ผลของอนุภาคนาโนซิลเวอร์ต่อการอยู่รอดของเซลล์ปกติและเซลล์มะเร็งผิวหนังของมนุษย์

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2556

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EFFECTS OF SILVER NANOPARTICLES ON CELL VIABILITY OF NORMAL AND CANCER
HUMAN SKIN CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

Faculty of Science

Chulalongkorn University

Academic Year 2013

Thesis Title	EFFECTS OF SILVER NANOPARTICLES ON CELL VIABILITY OF NORMAL AND CANCER HUMAN SKIN CELLS
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พรสวรรค์ เนตรเจริญสิริสุข : ผลของอนุภาคนาโนซิลเวอร์ต่อการอยู่รอดของเซลล์ปกติ และเซลล์มะเร็งผิวหนังของมนุษย์. (EFFECTS OF SILVER NANOPARTICLES ON CELL VIABILITY OF NORMAL AND CANCER HUMAN SKIN CELLS) อ.ที่ปรึกษา วิทยานิพนธ์หลัก: อ. ดร.กิตตินันท์ โกมลภิส, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร.ธนา ภัทร ปาลกะ, ดร.สเตฟาน ดูบาส, 88 หน้า.

ในปัจจุบันอนุภาคซิลเวอร์นาโน (AgNPs) ได้ถูกนำมาใช้อย่างหลากหลาย เช่น ใน ผลิตภัณฑ์ทางการแพทย์และเครื่องอุปโภคหลายชนิด แต่ความปลอดภัยของ AgNPs ยังคงเป็นที่ น่าสงสัย มีงานวิจัยจำนวนมากได้รายงานว่า AgNPs มีความเป็นพิษต่อเซลล์ต่างๆ ดังนั้น งานวิจัย นี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของ AgNPs ต่อการอยู่รอดของเซลล์ไลน์ผิวหนังปกติของมนุษย์ (CCD-986SK) และเซลล์ไลน์มะเร็งผิวหนังของมนุษย์ (A375) AgNPs สังเคราะห์ได้จากการใช้ $AgNO_3$ เป็นแหล่งของ Ag และใช้ $NaBH_4$ เป็นสารรีดิวซิ่ง โดยมีอัลจิเนตและ poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (Copss) เป็นสารเคลือบอนุภาค ผลการ ทดลองการสังเคราะห์ AgNPs พบว่าอนุภาคมีการดูดกลืนแสงสูงสุดอยู่ในช่วง 400-450 นาโน เมตร และมีขนาดอนุภาค 5-15 นาโนเมตร วัดค่า zeta potential ของอนุภาคที่เคลือบด้วยอัลจิ เนตและ Copss ได้ค่าประจุลบอยู่ในช่วง -36.0 ถึง -31.3 mV และ -32.05 ถึง -26.4 mV ตามลำดับ ซึ่งแสดงถึงการมีประจุลบของอัลจิเนตและ Copss ที่ผิวของอนุภาค และความเป็นพิษ ต่อเซลล์ CCD-986SK และ A375 ประเมินได้ด้วยวิธี 3-(4-,5-Dimethylthiazol-2-yl)-2, 5-diphenyltertrazolium bromide (MTT) assay พบว่า $AgNO_3$ มีความเป็นพิษต่อเซลล์ทั้งสอง ในขณะที่อัลจิเนตและ Copss ไม่มีความเป็นพิษต่อเซลล์ทั้งสองชนิด และพบว่า AgNPs ที่เคลือบ ด้วย Copss มีความเป็นพิษต่อเซลล์ A375 และมีความเป็นพิษต่อเซลล์ CCD-986SK ที่ความ เข้มข้นต่ำของ Copss และพิจารณาจากค่าความเข้มข้นที่ทำให้เซลล์ตาย 50% (IC_{50}) พบว่า เซลล์ ไลน์ A375 มีความไวต่อ AgNPs มากกว่าเซลล์ CCD-986SK ส่วนการศึกษารูปแบบการตายของ เซลล์โดยการย้อมสีด้วย Annexin V และ propiodium iodide พบว่า AgNPs ที่เคลือบด้วยอัลจิ เนตและ Copss ชักนำให้เซลล์มะเร็งผิวหนัง A375 ตายแบบอะพอพโทซิส 84-90% และตาย แบบเนโครซิส 8-12% จากงานวิจัยนี้สามารถสรุปได้ว่า AgNPs ที่เคลือบด้วยอัลจิเนตมีความเป็น พิษน้อยกว่า AgNPs ที่เคลือบด้วย Copss และที่สำคัญ AgNPs ที่เคลือบด้วยอัลจิเนตมีความเป็น พิษเฉพาะต่อเซลล์มะเร็งผิวหนังเท่านั้น ซึ่งอาจนำ AgNPs ไปพัฒนาเพื่อใช้ในการบำบัดมะเร็งแบบ จำเพาะต่อไปได้

สาขาวิชา เทคโนโลยีชีวภาพ	ลายมือชื่อนิสิต ....
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5472215223 : MAJOR BIOTECHNOLOGY
KEYWORDS: SILVER NANOPARTICLES / CYTOTOXICITY / APOPTOSIS / HUMAN SKIN CELL / SYNTHESIS OF SILVER NANOPARTICLES

PONSAWAN NETCHAROENSIRISUK: EFFECTS OF SILVER NANOPARTICLES ON CELL VIABILITY OF NORMAL AND CANCER HUMAN SKIN CELLS. ADVISOR: KITTINAN KOMOLPIS, Ph.D., CO-ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., STEPHAN DUBAS, Ph.D., 88 pp.

Silver nanoparticles (AgNPs) are widely used in many areas such as medical and consumer products. The safety of the AgNPs however, is still questionable. Several studies reported that AgNPs are toxic to various cell types. Therefore, this study aimed to evaluate the effect of the AgNPs on viability of human skin normal cell line (CCD-986SK) and human skin cancer cell line (A375). The AgNPs were prepared by using silver nitrate ($AgNO_3$) as the silver ion source, sodium borohydride ($NaBH_4$) as the reducing agent and either alginate or poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (Copss) as the stabilizing agent. The result showed that the synthesized AgNPs had the maximum absorbing wavelength in the range of 400-450 nanometers and 5-15 nanometers in size. Their zeta potential values were negative charge of -36.0 to -31.3 mV for AgNPs capped with alginate and -32.0 to -26.4 mV for AgNPs capped with Copss, indicating the presence of the anionic sodium alginate or Copss at the surface. The toxicity against CCD-986SK and A375 cells was assessed by the conventional 3-(4-,5-Dimethylthiazol-2-yl)-2, 5-diphenyltertrazolium bromide (MTT) assay. It was found that $AgNO_3$ alone was highly toxic to both cell types while either alginate or Copss alone was not toxic. However, the alginate capped AgNPs were toxic to A375 cell line but not CCD-986SK cell line. In addition, the Copss capped AgNPs were toxic to A375 cell line and CCD-986SK only at low concentration of the Copss. Judging from the 50% inhibition concentration (IC_{50}), it was found that A375 cell line was more sensitive to the AgNPs than the CCD-986SK cell line. The mode of cell death was investigated by Annexin V and propiodium iodide staining. AgNPs capped with both alginate and Copss was found to induce late apoptosis (84-90%) and necrosis (8-12%) in A375 cell line. Taken together, the AgNPs capped with alginate were less toxic than the AgNPs capped with Copss and the AgNPs capped with alginate were toxic to the skin cancer cell only. These results suggest that AgNPs may be useful as selective cancer therapeutice agent.

Field of Study: Biotechnology   Student's Signature ....

Academic Year: 2013   Advisor's Signature ....

Co-Advisor's Signature ....

Co-Advisor's Signature ....

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AgNPs	Silver nanoparticles
%	Percent
nm	Nanometer
µg	Microgram
µl	Microlitre
g	Gram
ml	Millilitre
L	Litre
mg	Milligram
mM	Millimolar
$^{\circ}$C	Degree Celsius
IC_{50}	50% inhibition concentration
EC_{50}	50% effective concentration
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
RPMI	Roswell Park Memorial Institute
IBM	Iscove basal medium
FCS	Fetal calf serum
PPM	Part per million
TEM	Transmission electron microscopy

MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrasolium bromide
PBS	Phosphate buffer saline
DDI	Double deionized water
PI	Propiodium iodide
CO_2	Carbon dioxide
S.D.	Standard deviation
ANOVA	Analysis of variance
λ_{max}	Lambda max
h	Hour

CHAPTER I

INTRODUCTION

Nanotechnology is the application of scientific knowledge in the synthesis and manipulation of matter at the nanoscale. Several groups of scientists are searching for the ways to take advantage of the structural phenomena of range small particles of about 1 to 100 nm, which have characteristic of the properties of matter such as atoms, molecules or materials (Roco, 2007). The nanoparticles can be applied to achieve the most benefit in various fields such as physics, chemistry, biology, computer science, biotechnology, material engineering and medicine (Colvin, Schlamp, & Alivisatos, 1994; Hamilton & Baetzold, 1979; Hoffman, Mills, Yee, & Hoffmann, 1992; Mansur et al., 1995; Schmid, 1992; Wang & Herron, 1991).

Among nanoparticles, silver nanoparticles can be considered as the most interesting materials (X. Chen & Schluesener, 2008; P. Mukherjee et al., 2001; Sondi & Salopek-Sondi, 2004). The unique physicochemical properties of silver nanoparticles (nanosilver) have brought them to the foreground of such nanotechnology-based products and applications. Silver nanoparticle is an effective killing agent against a broad spectrum of Gram-negative and Gram-positive bacteria, including antibiotic-resistant, antiviral, and antifungal strains (R. Burrell, Heggers, Davis, & Wright, 1999; Wijnhoven et al., 2009; Yin, Langford, & Burrell, 1999). Recently, it has been shown that silver nanoparticles (diameter 5-32 nm, average diameter 22.5 nm) enhance the antibacterial activity of various antibiotics (Shahverdi, Fakhimi, Shahverdi, & Minaian, 2007). The antibacterial activities of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin against Staphylococcus aureus and Escherichia coli were increased in the presence of silver nanoparticles. In addition, silver nanoparticles showed high activity against HIV-1 virus (Wijnhoven et al., 2009). Consequently, research and development of products containing silver nanoparticles is increasing in terms of application device in household such as air spray, washing detergent, air filter, air conditioning, mask, washer food packaging equipment or disinfectant in medical devices (Kowalski, 2010).

However, applications of silver nanoparticles have some dispute in terms of the toxicity on the environment and human health. They can enter into the aquatic environment via municipal and industrial water treatment plants including the product containing silver nanoparticles such as textiles, plastics, and medical industries. The silver nanoparticles, thus affect organisms that live in the environment. Smith and Carson (1977) found that 150,000 kg of silver enter the aquatic system every year from industry; as the world production of silver has almost doubled since; up until 2000 close to 300,000 kg of silver probably enter the aquatic system every year (Smith, 1977). Recent research with zebra fish showed that silver nanoparticles (12 nm) affected early development of fish embryos(Lee, Nallathamby, Browning, Osgood, & Xu, 2007). The silver nanoparticles can pass through biological membranes. After administration, silver nanoparticles are small enough to penetrate even very small capillaries throughout the human body in several ways such as inhalation, ingestion and dermal absorption. The health effect of silver nanoparticles on human has been reported, such as argyria (a condition in which the skin becomes blue or bluish-grey colored) which mainly found in worker manufacturing of silver nanoparticles. Several cross-section studies reported that argyria is the most frequent adverse outcome from exposure to silver nanoparticles. Prolonged ingestion of colloidal silver can change the color of skin and cause blue-grey appearance on face (Chang, Khosravi, & Egbert, 2006). The silver nanoparticles can bind to different tissues and cause toxic effect, such as adhesive interactions with cellular membrane and production of toxic radicals like reactive oxygen species (ROS) which can cause toxic effects through the production of free radicals that influence the redox potential of the cell thus damaging proteins, lipids and DNA (Y. S. Kim et al., 2008). Moreover, silver nanoparticles (10 µg/ml and above concentration) showed dramatic changes such as necrosis and apoptosis of the cell and at 5-10 µg/ml, they drastically reduced mitochondrial function and cell viability (A. Lansdown, 2007). Therefore, the use of products containing silver nanoparticles can be contaminated to human body and affect the cells. Dermal exposure represents an important potential absorption route for nano-silver. Antibacterial textiles and wound dressing that contain silver nanoparticles are directly contact to skin which is the direct path

to the epithelia and entry the systemic circulation (Wijnhoven et al., 2009). Moreover, the respiratory system represents a major port of entrance for silver nanoparticles. Sprays containing nano-silver are already on a market, indicating that this is a relevant exposure route. In addition, due to the small diameter of the silver nanoparticles, Brownian diffusion also determines deposition, resulting in a deep penetration of silver nanoparticles in the lungs and diffusion to the high lung surface area presented in the alveolar region (Wijnhoven et al., 2009).

