the best condition of each nanoparticles were vary, as described in the result session. The release of ions (Ag, Cu and Sn ions) demonstrated that very small amount of Ag ion was released from AgNPs and AgCuNPs whereas no Ag ion release was detected from AgSnNPs. Thus, the subsequent tested effects were contributed from the nanoparticles themselves not from the Ag ion released from the nanoparticles. Ag ion release from the AgNPs was shown to the responsible for the toxicity of AgNPs in some studies [102]. However, Cu ion was shown to be released from AgCuNPs. Thus, the more cytotoxic of AgCuNPs than AgNPs and AgSnNPs may be contributed from the Cu ion beside the influence from the nanoparticle itself.

The THP-1 differentiated macrophage cells used in this study were differentiated from THP-1 cells using PMA according to the reports from many studies [95]. This study found that PMA at 100 μg/ml and incubation time of 72 h were the appropriate conditions for differentiation of THP-1 to macrophage cells, the conditions which were consistent to the findings of others [3]. Morphology of the obtained macrophages were checked under light microscope and the surface marker protein, CD11b was determined using flow cytometry. CD11b was known to be the specific surface marker protein of macrophage cells [103].

In conclusion, AgNPs, AgCuNPs and AgSnNPs were investigated for their toxicity and their influence on intracellular ROS generation and cytokine release in THP-1 and THP-1 differentiated macrophage cells. All three nanoparticles were not cytotoxic to THP-1 cells but AgNPs and AgCuNPs decreased cell viability of macrophage cells only at high concentrations. Intracellular ROS generation was not the solely cause of cell injury. Regarding the release of three cytokines (TNF-α, IL-1β and IL-8), all three nanoparticles significantly impaired the release of TNF-α in THP-1 cells but caused significant increase of IL-8 release in THP-1 differentiated macrophage cells. Cytotoxic effects and the impact of the nanoparticles on immune functions should be concern when they are incorporated into the human health products or contaminated into the environment.

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APPENDIX

VITA

Miss Patamaporn Monprasit was born on August 4, 1983 in Rayong. She received her B.Sc in Pharm from the Faculty of Pharmacy, Huachiew Chalermprakiet University in 2006.