Inevitably, it is essential to study the effect of silver nanoparticles on human cells as a precaution to prevent or avoid exposure to silver nanoparticles if it is found that the silver nanoparticles are toxic to cells. In addition, the effect of silver nanoparticles on cancer cells is also important and is particularly useful in medical treatment. Therefore, the scope of this research is to study the effect of silver nanoparticles coated with either poly (4-styrenesulfonic acid-co-maleic acid) sodium salt or alginate on normal and cancer skin human cells.

Objective

1.   Study the cytotoxicity of silver nanoparticles to human skin normal and cancer cells

2.   Investigate the mode of human skin cell death caused by the silver nanoparticles

Outcome

Information on the cytotoxicity of silver nanoparticles to skin normal and skin cancer human cell

CHAPTER II

LITERATURE REVIEWS

2.1 Nanoparticles (NPs)

Nanotechnology is an important field of modern creation and manipulation of particles ranging from 1 nm to100 nm. There are widely interesting application in a number of areas such as chemical industries, medical device, mechanics, health care, cosmetics and consumer products etc. The unique function of nanomaterial depends on their size and structure-dependent properties (Wijnhoven et al., 2009).

Nanoparticles have a high surface area to volume ratio that can interact with the environment and health. Because of silver nanoparticles are very small which increase contact and interact with tissues, organs and cell, for instance. Figure 1 show how a nanoparticles size compares to other material. According to Figure 2.1, a human hair is 100,000 times the width of a nanometer

2.2 Silver and silver nanoparticles

2.2.1 History of silver

Metallic silver has been known since ancient times as a valued metal; it is used to make jewelry, tableware and widely used in conductors. The ancient Phoenicians, Greeks, Romans, Egyptians, and others have been used silver to preserve food and water, and this was practiced through World War II. The Macedonians used silver plates wound healing, perhaps the first endeavor to prevent and treat surgical infections. Moreover, Hippocrates used silver for the treatment of abscess and to help wound healing. Medical uses of silver nitrate was mentioned in a pharmacopeia published in Rome in 69 B.C.E. (Alexander, 2009).

Silver has been used by mankind for 7000 year. It is a metallic element which white lustrous transitional found widely in the environment. Silver was used increasing of effective chemotherapeutic antibacterial and antifungal agent in wound care products, textiles, cosmetics, medical devices (bone cements, catheters, surgical sutures, cardiovascular prostheses, and dental fillings). The metal silver was used in
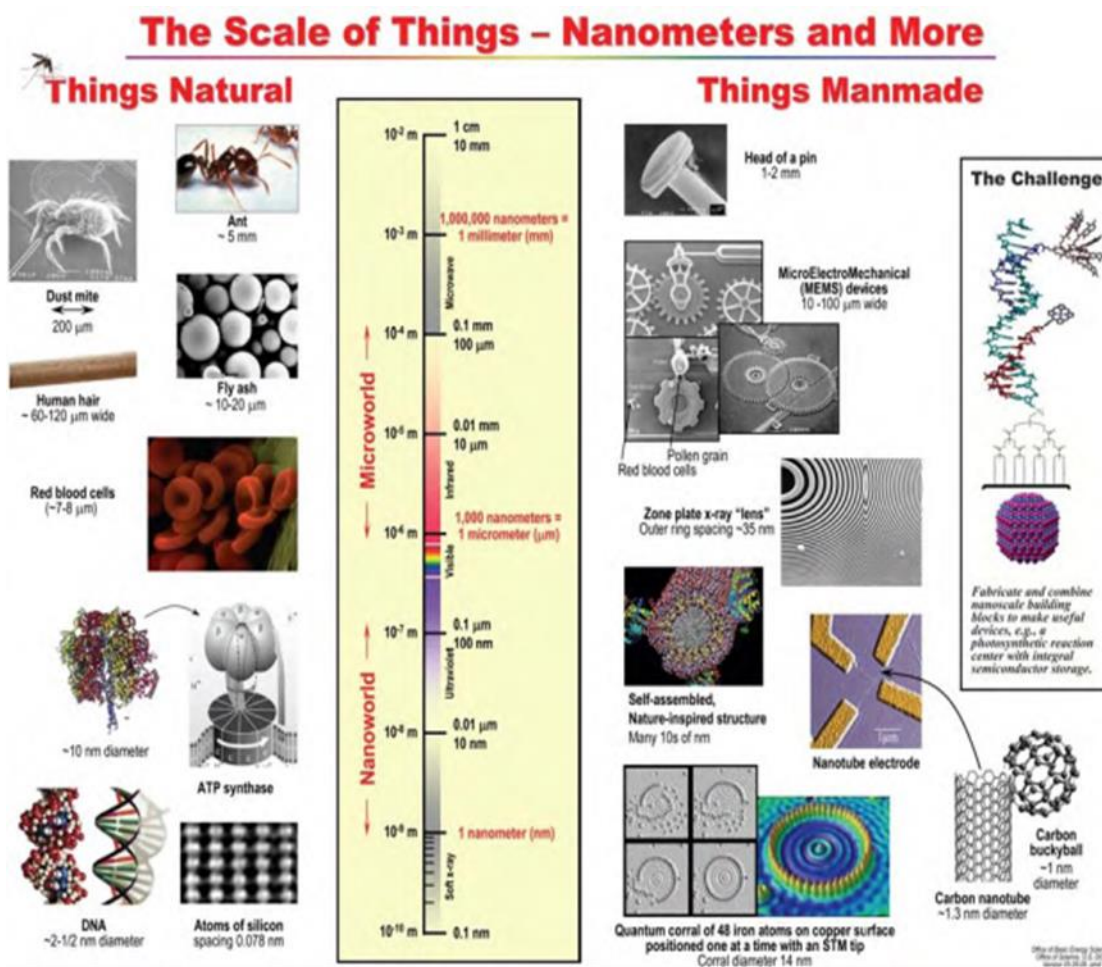
Figure 2.1 Nanomaterial dimensions on the metric scale (in nm) (EI-Badawy, 2010)

many shapes, including vessels or containers for liquid, coins, shavings, foils, sutures, solutions (e.g., nitrate, oxide, bromide, chloride, and iodide), colloids providing fine particles, and electric colloids. Electric colloids of silver became the main stay of antimicrobial therapy in the first part of the 20th Century until the introduction of antibiotics in the early 1940s (Alexander, 2009).

2.2.2 Absorption and Metabolism of silver

Metallic silver can be absorbed into the body and compete for binding sites of carrier proteins like metallothioneins, protective mechanisms of key metal-binding proteins (Idson, 1977; A. B. Lansdown, 1995; A. B. Lansdown, Sampson, & Rowe, 2001). Silver can enter the human body through inhalation, ingestion, dermal contact, and through intraparenteral insertion of medical devices. The data on the uptake of silver as a cause of argyria and silver found in raised blood from workers

exposed to silver and silver compounds over many years (DiVincenzo, Giordano, & Schriever, 1985; Drake & Hazelwood, 2005; Rosenman, Moss, & Kon, 1979; Rosenman, Seixas, & Jacobs, 1987). However, the amounts of silver absorbed were not determined (Klaassen, 1979; Wan, Conyers, Coombs, & Masterton, 1991; Williams & Gardner, 1995). The maximum capacity of silver in blood was not known but was estimate associated to albumin and macroglobulin concentrations. It was found that blood silver levels from 98 occupationally exposed workers involved in silver production and silver compound were ranged from 0.1 to23 g/l (Armitage, White, & Wilson, 1996). The absorption and elimination of silver by 37 workers exposed to silver in smelting and refining was studied which revealed that silver concentration in blood; urine and feces were 11 g/l, 0.005 g/g, and 15 g/g, respectively (DiVincenzo et al., 1985). Because silver is importantly in the feces, fecal measurement were used as an index of exposure in body burdens and expected to fecal excretion of about 1 mg of silver per day.

2.2.3 Biological action of silver

Silver in a form of cation is a soft Lewis acid which has high possibility to perturb biochemical process. The toxic effect of silver ion is due to reactions with thiol and amino groups of proteins, with cell membrane, and with nucleic acid (Brett, 2006; Choi et al., 2008; Feng et al., 2000; A. B. Lansdown, 2010; Powers, Badireddy, Ryde, Seidler, & Slotkin, 2010). Normally, eukaryotic cells easily take up silver nanoparticles by macropinocytosis and endocytosis (Kittler, Greulich, Diendorf, Koller, & Epple, 2010). As all nanoparticles, silver nanoparticles are promptly coated by peel ("corona") of proteins in a biological medium. This means that corona clearly affects the interaction with cells. Silver nanoparticles, metallic silver and soluble silver salts discharge silver ion when they contact with water. Silver ion will react to sparingly soluble salt to cause precipitate or are in colloidal and will also incur complexion with proteins and biomolecules. It has been recently shown that the toxicity was obviously correlated to the amount of released silver ion and nanoparticles with sizes of 5 and 11 nm in diameter alone were not toxic up to 200 mg/l (Chernousova & Epple, 2013).

The cation of silver on single-celled organism is shown in the table 2.1. Although the results were done in different conditions in both organism and silver species, it could be concluded that inhibitory concentration was in the range of 0.1 to 20 mg/l. Silver concentration of 0.1 mg/l was quickly killed *L. pneumophila* and *P. aeruginosa* (Hwang, Katayama, & Ohgaki, 2006).

Table 2.1 The biological effect of silver on single-celled organism

Organism	Silver species	Particles diameter	Functionalization	Effect
Bacillus subtilis ATTC 6633	Ag nanoparticles	6.5-43.8 nm	not reported	MIC_{90}=6.25 mgL^{-1}; $MBC_{99.9}$=12.5 mgL^{-1}
Bacillus subtilis	Ag nanparticles	10 nm	Citrate	No growth inhibition at 50 mgL^{-1}; EC_{50}(CFU assay)>10 mgL^{-1}; EC_{50} (LTP assay; 1h)=0.06-0.09 mgL^{-1}; EC_{50}(LTP assay; 2 and 3 h)<0.025mgL^{-1}
Bacillus subtilis	Ag_2S nanoparticles	9±3.5 nm	unfunctionalized	not toxic at 150 mgL^{-1}
Candida albicans I	Ag^+	-	-	MIC=0.42 mgL^{-1}
Candida albicans II	Ag nanopaarticles	not reported	unfunctionalized	MIC=0.21 mgL^{-1}
Candida albicans II	Ag nanopaarticles	25 nm	PVP	MIC= 0.21 mgL^{-1}
Candida parapsilosis	Ag nanoparticles	25 nm	PVP	MIC= 0.84 mgL^{-1}
Candida tropicalis	Ag^+	-	-	MIC= 0.84 mgL^{-1}
Escherichia coli ATTC 117	Ag nanoparticles	6.5-43.8 nm	not reported	MIC= 6.25 mgL^{-1}; $MBC_{99.9}$= 12.5 mgL^{-1}

Table 2.1 (Continued)

Organism	Silver species	Particles diameter	Functionalization	Effect
Escherichia coli DH5α	Ag nanoparticles	75±20 nm	PVP	MBC= 12.5-20 mgL^{-1} for 10^3 cell at cultivation in RPMI/FCS
Escherichia coli	Ag nanoparticles	10 nm	citrate	30 and 50 mgL^{-1} inhibit bacteria growth EC$_{50}$ (CFU assay)=3.2-4.2 mgL^{-1}; EC$_{50}$ (LTP assay 0 h.)>0.25 mgL^{-1}; EC$_{50}$ (LTP assay ; 2 and 3 h)<0.025 mgL^{-1}
Escherichia coli	Ag$_2$S nanoparticles	9±3.5 nm	unfunctionalized	not toxic at 150 mhL-1
Escherichia coli	Ag nanoparticles	7 nm	gallic acid	MIC= 6.25 mg/L^{-1}
Escherichia coli	Ag nanoparticles	29 nm	gallic acid	MIC= 13.02 mgL^{-1}
Escherichia coli	Ag nanoparticles	89 nm	gallic acid	MIC= 11.79 mgL^{-1}
Proteus vulgaris NCIB 4157	Ag nanoparticles	6.5-43.8 nm	not reported	MIC$_{90}$=6.25 mgL^{-1}; MBC$_{99.9}$=12.5mgL^{-1}
Pseudomonas aeruginosa ATTC 33152	Ag nanoparticles	6.5-43.8 nm	not reported	MIC$_{90}$=6.25 mgL^{-1}; MBC$_{99.9}$= 12.5 mgL^{-1}
nitrifying bacteria	Ag nanoparticles	14-16 nm	PVA	1 mgL^{-1} inhibits respiratory activity by 86±3 %
Salmonella typhimurium ATCC 23564	Ag nanoparticles	6.5-43.8 nm	not reported	MIC$_{50}$ = 6.25 mgL^{-1}
Staphylococcus aureus	Ag nanoparticles	29 nm	gallic acid	MIC = 7.5 mgL^{-1}
Staphylococcus aureus ATCC 6538	Ag nanoparticles	6.5-43.8 nm	not reported	MIC$_{90}$=12.5 mgL^{-1}

Source: (Chernousova & Epple, 2013)

The biological effect of silver and silver nanoparticles on eukaryotic cells in vitro was shown in the table 2.2 the toxic concentrations of silver ion and silver nanoparticles were in the range of 1-10 mg/l and 10-100 mg/l for respectively. Silver ion can interact with complexion of viruses.

Table 2.2 The biological effect of silver on eukaryotic cell in vitro

Organism	Silver species	Particles diameter	Functionalization	Effect
alveolar epithelial cells A549 (adenocarcinome, human)	Ag^+	-	-	mitochondrial function was reduced at 4-10 mgL^{-1}
alveolar epithelial cells A549 (adenocarcinome, human)	Ag^+	-	-	after 24 h, the cells morphology was changed at 3.24 mgL^{-1}
alveolar epithelial cells A549 (adenocarcinome, human)	Ag nanoparticles	30-50 nm	PVP	Mitochondrial function was reduced at 10-20 mgL^{-1}; necrosis/apoptosis at 2.5-15 mgL^{-1}
alveolar epithelial cells A549 (adenocarcinome, human)	Ag nanoparticles	82±1 nm	glucose	increase of the vitality at 7.5 and 15 mgL^{-1} (1st -3rd day); toxic at 7.5 and 15 mgL^{-1} (after 4 day); toxic at 30 mgL^{-1} (after 2 days)
alveolar epithelial cells A549 (adenocarcinome, human)	Ag nanoparticles	95±1 nm	glucose and oligonucleotide	toxic at 7.5, 15 and 30 mgL^{-1} (after 4 day)

Table 2.2 (Continued)

Organism	Silver species	Particles diameter	Functionalization	Effect
alveolar epithelial cells A549 (adenocarcinome, human)	Ag nanoparticles	99±1 nm	lactose and oligonucleotide	toxic at 7.5, 15 and 30 mgL^{-1} (after 4 day)
embryonal stem cells D3 (mouse)	Ag nanoparticles	20 nm	not reported	EC_{20} (WST test)=21 mgL^{-1}; EC_{20} (LDH assay)=3 mgL^{-1}
embryonal stem cells D3 (mouse)	Ag nanoparticles	80 nm	not reported	EC_{20} (WST test)=31 mgL^{-1}; EC_{20} (LDH assay)=33 mgL^{-1}
epithelial cells Hela S3 (human)	Ag nanoparticles	113 nm	not reported	EC_{20} (WST test)=29 mgL^{-1}; EC_{20} (LDH assay)=43 mgL^{-1}
epithelial cells Hela S3 (human)	Ag^+	-	-	cytotoxic at 12 mgL^{-1}; IC_{50} at 17 mgL^{-1}
epithelial cells Hela S3 (human)	Ag nanoparticles	2-5 nm	not reported	cytotoxic at 80-120 mgL^{-1}; IC_{50} at 92 mgL^{-1}
fibroblasts L929 (mouse)	Ag nanoparticles	20 nm	not reported	EC_{20} (WST test)= 2.8 mgL^{-1}; EC_{20}(LDH assay)=0.2 mgL^{-1}
fibroblasts L929 (mouse)	Ag nanoparticles	79±1 nm	unfunctionlized	toxic at 15 and 30 mgL^{-1}
fibroblasts L929 (mouse)	Ag nanoparticles	82±1 nm	glucose	increase of the viability at 7.5, 15 and 30 mgL^{-1} (1st-2nd day); toxic at 30 mgL^{-1}(after 3 day)

Table 2.2 (Continued)

Organism	Silver species	Particles diameter	Functionalization	Effect
fibroblasts L929 (mouse)	Ag nanoparticles	88±1 nm	lactose	increase of the viability at 7.5, 15 and 30 mgL^{-1} (1st-2nd day); toxic at 30 mgL^{-1} (after 3 day)
hepatocellular carcinoma cells Hep G2 (human)	Ag nanoparticles	6.5-43.8 nm	not reported	IC$_{50}$251 mgL^{-1}
hepatocellular carcinoma cells C3A (human)	Ag nanoparticles	35 nm	unfunctionalized	cytotoxicity ca. 90% at≥ 100 mgL^{-1}
hepatocytes (primary, fish)	Ag nanoparticles	35 nm	unfunctionalized	cytotoxicity ca. 30% at≥ 800 mgL^{-1}
Testicular cells (primary, C57BL6 mouse)	Ag nanoparticles	20 nm	BSA	metabolism reduced by 50% at 10 mgL^{-1}
lung cells HLF (human)	Ag nanoparticles	25 nm	PVP	toxic at 62.5 mgL^{-1}
lung cells HLF (human)	Ag nanoparticles	35 nm	PVP	toxic at 62.5 mgL^{-1}
lung cells HLF (human)	Ag nanoparticles	45 nm	PVP	toxic at 62.5 mgL^{-1}
lung cells HLF (human)	Ag nanoparticles	60 nm	PVP	toxic at 125 mgL^{-1}
macrophages U937 (human)	Ag nanoparticles	4 nm	PVP	cell viability 36% at 3.12 mgL^{-1}
macrophages U937 (human)	Ag nanoparticles	20 nm	PVP	cell viability 6% at 25 mgL^{-1}
macrophages U937 (human)	Ag nanoparticles	100 nm	PVP	cell viability 100% at 25 mgL^{-1}
mesenchymal stem cells (human)	Ag^{+}	-	-	toxic at 2.5 mgL^{-1}

Table 2.2 (Continued)

Organism	Silver species	Particles diameter	Functionalization	Effect
macrophages THP-1 (human)	Ag nanoparticles	20 nm	peptide	IC_{50}(24 h)=110 mgL^{-1} IC_{50}(48 h)= 18 mgL^{-1}
macrophages THP-1 (human)	Ag nanoparticles	40 nm	peptide	IC_{50}(24 h)=140 mgL^{-1} IC_{50}(48 h)= 30 mgL^{-1}
mesenchymal stem cells (human)	Ag nanoparticles	75±20 nm	PVP	toxic at 50 mgL^{-1}
monocytes (human)	Ag$^+$	-	-	toxic at 1 mgL^{-1}
monocytes (human)	Ag nanoparticles	75±20 nm	PVP	toxic at 30 mgL^{-1}
adrenal medulla cells PC-12 (rat)	Ag nanoparticles	46±8 nm	not reported	cell viability ca. 60% at 10 mgL^{-1} after 72 h.
preosteoblasts MC3T3-E1 (mouse)	Ag nanoparticles	8.6±3.2 nm	not reported	cell viability ca. 70% at 10 mgL^{-1} after 72 h.

Source: (Chernousova & Epple, 2013)

The effect of silver on higher organism is shown in the table 2.3. The lethal concentration of all listed of 0.1-13 mg/l. This concentration stressed only is decent for sea-living organism. Because of colloidal particles when dispersed in water will likely change to release silver ion.

Table 2.3 Biological effect of silver on multicellular organism in vivo

Organism	Silver species	Particles diameter	Functionalization	Effect
Capoeta fusca (fish)	Ag^+	-	-	LC_{50} = 0.014±0.013 mgL^{-1}(24-96 h)
Danio rerio (zebrafish)	Ag^+	-	-	LC_{50} = 28 mgL^{-1}(24 h) LC_{50} = 25 mgL^{-1}(48 h)
Aedes aegypti	Ag nanoparticles	3-21 nm	not reported	LC_{50} (II. Larval instar)= 1.29±0.09 mgL^{-1}; LC_{90} (II. Larval instar)= 3.08±0.21 mgL^{-1}; LC_{50} (II. Larval instar)= 1.48±0.09 mgL^{-1}; LC_{50} (II. Larval instar)= 1.29±0.09 mgL^{-1}; LC_{50} (II. Larval instar)= 1.58±0.07 mgL^{-1}; LC_{90} (II. Larval instar)= 3.41±1.23 mgL^{-1}
Danio rerio (zebrafish embryo)	Ag nanoparticles	20 nm	PVP	Lethal for ca. 16% at 2.5 mgL^{-1}; for ca. 17% at 5 mgL^{-1}
Danio rerio (zebrafish embryo)	Ag nanoparticles	10 nm	PVP	Lethal for ca. 12% at 2.5 mgL^{-1}; for ca. 22% at 5 mgL^{-1}
Danio rerio (zebrafish embryo)	Ag nanoparticles	40 nm	PVP	Lethal for ca. 16% at 2.5 mgL^{-1} and 5 mgL^{-1}

Table 2.3 (Continued)

Organism	Silver species	Particles diameter	Functionalization	Effect
Danio rerio (zebrafish embryo)	Ag nanoparticles	32 nm	PVP	Lethal for ca. 34% at 2.5 mgL^{-1}; for ca. 42% at 5 mgL^{-1}
Daphnia magna (crustacean)	Ag^{+}	-	-	LC$_{50}$=0.4± 0.12 µgL^{-1} (24 h)
Daphnia magna (crustacean)	Ag nanoparticles	5-25 nm	citrate	EC$_{10}$=3 µgL^{-1} EC$_{50}$= 5 µgL^{-1}
Daphnia magna (crustacean)	Ag nanoparticles	35 nm	PVP	LC$_{50}$=10.6±5.2 µgL^{-1} (24 h)
Daphnia magna (crustacean)	Ag nanoparticles	36 nm	citrate	LC$_{50}$=3-4 µgL^{-1}
Daphnia magna (crustacean)	Ag nanoparticles	40 nm	citrate	LC$_{50}$=1.8±0.96 µgL^{-1} (24 h)
Drosophila melanogaster (fruit fly)	Ag nanoparticles	29±4 nm	maltose	acute toxicity at 20 mgL^{-1}; effect on fertility at 5 mgL^{-1}
Drosophila melanogaster (fruit fly eggs)	Ag nanoparticles	20-30 nm	not reported	57±48% of the eggs reached the adult stadium at 10 mgL^{-1}
Mouse C57Bl/6	Ag nanoparticles	5±2 nm, 22±4 nm	not reported	inhalation of 3.3 mg m^{-3} Ag nanoparticles for 40 h induced minimal lung toxicity and inflammation.
Nereis diversicolor (ragworm)	Ag^{+}	-	-	1250 ng Ag^{+} per worm for 10 days were given. The silver was bound by metallothioneins.

Table 2.3 (Continued)

Organism	Silver species	Particles diameter	Functionalization	Effect
Hartley albino guinea pig	Ag nanoparticles	<100 nm	not reported	acute dermal toxicity (10 mg mL^{-1}): no change in the weight of organs and no macroscopic changes; histopathologic anomalies in skin, liver and spleen; subchronic dermal toxicity (10 mg mL^{-1}, 5 times per week for 13 weeks): histopathologic anomalies in skin, liver, and spleen
Pimephales promelas (fish embryo)	Ag nanoparticles	35 nm	not reported	LC_{50}= 9.4 mgL^{-1}
Mouse (balb/c)	Ag microparticles	<20 mm	sodium hyaluro-nate	1.18 mg Ag were injected into the brain. After 9 months, neural inflammation and tissue loss in the brain.
Pimephales promelas (fish embryo)	Ag nanoparticles	35 nm	not reported	LC_{50}= 9.4 mgL^{-1}
Ulva lactuca (makroalga)	Ag+	-	-	toxic at 2.5 μgL^{-1}

Source: (Chernousova & Epple, 2013)

However, the information in table 2.1-2.3 do not clearly show that the trend in toxicity of silver nanoparticles as a function of the particle size. This is due to the differences in the species, functionalization charge of the nanoparticles and the types of the biological system. It is also difficult to explain the relationship between the particle morphology and the biological effect. Only a few studies have been published. In addition, the release of silver ion, silver nanoparticles and silver compound depends on the kinetic of cellular uptake which is an important role. The coalescence in biological media is also influence the bioavailability (Kittler et al., 2010; Teeguarden, Hinderliter, Orr, Thrall, & Pounds, 2007).

2.3 Silver nanoparticles

Silver nanoparticles are of most interest when compared to other nanomaterials due to silver nanoparticles have been popularly used for theirs antimicrobial properties in a large number of consumer and medical products (X. Chen & Schluesener, 2008; Kamyshny, Ben‑Moshe, Aviezer, & Magdassi, 2005). Silver has been suggested to be a therapeutic agent for a long time in the management of open wound and burns. Moreover, silver (and its ions and compounds) is efficient in killing about 650 types of the disease-causing microorganism (Raffi et al., 2010).

2.3.1 Uses of silver nanoparticles

Silver nanoparticles are pored to be an effective antibiotics against both Gram negative and Gram positive bacteria (Alt et al., 2004; J. S. Kim et al., 2007). They also act as a fungicidal agent against Aspergillus, Candida and Saccharomycees (Wright, Lam, Hansen, & Burrell, 1999). Importantly, they are also effective against some antibiotic resistant bacteria such as vancomycin and methicillin resistant bacteria (R. Burrell et al., 1999; Percival, Bowler, & Dolman, 2007; Yin et al., 1999). Because of their anti-microorganism properties, they are applied to be used in many products.

2.3.1.1 Silver nanoparticles in medical, personal care and consumer product

Silver has been used in medical for a long period. In hospital, it was used extensively for wound management in 18th century, especially for cure of burns and various ulcers such as Diabetic ulcers, rheumatoid arthritis-associated (A. Lansdown,

2007). It is also used as a coating agent for medical devices in order to prevent bacteria-associated problems such as colonization, biofilm formation and bacterial adhesion (EI-Badawy, 2010).

Table 2.4 Medical use of silver nanoparticles

Medical domains	Examples
Wound care	Hydrogel for wound dressing
Surgery	Coating of hospital textile (face mask, surgical gowns)
Anesthesiology	Coating of breathing mask Coating of endotracheal tube for mechanical ventilatory support
Dentistry	Additive in polymerizable dental materials silver-loaded Sio_2 nanocomposite resin filler
Orthopedics	Additive in bone cement Implantable material using clay-layers with starch-stabilized silver nanoparticles coating of implant for joint replacement
Drug delivery	Remote laser light-induced opening of microcapsules
Cardiology	Coating of driveline for ventricular assist devices
Diagnostics	Nano-silver Pyramids for enhanced biodetection Ultrasensitive and Ultrafast platform for clinical assays for diagnosis of myocardial infarction
Eye care	Coating of contact lens

Source: (Wijnhoven et al., 2009)

The main purpose of nano-packaging is to carry longer shelf-life by enhancement the barrier functions of packaging and to reduce gas, exchange moisture and exposure of UV light (Sorrentino, Gorrasi, & Vittoria, 2007). Nano-packaging can be designed to release biocide response to the growth of microbial population. Several nanomaterials such as gold, nickel, zinc oxide, titanium dioxide

and silica are used in many consumer products. Among these nanoparticles silver nanoparticles have been reported to be the most widely used particles in the highest number of products (233 consumer products and 33 food products) (Wijnhoven et al., 2009). The silver nanoparticles are used in the purification of drinking water and in cleaning of water in swimming pool. In addition, they are used in textile products such as apparel, towel and swimwear.

Moreover, they are used in cosmetics such as face mask, skin whitener and cream. Besides silver, other noble metals, for example gold and platinum, are also used. The metal contents in some products were measured by atomic absorption spectroscopy as shown in table 2.5 (Chernousova & Epple, 2013).

Table 2.5 Noble metal concentration in some cosmetics, determined by atomic absorption spectroscopy

Sample	Noble metal concentration (PPM)	Packaging size (mL)	Noble metal per package (mg)
Silver toothpaste	0.1	75	0.0075
Silver shower gel	2.7	200	0.54
Silver hand cream	2700	75	202.5
Silver deodorant (roller)	950	50	47.5
Gold night cream	2.4	50	0.12
Platinum anti-wrinkle cream	<15	50	<0.75

Source: (Chernousova & Epple, 2013)

2.3.2   Exposure to silver nanoparticles

Since, silver nanoparticles are presented in many consumer products, there is several ways that they can enter to human body and affect human health.

2.3.2.1 Pulmonary exposure

It is possible that the silver nanoparticles can enter the body by inhalation. The pulmonary retention and distribution of inhale silver nanoparticles was investigated. Rats were exposed to silver nanoparticles (4-10 nm in size) for 6 h and the amount of the nanoparticles was measured after the exposure. It was demonstrated that silver was present in lungs post exposure (1.7 µg) and decreased with time to 4% after exposure to silver within 7 days (Takenaka et al., 2001).

Hyun et al. (Hyun et al., 2008) examine the exposure to silver nanoparticles (13-15 nm) on the olfactory respiratory mucosa of rats by exposing the rat in inhalation chamber for 6 hours per day, 5 time a week, for 28 weeks at low (0.5 µg/m3), medium (3.5 µg/m3) and high (61 µg/m3) concentration of silver nanoparticles. They found that silver nanoparticles did influence the neutral mucins in the respiratory mucosa.

Sung et al. (Sung et al., 2009) investigated the inhalation of silver nanoparticles (18-19 nm) by inhalation study 6 hours/day at low (49 µg/m3), medium (133 µg/m3) and high (515 µg/m3) doses. The exposure of all concentrations of silver nanoparticles demonstrates inflammatory response within alveoli and induces differentiation in lung function. They indicated that silver was increasing the blood and transferred into the circulation from lung. Silver was accumulated within the liver, olfactory bulb, brain and kidneys. These results were evidence that nasal inhalation can cause an accumulation of silver.

2.3.2.2 Dermal exposure

Currently, silver nanoparticles are applied and used in many skin care and treatment products. Therefore, they are inevitably come into contact with skin. An example of the most widely used products is an antimicrobial wound dressing. The ability of silver nanoparticles in wound dressing for the treatment of skin burn was studied by using thermal injury mouse model (Johnston et al., 2010).

The recovery of burn wound in mice occurred more rapidly when treated with silver nanoparticles as compare to silver sulfadiazine which is generally used in

burn treatment. The appearances of wound look better with silver nanoparticles and with minimum scarring obvious. Moreover, silver nanoparticles can heal the wound better than antibiotics amoxicillin and metronidazole. These indicate that silver nanoparticles exhibited more potent antibacterial and their ability to improved wound healing. These might be due to the reason that silver nanoparticles improve cytokine production to reduce inflammation burn which was confirmed by the reduction of neutrophil infiltration within the wound (Tian et al., 2007).

The availability of silver nanoparticles from Actocoat, a commercial wound dressing in 30 burn patients was investigated (Vlachou et al., 2007). In this study, the patients were treated with Acticoat which was changed every nine days of treatment, the maximum concentration of silver was found at 56.8 µg/ml. moreover, it was found that the level of silver increased with respect to the burn size. This result suggested that the silver nanoparticles can transfer from the wound dressing to the patient skin. Another study of wound dressing containing silver nanoparticles was performed by Trop et al. (Trop et al., 2006) They showed that the burn wounds were healed quickly with the dressing. However, discoloration of skin to gray color called argyria was observed. The change in color is a regular side effect due to the silver deposition in the skin. This could be due to the reason that silver stimulates the melanocyte of the skin (Chang et al., 2006). Figure 2.2 show an example of conjuctival-corneal argyrosis in the craftsman occupationally exposed to silver.
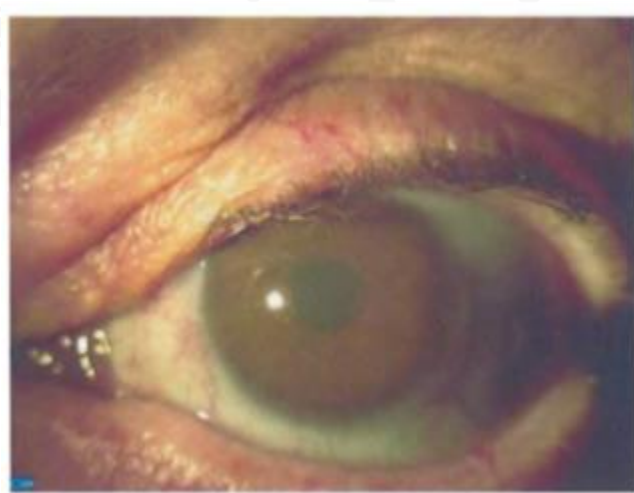
Figure 2.2 Conjuctival-corneal argyrosis in the craftsman occupationally exposed to silver (El-Badawy, 2010)

2.3.2.3 Oral exposure

Besides accidentally consumption of silver nanoparticles, the particles contained in the food packaging may contaminants the food, resulting in an unintentially consumption of the particles. The effect of silver nanoparticles after ingestion was studied by direct delivery of the particles into the stomach of mice. Since, the liver is the organ where most drug metabolism occurred, the liver tissure was examined after exposure to the particles for 3 day. The silver was considered especially because it involved in drug metabolism. It was found that there was inflammation of the lymphocyte associated with the change in the gene expression of four genes (Cha et al., 2008).

In another study, the toxicity of silver nanoparticles (60 nm in size) after oral exposure of rats at high (1000 mg/kg/day), medium (300 mg/kg/day) and low (30 mg/kg/day) concentration for 28 days was investigated. The particles were transfer into blood circulation and deposited in the brain, liver, kidney, lung and testes (Y. Kim et al., 2009). This indicated that silver nanoparticles can be distributed and absorbed at many secondary target sites.

2.4 Synthesis of silver nanoparticles

Synthesis of silver nanoparticles requires three chemical agents: silver nitrate, reducing agent and capping or coating substance. Silver nitrate is reduced by a reducing agent to form silver metal which is then stabilized by capping agent to prevent agglomeration of the nanoparticles (Kildeby NL, 2005). Silver nanoparticles can be synthesized by both chemical and biological methods.

2.4.1 Chemical approaches

The simplest method for synthesis of silver nanoparticles is a chemical reduction by organic and inorganic substance such as sodium citrate, ascorbate, sodium borohydride ($NaBH_4$) and elemental hydrogen. The following reaction shows a chemical reduction method by used ethanol.

$$Ag^+ (aq) \quad + \quad e^- \quad \longrightarrow \quad Ag\ (s)$$

$$2Ag^+ \quad + \quad C_2H_5OH \quad + \quad H_2O \quad \longrightarrow \quad 2Ag\ (s) + \quad C_2H_5O_2^- + \quad 3H^+$$
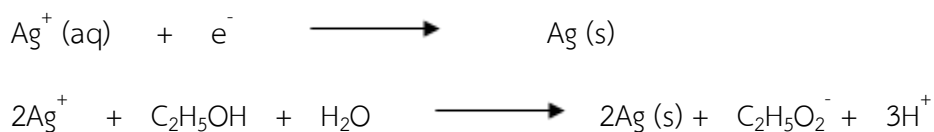
In an aqueous solution silver nitrate splits to positive silver ion (Ag+) and negative ion (NO_3^-). The positive silver ion is reduced by ethanol to metallic silver (Ag^0), which is then coalesces molecules leading to the formation of metallic colloidal silver nanoparticles (Evanoff & Chumanov, 2004; Merga, Wilson, Lynn, Milosavljevic, & Meisel, 2007; Wiley, Sun, Mayers, & Xia, 2005). It is important to use substances that protect agglomeration of silver nanoparticles. This substance is usually called capping agent or stabilizer (Oliveira, Ugarte, Zanchet, & Zarbin, 2005). The presences of surfactant with functionalities such as thiols group, amines group, acids and alcohols for interactions with particles surface, can stabilize particles cluster and prevent sedimentation or losing surface properties of particles. Polymeric compound such as poly (vinylpyrrolidone), poly (vinyl alcohol), poly (ethylene glycol) have been reported to be effective capping agent of nanoparticles (Oliveira et al., 2005).

Preparation of silver nanoparticles capped with dodecanethial followed by reduction with sodium borohydride dodecanethial bind to the nanoparticles has been reported Oliveira et al (2005). Small changes in the synthesis factors resulted in modification in structure, size, stability and self-assemble patterns. In another study, silver nanoparticles were prepared by using poly (vinyl pyrrolidone) (PVP) as surfactant (L. Li et al., 2012). It was found that the reaction temperature, reaction time, concentration of PVP and reactants were important in determining size of silver nanoparticles. Monodispersion of silver nanoparticles in a wide range from 25 to 70 nm have been successfully obtained by using different surfactant concentrations of PVP and different times of reaction.

2.4.1.1 Stabilization of the nanoparticles (Capping or coating agent)

Capping agent is the substance that controls the particle size and morphology and there acquisition could be added to prevent aggregation of the particles. The properties of the protective ligands strongly influence the particle size and the

dispersity of metal nanoparticles (Korbekandi, Iravani, & Abbasi, 2009). Differences of capping agent affect to size and stability of the particles (Korbekandi et al., 2009). Studies the synthesis of silver nanoparticles by used both dextran and polyvinylpyrrolidone (PVP) which was a natural polymer and synthetic polymer respectively as a stabilizing agent and used sodium borohydride as reducing agent. It was found that the dextran-capped silver nanoparticles ones possessed better stability (Yang et al., 2012). Various substances were used to synthesize of silver nanoparticles such as citrate, alginate, Poly (4-styrenesulfonic acid-co-maleic acid), polyvinylpyrrolidovne, glucose, alginate, chitosan, dextran and starch (Korbekandi et al., 2009).

Poly (4-styrenesulfonic acid-co-maleic acid) (Copss) is an anionic synthetic polymer has both weak (maleic acid) and strong (sulfonic acid) charge groups, in which the strong charge group (sulfonic acid) can increase the stability of the multilayers and the weak charge groups (maleic acid) can be adjusted by pH value (Quinn, Tjipto, Yu, Gengenbach, & Caruso, 2007; Tjipto, Quinn, & Caruso, 2005).
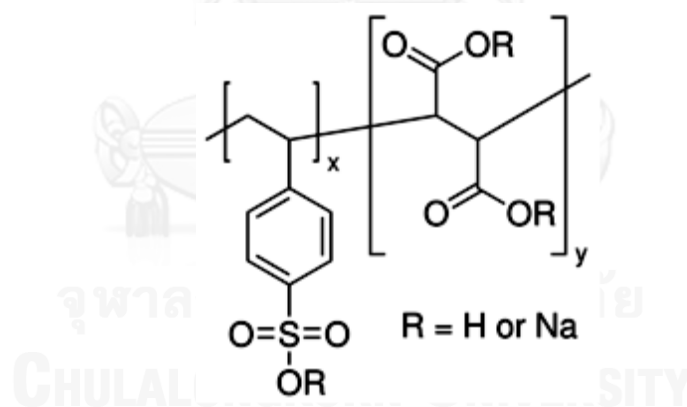
Figure 2.3 Poly (4-styrenesulfonic acid-co-maleic acid) sodium salt

Available from:

http://www.sigmaaldrich.com/catalog/product/aldrich/434558?lang=en&region=TH [2014, February 24]

Alginic acid is a natural polymer, also called align or alginate. It is an anionic polymer in polysaccharide group, where through binding with water it forms a viscous gum. It has color range from white to yellowish-brown. Alginates are polished from

brown seaweeds and have a wide use variety industry including food, textile, pharmaceutical and use for capping agent (Raymond C. Rowe 2009).

Alginic acid is a linear copolymer (figure 9) with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G) residues, respectively.

Several studies have reported the use of alginate as capping agent in the synthesis of silver nanoparticles. Dubas et al (2011) prepared silver nanoparticles capped with sodium alginate for their anti-microbial against Staphylococcus aureus in surgical sutures. They synthesized silver nanoparticles by using alginate varied from 5 mM to 0.1 mM. They found that the silver nanoparticles capped with alginate have an average size of 8 nm and they observed that the lower alginate concentration, the higher the antimicrobial efficiency.
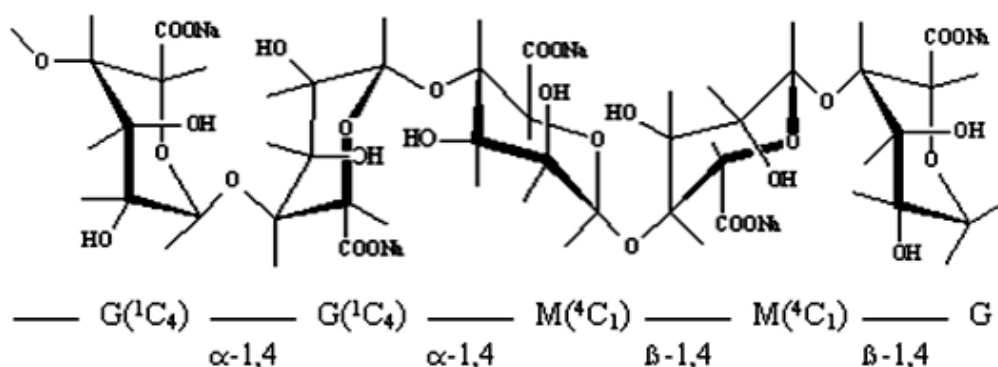
Figure 2. 4 Structural formula of alginate (Phillips et al., 1990)

2.4.2 Biological approaches

Biological methods can be used to synthesize silver nanoparticles without use of harsh, toxic and expensive substances (Ahmad et al., 2003; Ankamwar, Damle, Ahmad, & Sastry, 2005; Huang et al., 2007; Shankar et al., 2004). The biological methods concern with the use of biomolecule found in the extract from bacteria, fungi and plant (e.g., enzymes/proteins, amino acid, polysaccharide and vitamin). These extracts are capable to use as reducing and stabilizing agent in the synthesis.

2.4.2.1 Synthesis of silver nanoparticles by bacteria

It was reported that high stability of silver nanoparticles could be obtained by bioreduction of silver ion and culture supernatant of *Bacillus licheniformis* (Kalishwaralal, Deepak, Ramkumarpandian, Nellaiah, & Sangiliyandi, 2008). From research of Saifuddin et al. (Saifuddin, Wong, & Yasumira, 2009) the synthesis of silver nanoparticles was performed by using an integration of culture supernatant of *B. subtilis* and microwave irradiation methods. They reported extracellular biosynthesis of monodispersed silver nanoparticles with no aggregation of particles.

In another study, biomolecular from culture supernatant of *Klebsiella pneumonia*, *E. coli* and *Enterobacter cloacae* were used to biosynthesis by using culture supernatant reduce aqueous Ag^+ to silver nanoparticles (Shahverdi, Minaeian, Shahverdi, Jamalifar, & Nohi, 2007).

2.4.2.2 Synthesis of silver nanoparticles by fungi

The extracellular biomolecules such as proteins and amino acids from *Fusarium oxysporum* can be used to synthesize silver nanoparticles (5-50 nm). The long-term stability of nanoparticles with no aggregation due the particle capping ability of protein from *F. oxysporum* extract was obtained. These proteins can be self-assembled on citrate-reduced silver colloid surface (Macdonald and Smith 1996). Stability of the capping protein was found to be pH dependent. Nanoparticles solution remained stable at higher pH values (>12) and they had aggregation at lower pH values (<2) due to denaturation of protein (Kumar et al., 2007).

In another study, stable silver nanoparticles could be prepared by using *Aspergillus flavus*. They found that the silver nanoparticles were stable in water for more than 3 months with no aggregation due to stabilizing materials from *A. flavus* (Vigneshwaran et al., 2007).

The extracellular filtrate of *Cladosporium cladosporioides* biomass can be also used to synthesize silver nanoparticles. Proteins and polysaccharides released from *C. cladosporioides* were chargeable for the formation silver nanoparticles (Balaji et al., 2009).

2.4.2.3 Synthesis of silver nanoparticles by plants

The extract of *Camellia sinensis* (green tea) such as caffeine and theophylline, an phenolic compound, can be responsible for the formation and stabilization of silver nanoparticles. It was observed that the nanoparticles were larger and more spherical when the quantity of *C. sinensis* extract increased (Vilchis-Nestor et al., 2008).

Harris et al. (Harris & Bali, 2008) examined the limits of silver uptake in two common metallophytes, *Brassica juncea* and *Medicago sativa*. They showed that *B. juncea* and *M. sativa* can be used in the phytosynthesis of silver nanoparticles. *B. juncea* accumulated up to 12.4 wt. % silver when exposed to 1000 ppm of silver nitrate for 72 h. *M. sativa* accumulated up to 13.6 wt. % silver when exposed to 1000 ppm silver nitrate for 24 h. In both cases, TEM analysis showed the particles with an average size of 50 nm.

Pure natural composition can be used to bioreduction and stabilize silver nanoparticles. Kasthuri et al. (Kasthuri, Veerapandian, & Rajendiran, 2009)have demonstrated the use of apiin extract from henna leave to synthesize gold and silver nanoparticles. They found that the size and shape of the particles depends on the concentration of the apiin extract and the particles are stable for 3 months.

2.5 Toxicity of silver nanoparticles

Although silver nanoparticles are commonly not available at high concentration which is sufficient enough to pose a risk to human health, they have unique physical properties which could deposit a treat to human and environment health (Lee et al., 2007). Silver nanoparticles have a special characteristic such as size, surface area, solubility, chemical composites, surface chemistry and their capability to assemble that are different from bulk silver. Because silver nanoparticles have large surface area when compare with normal metal, they might be more toxic due to the activity of free silver ion released from the nanoparticles

The health impact of the silver nanoparticles in consumer products has not yet well studied. However, many studied reported that silver nanoparticles have

adverse health effect. Inhalation of silver nanoparticles leads to their rowing to olfactory bulb and translocate to the circulation system, heart, kidney, lung and liver (Oberdörster et al., 2005b; Oberdörster, Oberdörster, & Oberdörster, 2005a; Takenaka et al., 2001).

Silver nanoparticles can be translocate through the circulation lymphatic and nervous system to many organs and penetrate to cellular and tissue. This cause dysfunction of cellular and alter a redox balance toward oxidation, thus causing cell death (El-Badawy, 2010).

2.5.1 Toxicity of silver nanoparticles to microorganism

Silver is known to inhibit bacteria, but may render toxic to human cells. Concentration of silver nanoparticles that are lethal for bacteria, are also lethal for keratinocytes and fibroblasts (Poon and Burd 2004). Silver nanoparticles with the size of 1-10 nm interact with HIV-1 virus at the sulfur-bearing residues of the gp 120 glycoprotein knobs. This inhibits virus from binding to host cell (Elechiguerra et al., 2005).

Effect of silver nanoparticles on Escherichia coli was studies by focusing on the properties of the cell membrane after the treatment. The membrane was destroyed, thus causing loss of function in the ATP synthesis. This was due to the destruction of proton force and decoupling of oxidative phosphorylation (Lok et al., 2006).

2.5.2 Toxicity of silver nanoparticles to human health

Effect of silver nanoparticles on human health depend on several factors such as genetics, existing disease, exposure time, size , shape, coalescence stat of the particles. Studies in both human and animals demonstrated that inhalation of silver nanoparticles with the size larger than the size which can be eliminated by the macrophage purging mechanism can cause lung damage (Asgharian & Price, 2007; Card, Zeldin, Bonner, & Nestmann, 2008; Oberdörster, Stone, & Donaldson, 2007). Most studies have focused on the penetration of silver nanoparticles through the ingestion, skin and liver. Schematics of human body with pathways of exposure to

nanoparticles and associated diseases from epidemiological shown in figure 2.5. Diseases related with inhalation of nanoparticles such as asthma, bronchitis, emphysema and lung cancer are found. Ingestion of nanoparticles can lead to crohn's disease and colon cancers in gastrointestinal tract. Effect of nanoparticles on the circulation system relates to blood clot, arrhythmia, heart diseases and arteriosclerosis (Buzea et al., 2007).
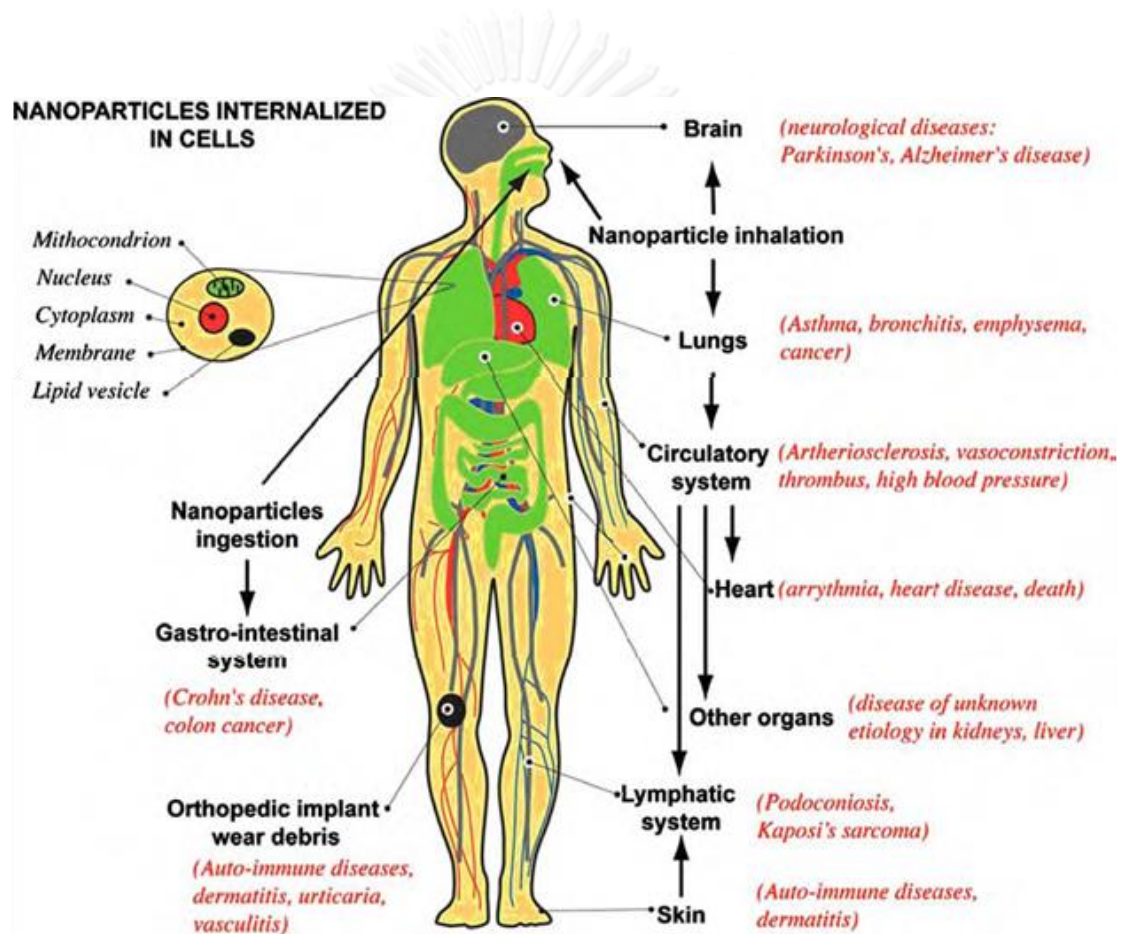
Figure 2.5 A schematic of the human body with pathways of exposure to nanoparticles and affected organs with associated diseases (Buzea, Pacheco, & Robbie, 2007)

Asharani et al. (AshaRani, Low Kah Mun, Hande, & Valiyaveettil, 2008) has demonstrated the toxicity of silver nanoparticles coated with starch (6-20 nm) to normal human lung fibroblast cell (IMR-90) and human glioblastoma cell (U251). The silver nanoparticles coated with starch were found to cause reactive oxygen species

and mitochondrial damage, leading to an interruption of ATP synthesis and DNA damage.

2.5.3 Toxicity of silver nanoparticles to ecosystem

Lee et al. (Lee et al., 2007) studied the effect of silver nanoparticles on zebrafish embryos, are found that accumulation of silver nanoparticles causes Brownian motion of embryos through chorion pore canals due to the viscosity inside the embryo. Moreover the deformities of the embryos increased with the silver nanoparticles concentration up to 0.19 nM.

The toxicity of silver nanoparticles to zebrafish was also studied. The result of transmission electron microscopy (TEM) and electron dispersive X-ray analysis (EDX) show that silver nanoparticles are accumulated in the brain, heart, yolk and blood of embryo. In addition, silver nanoparticles affect normal embryo development and are dose-dependent toxicity in embryos (AshaRani et al., 2008).

Carlson et al. (Carlson et al., 2008) examined the capability of silver nanoparticles (15, 30 and 55 nm) to induce oxidative stress in NR8383 rat alveolar macrophages. The particles at the concentration higher than 100 µg/ml increase reactive oxygen species production and lack of cellular GSH. This indicated that the silver nanoparticles caused exhibited an oxidative response. Moreover, the increase in the production of tumor necrosis alpha (TNFα), IL-1β and macrophage inflammatory protein (MIP)-2 was observed. In addition, silver nanoparticles decreased cell viability. The response was found to depend on size of the particles.

The exposure of silver nanoparticles in normal human lung fibroblasts (IMR-90) and human glioblastoma cell (U251) at different doses was studied (AshaRani et al., 2008). The uptake of silver nanoparticles emerged mostly through endocytosis, regulated by a time dependent increase in exocytosis rate. The electron micrographs showed an intercellular spreading of silver nanoparticles in cytoplasm and nucleus. Both cells exposed to silver nanoparticles exhibited chromosome instability and mitotic arrest. There was proficient revival from arrest in normal human fibroblasts whereas the cancer cells broke to proliferate.
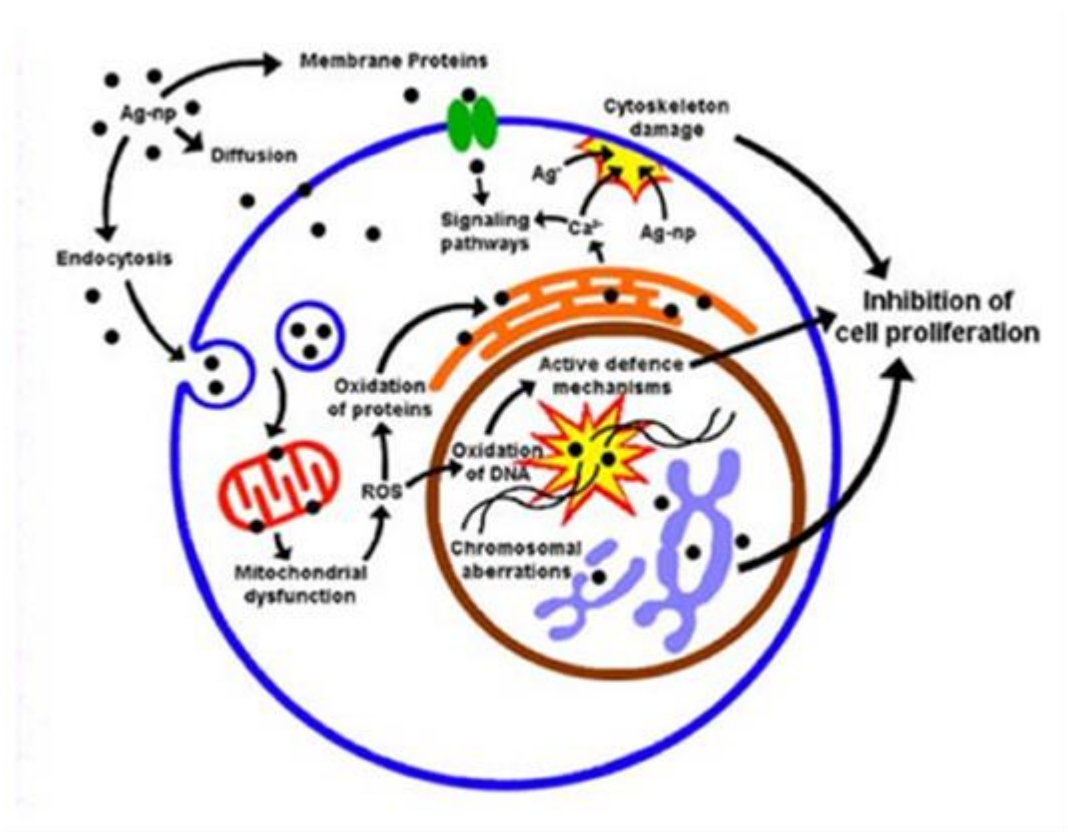
Figure 2.6 The proposed mechanism of silver nanoparticles toxicity based on the experimental data obtained in the present study (AshaRani et al., 2008)

Figure 2.6 shows the proposed Mechanism of silver nanoparticles toxicity based on the result from this study. Toxicity of silver nanoparticles is conducted through intracellular calcium (Ca^{2+}) short-lived along with significant adaptations in cell morphology, distribution and surface roughening. This affects the regulation of action binding protein. It was suggested that cancer cell are weakened to damage by induced stress, thus causing chromosomal aberration and finally apoptosis cell death.

Silver nanoparticles potentially damage mitochondrial structure by oxidative stress (Pan et al., 2009; Sun et al., 2011).This damage is caused by the loss of integrity of mitochondrial membrane permeability transition pore (PTP) leading the ROS production and cell death (N. Li et al., 2003; Xia, Kovochich, & Nel, 2006). Furthermore, it was found that nanoparticles could induce mitochondrial dysfunction

leading to increase of ROS production, decrease of mitochondrial membrane potential and induction of apoptosis (Upadhyay, Panduri, Ghio, & Kamp, 2003).

2.6 Modes of cell death

Cell death could be happen by either apoptosis or necrosis, which both cell deaths are differences of mechanism. In addition definite chemical compound and cells are called to be cytotoxic to the cell, that is, to cause its death (Studzinski, 1995).

2.6.1 Apoptosis

Apart from that to cell-cycle arrest and repair machinery, the damaged cells, where damage is beyond repair, may induce an apoptotic ("normal" or programmed cell death; PCD) response that is highly cell-specific and is the most common form of physiologic cell death in multicellular forms. Apoptosis is involves the stimulation of a set of cysteine proteases (caspases) and a complex cascade of events (Franklin, Brussaard, & Berges, 2006). Because of quick cell dehydration, cells which were formerly rounded frequently become elongated twist shape and depreciate in size. Chromatin condensation and the loss of distinct chromatin structure, which happen in semblance with cell shrinkage, begin at the nuclear rim and imitate by nuclear fragmentation. Nuclear fragment, condensed with the complement of the cytoplasm, are then pack into called apoptotic bodies, which enveloped in plasma membrane. They are phagocytosed by neighboring cells without an inflammatory response (Franklin et al., 2006).

2.6.2 Necrosis

Necrosis ("accidental" cell death) is the pathological process, which they are happen by exposure of a serious physical or chemical stimulate. It does not involve gene expression and is a passive externally driven process that results after cell death in the absence of any metabolic self-involvement. The firstly event of necrosis is swelling of cell mitochondria, plasma membrane are broken and release of cell component, which include many proteolytic enzymes. Necrosis caused inflammatory

reaction in the tissue, they are generally represents a cell response to whole injury and is often induced by an overdose of cytotoxic agents (Studzinski, 1995).

2.6.3 Morphological of apoptosis and necrosis cell death

They are difference to morphology between apoptosis cell death and necrosis cell death in many characteristic such as cytoplasmic condensation, DNA fragmentation, chromatin condensation, nuclear fragmentation and plasmic membrane blebbing; cell shrinkage and formation of apoptotic bodies was observed in apoptosis cell. While necrosis cell death has many features distinct such as loss of membrane integrity, cell swelling, formation of cytoplasmic vacuoles, swellen endoplasmic reticulum leading to inflammatory which is not found in apoptosis cell. Figure 10 shows the difference morphological between apoptosis and necrosis cell death. From the figure, apoptosis cell decrease cell volume, nuclear changes which chromatin condensation and fragmentation. Then plasma membrane blebbing occur and cause apoptotic bodies. After that apoptotic bodies are removed by the process of phagocytosis in extracellular environment. Necrosis is unintended process cause external injury and increase cell volume, loss of membrane integrity and release cellular component which consist of enzyme hydrolase and formation cell debris that cell leading to inflammation reaction (Rastogi & Sinha, 2010).
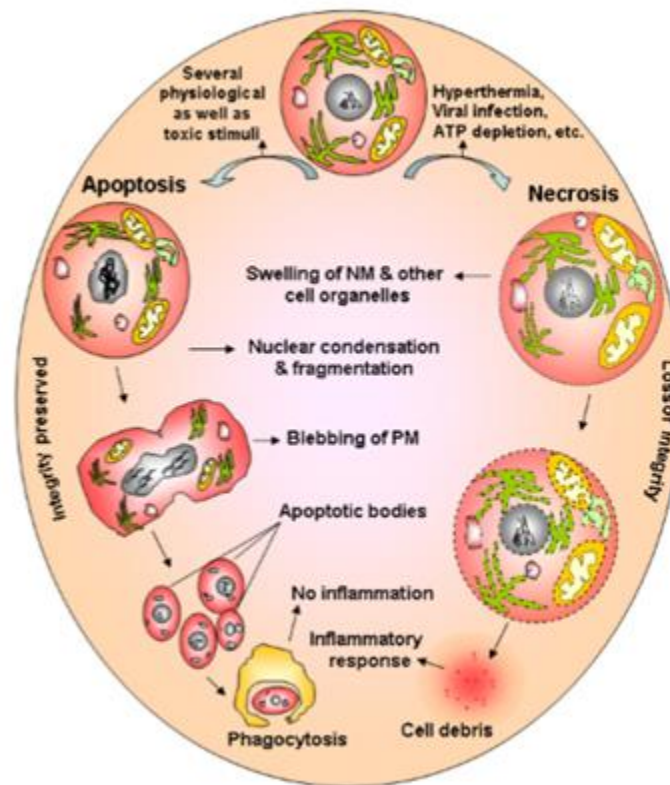
Figure 2.7 Diagrammatic illustrations showing the morphological distinctiveness occurring during apoptosis and necrosis (Rastogi & Sinha, 2010)

2.6.4 Determination of apoptosis and necrosis cell death

Flow cytometry is the appliances of investigation of cell death by both staining of Annexin V and propidium iodide. Apoptosis and necrosis cell death had distinct of cell membrane thus resulting different of staining.

2.6.4.1 Hoechst and propidium iodide staining

Hosechst and propidium cannot through life-cell but both Hosechst and propidium can through the necrosis cell death. Moreover, Hosechst can enter the cell membrane of apoptosis, leak to binding with A-T rich of DNA but propidium iodide cannot through cell membrane of apoptosis. These two compounds have the ability to emit light at different wavelength, necrotic and apoptotic cells are analyses by cell dying with them (Vermes, Haanen, & Reutelingsperger, 2000).

2.6.4.2 Annexin V staining

Membrane alteration causes a first of apoptosis cell death, normally phosphotidylserine (PS) stay in an inside of plasma membrane but they translocate to outside of plasma membrane when apoptosis cell death occurred. Phosphotidylserine was anionic phospholipids properties which can bind to Annexin V labeled with fluorochromes. So, both together staining with Annexin V and PI can be separate the different of apoptotic and necrotic cells (Van Engeland, Nieland, Ramaekers, Schutte, & Reutelingsperger, 1998).
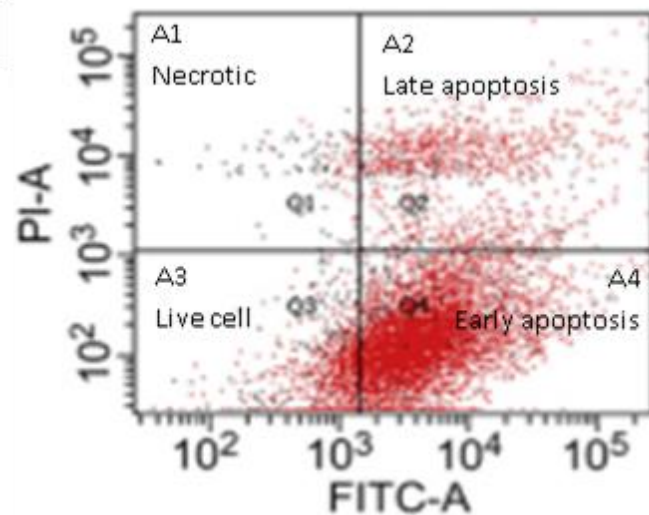
Figure 2.8 Annexin V/PI staining (L. Li et al., 2012)

The cytotoxicity of silver nanoparticles capped with polyvinylpyrrolidone (PVP) at 250 µg/ml for 72 h (70 nm) to human lung fibroblast (HLF) was investigated apoptosis and necrosis cell death by flow cytrometry which Annexin V and PI staining. It was found that percent cell were an early apoptosis, which shown in the figure 2.7 (L. Li et al., 2012).

Apoptosis and necrosis cell death after exposed with silver nanoparticles were synthesized by hydroxylamine hydrochloride to MCF-7 cell. Double staining of Hoechst and PI as well as from Annexin V FLUOS staining was detected. It was found

that silver nanoparticles at 120 µg/ml induced necrotic MCF-7 cell death (Ciftci, TÜRK, TAMER, Karahan, & Menemen, 2013).

CHAPTER III

MATERIALS AND METHODS

3.1 Material

3.1.1 Chemical

Roswell Park Memorial Institute medium (RPMI1640)	Biochrom
Iscove Basal medium (IBM)	Biochrom
Fetal calf serum (FCS)	Biochrom
Trypsin	Sigma
3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrasolium bromide (MTT)	Bio Basic INC
Silver nitrate (AgNO$_3$) 99.8 %	Carloerba reagents
Sodium borohydride (NaBH$_4$)	Fisher Scientific
Sodium alginate	Aldrich Chemistry
Poly (4-styrenesulfonic acid-co-maleic acid) - sodium salt (Copss)	Aldrich Chemistry
Alexa fluor 488 annexin V/Dead Cell Apoptosis Kit- with Alexa fluor 488 annexin V and PI	Invitrogen
Glycine	Sigma
Dimethyl Sulfoxide (DMSO)	ACI labscan limited
Doxorubicin hydrochloride 98.0-102.0 % (HPLC)	Sigma

3.1.2 Instrument

96-well plate	Corning, NY
Flask 25 Filt	Nunc, Denmark
Centrifuge machine	Universal 320
Centrifuge machine	5424R
Centrifuge machine	Kubota KR 20000T
Light Microscope	Nikon TMS
Incubator	Thermo scientific
UV-visible spectrophotometer	Thermos cientific Multskaan FC
Transmission Electron Microscopic (TEM)	H-765 Hitachi
	at an operating voltage of 100 kV
Zetasizer	Malvern Instrument, UK
Flow cytometry	Beckman coulter FC 500 MPL
Inverted Fluorescent microscope	Olympus DP71
Laminar flow Cabinet Model HS 124	ISSCO
Haemocytometer	Boeco

3.1.3 Cell line and medium

Cells line were obtained from the American Type Culture Collection (Rockville, MD)

- A375 (Human skin malignant melanoma cell line (Skin cancer cell)

  ATTC no. CRL-1619)

- CCD-986SK (Human skin fibroblast cell line (Skin normal cell)

  ATTC no. CRL1947)

3.2 Methods

3.2.1 Preparation of silver nanoparticles

In this experiment, sodium borohydride ($NaBH_4$) was used for the chemical reduction of silver salt into silver nanoparticles. Poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (Copss) and sodium alginate was used for capping agent. The synthesis of particles can be described as follow; 10 ml of 3.8 mM silver nitrate ($AgNO_3$) was mixed with 10 ml of various concentration of alginate and copss (0.04-4.56 mM) and then 10 ml of various concentration of sodium borohydride (3.8, 19 and 38 mM) which prepared freshly was rapidly added into the mixture solution and stirred continuously 5 minutes for reduce silver ion and kept overnight at room temperature to obtain a yellow solution of silver nanoparticles. The solution was centrifuged at 35,000 x g for 30 minutes to separate the particles. The pellet of capped silver nanoparticles was washed with distilled water and centrifuged again to remove excess chemical for further use.

3.2.2 Characterization of silver nanoparticles

The synthesis of silver nanoparticles was confirmed by verification surface plasmon absorbance band with a UV-vis spectrophotometer. The morphology of silver nanoparticles was visualized using a transmission electron microscopy (TEM) and determined size distribution of the particles by Image J analysis. The zeta potential of the particles was measured using a zetasizer.

3.2.3 Cell culture and treatment condition

Skin cancer cell (A375) were cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplement with 5 % fetal calf serum (FCS). While skin normal cell (CCD-986SK) were cultured in Iscove basal medium (IBM) supplement with 10 % fetal calf serum (FCS). Cells cultured were incubating at 37 $^{\circ}$C in a 5 % CO_2 atmosphere. After 24-48 h incubation period, the attached cell was trypsinized by trypsin 0.25% (1-2 ml) for 3-5 minutes and centrifuges a 380 x g for 5 minutes. The cell were counted and distributed in 96-well plates at 5×10^3 cell/well for further treatment.

Both normal cell and cancer cell lives was treated with different concentration (0-600 µg/ml) of silver nanoparticles capped with both (0.228, 1.14 and 4.56 mM) alginate and Copss and with different concentration of capping agent and silver nitrate alone. The all samples were diluted by serial dilution of culture media. The cells were counted and distributed in 96-well plates at 5×10^3 cell/well and incubate for 24 h. After that culture medium were remove from 96-well plates and added 200 µl, serial dilution of silver nanoparticles, silver nitrate and capping agent for each well. The plate was incubating 24-72 h at 37 $^{\circ}$C in 5 % CO_2 atmosphere.

3.2.4 Cytotoxicity test by MTT assay

Cytotoxicity was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrasolium bromide (MTT), a mitochondrial-based cell viability assay. This assay is a colorimetric assay for measuring the activity of enzyme that reduce MTT to produce a blue formazan dyes by viable cells and give a purple color, which allows to assess the viability and the proliferation of cells. The procedure is as following: 5×10^3 cells/well was plate in 96-well plate and, after 24 h treated with different concentration of silver nanoparticles. The plate was incubated 24-72 h at 37 $^{\circ}$C in 5 % CO_2 atmosphere. After that cell was incubated with 10 µl of 5 mg/ml MTT solution for 4 h at 37 $^{\circ}$C in 5 % CO_2 atmosphere, the MTT solution was discarded and 25 µl of glycine (pH 10.5), 150 µl of DMSO was added to each well. Optical density was read on a microplate reader at 540 nm. The percentage of cell viability was calculated according to the following formula.

$$\text{The \% of cell viability} = \frac{\text{OD of treated cell} \times 100}{\text{OD of control cells}}$$

The IC_{50} values were obtained by plotting the percentage of cell viability versus the concentration.

3.2.5 Detection of apoptosis and necrosis

Annexin V/Propidium iodide staining was performed to distinguish apoptosis from necrotic cell death induced by silver nanoparticles. Annexin V has a high affinity for phosphotidylserine, which is translocated from the inner to the outer of the plasma membrane at an early stage of apoptosis. Annexin V was conjugated with the fluorescent probe FITC measurement by flow cytometric analysis. Using propidium iodide (PI) staining helps distinguish necrosis from apoptosis due to the different in permeability of through the cell membranes of damaged and live cell. The detection methods were as follows. Cells were harvested after treatment with silver nanoparticles for 72 h. The cells washed with cold phosphate buffered saline (PBS) and centrifuge at 4° C, 3000 rpm for 5 minutes. Then remove supernatant and added 100 µl, 1x annexin V binding buffered. And then the cells was stained with 5 µl Alexa Fluor 488 annexin V and 1 µl propidium iodide (PI) at room temperature for 15 minutes, and then added with 400 µl 1x annexin V binding buffered, mixed gently and keep the samples on ice. Analyze the stained cells by flow cytometry, measuring the fluorescence emission at 530 nm and 575 nm (or equivalent) using 488 nm excitation.

3.2.6 Statistical analysis

All data were presented as means ± standard deviation (S.D) of three independent experiments. Data shown in the figure were a representation set of experiment. Different among group was analyzed by one way analysis of variance (ANOVA) followed by tukey test for multiple comparisons. The level of statistical

significant was set as p values <0.05. All data analyzed by SPSS program version 19.0 (Network licensa purchased by Chulalongkorn University.

CHAPTER IV

RESULTS AND DISCUSSION

In this study, silver nanoparticles (AgNPs) were prepared by using silver nitrate as the silver ion source, sodium borohydride ($NaBH_4$) as the reducing agent and either alginate or poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (Copss) as the stabilizing agent (also called coating agent or capping agent). Various concentrations of $NaBH_4$ and alginate or Copss were optimized to prepare AgNPs. The obtained AgNPs were characterized by TEM, UV-Vis spectroscopy and zeta potential measurement. Then, the cytotoxicity of the AgNPs to human skin normal and cancer cells was tested. In addition, program of cell death (apoptosis or necrosis) was also investigated.

4.1 Preparation and characterization of silver nanoparticles

Preparation of silver nanoparticles (AgNPs) in an aqueous solution requires three reagents: silver salt, reducing agent and stabilizing agent. In this study, two types of stabilizing agent were investigated to see if the type of coating agent relates to the toxicity of the AgNPs. Alginate and Copss were selected as a model for natural and synthetic stabilizing agent, respectively. The AgNPs were prepared by using $AgNO_3$ at 3.8 mM, and varied concentration of $NaBH_4$ (3.8-38 mM) and either alginate or Copss (0.04-4.56 mM). The AgNPs solutions were shown in Figure 4.1. It can be seen that the color of the AgNPs was different depending on the concentrations of the reducing agent and stabilizing agent. The results showed that the higher the concentrations of both reducing agent and stabilizing agent, the lighter the color of the AgNPs. This was correlated well with the report of Solomon et al. (Solomon, 2007) The aggregation of the nanoparticles can occur if the amount of coating agent is not enough to stabilize silver ions and the color of the solution can be used to qualitatively inform the stability of the particles. The solution with dark brown color indicates unstabilized and aggregated AgNPs while the solution with light or dark yellow indicates colloidal AgNPs.

Concentration of alginate

Concentration of NaBH₄	4.56 mM	2.28 mM	1.14 mM	0.38 mM	0.23 mM	0.04 mM
38 mM						
19mM						
3.8 mM						

Concentration of Copss

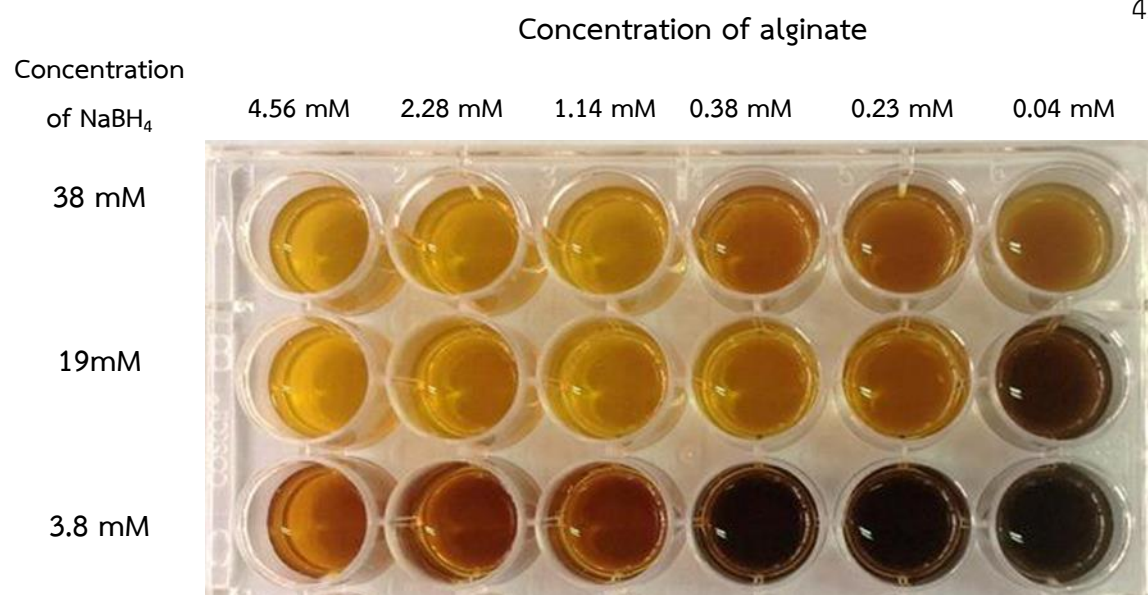| Concentration of NaBH₄	4.56 mM	2.28 mM	1.14 mM	0.38 mM	0.23 mM	0.04 mM
38mM						
19mM						
3.8 mM						

Figure 4.1 Solution of AgNPs prepared by using 3.8 mM AgNO₃, 3.8 - 38 mM NaBH₄ and (A) 3.8 - 38 mM alginate or (B) 3.8 - 38 mM Copss. The light yellow of AgNPs indicated that the stabilized particles

4.1.1 Characterization by UV-Vis spectrum

To confirm the colloidal state of the particles, UV-Vis spectrum of the solutions were scanned between 300nm and 700 nm and shown in Figure 4.2 for alginate and Figure 4.3 for Copss. The results showed that the symmetrical bell shape peaks were obtained when $NaBH_4$ at 19 mM and 38 mM was used. While at 3.8 mM $NaBH_4$, the peaks were not symmetrical. This is actually based on the fact that, degree of dispersion of the AgNPs was reported by Kim et al. (K. D. Kim, Han, & Kim, 2004) or it can easier way to say that uses of low concentration of capping agent and reducing agent lead to broad spectrum and low absorbance.

Moreover, from Solomon et al. (Solomon, 2007) which have been reported that a symmetrical bell shape spectra with the maximum wavelength 390-420 nm could be represented the states of the colloidal phase of AgNPs. This is corresponded well with our result which was shown the symmetrical bell shape when using $NaBH_4$ between 19 mM & 38 mM (as shown in figure 4.1). Therefore, based on the results of color observation and shape of spectrum, $NaBH_4$ at 38 mM was selected to prepare the particles. ($NaBH_4$ at higher concentration was not studied because $NaBH_4$ at 38mM was considered to be an excess concentration). At this concentration, the molar ratio of $NaBH_4$ to $AgNO_3$ was 10 to 1, that were agree well with these research from Song et al. (Song, Lee, Park, & Lee, 2009) though Song reported by varying the ratio from 0.5-15 mM, but molar ratio of $NaBH_4/AgNO_3$ at 10 to 1 is enough to stabilize the solution of AgNPs.

In addition, when the concentration of $NaBH_4$ was too high, a red shift of the spectrum (longer maximum wavelength) could be observed for higher alginate and Copss concentration. It could be due to the increasingly polar capping which tends to increase the dielectric constant on the surrounding of the particles (Dubas, Wacharanad, & Potiyaraj, 2011).

As a result, the AgNPs for cytotoxicity study were prepared by using $AgNO_3$ at 3.8 mM, $NaBH_4$ at 38 mM and either alginate or Copss at 0.23, 1.14 and 4.56 mM.
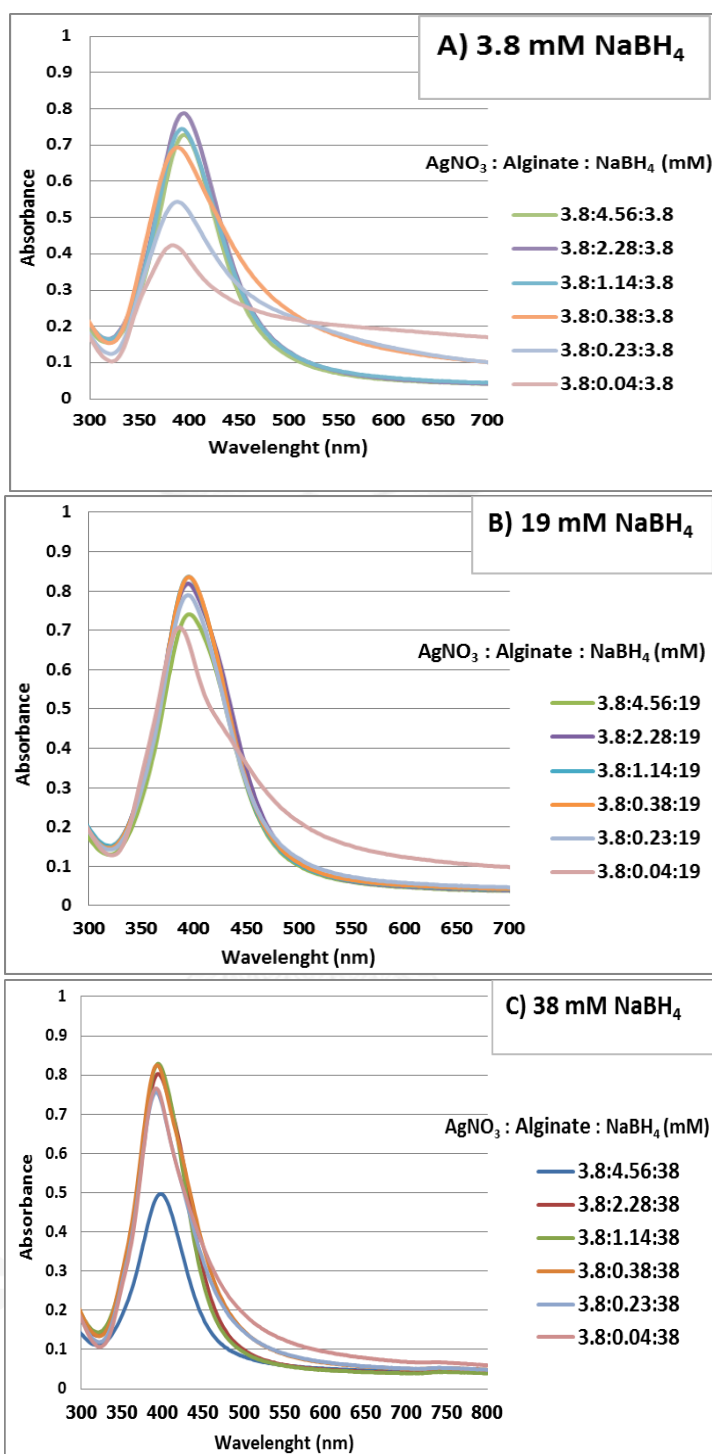
Figure 4.2 UV-Vis absorbance spectra of AgNPs solutions capped with Alginate (0.04 - 4.56 mM) using $NaBH_4$ at (A) 3.8 mM, (B) 19 mM and (C) 38 mM. Each line represents spectra of the AgNPs solution prepared at different molar ratio of $AgNo_3$ to alginate or Copss to $NaBH_4$.
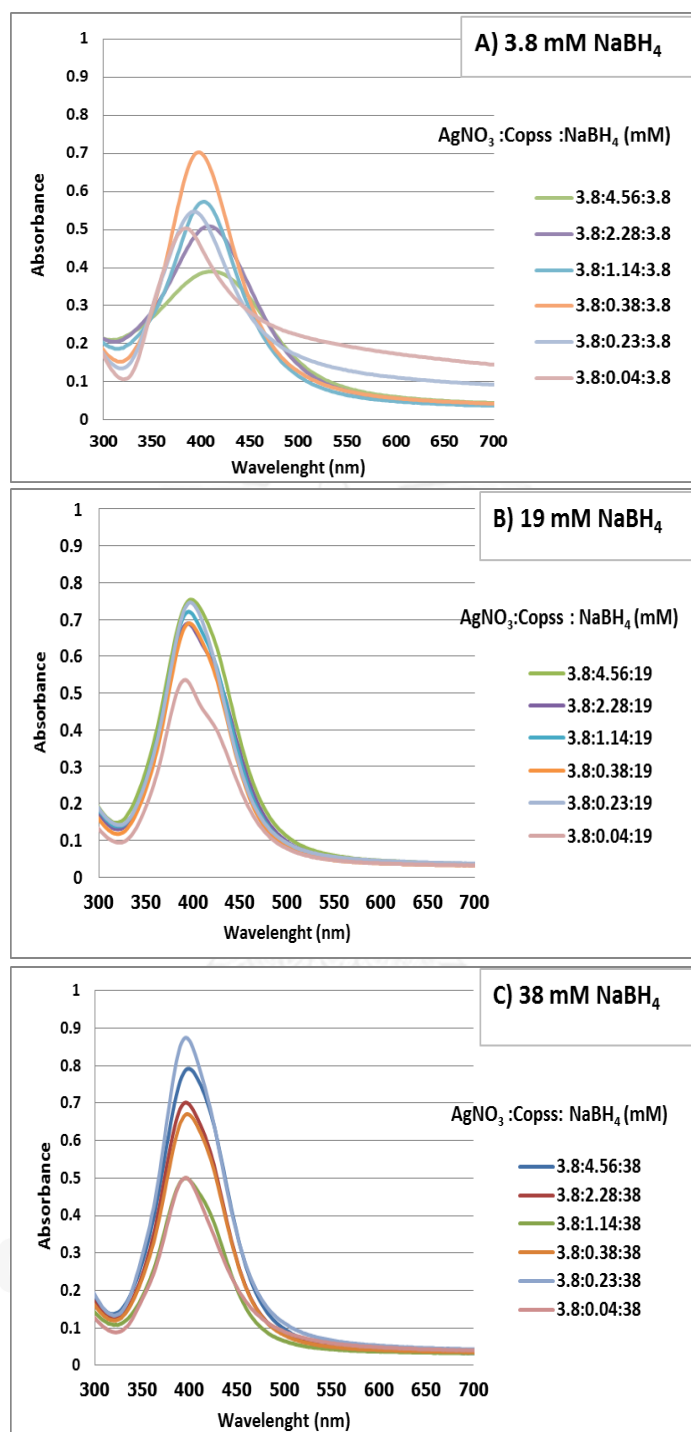
Figure 4.3 UV-Vis absorbance spectra of AgNPs solutions capped with Copss (0.04 - 4.56 mM) using $NaBH_4$ at (A) 3.8 mM, (B) 19 mM and (C) 38 mM. Each line represents spectra of the AgNPs solution prepared at different molar ratio of $AgNO_3$ to alginate or Copss to $NaBH_4$.

4.1.2 Characterization by Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to characterize the size, shape and morphology of the prepared AgNPs as shown in Figure 4.4 – 4.5. All concentration conditions yielded the particle size of 5-15 nm of which the size distribution was calculated by Image J analysis software. The results from TEM also showed that the morphology of the particles was nearly spherical in shape.
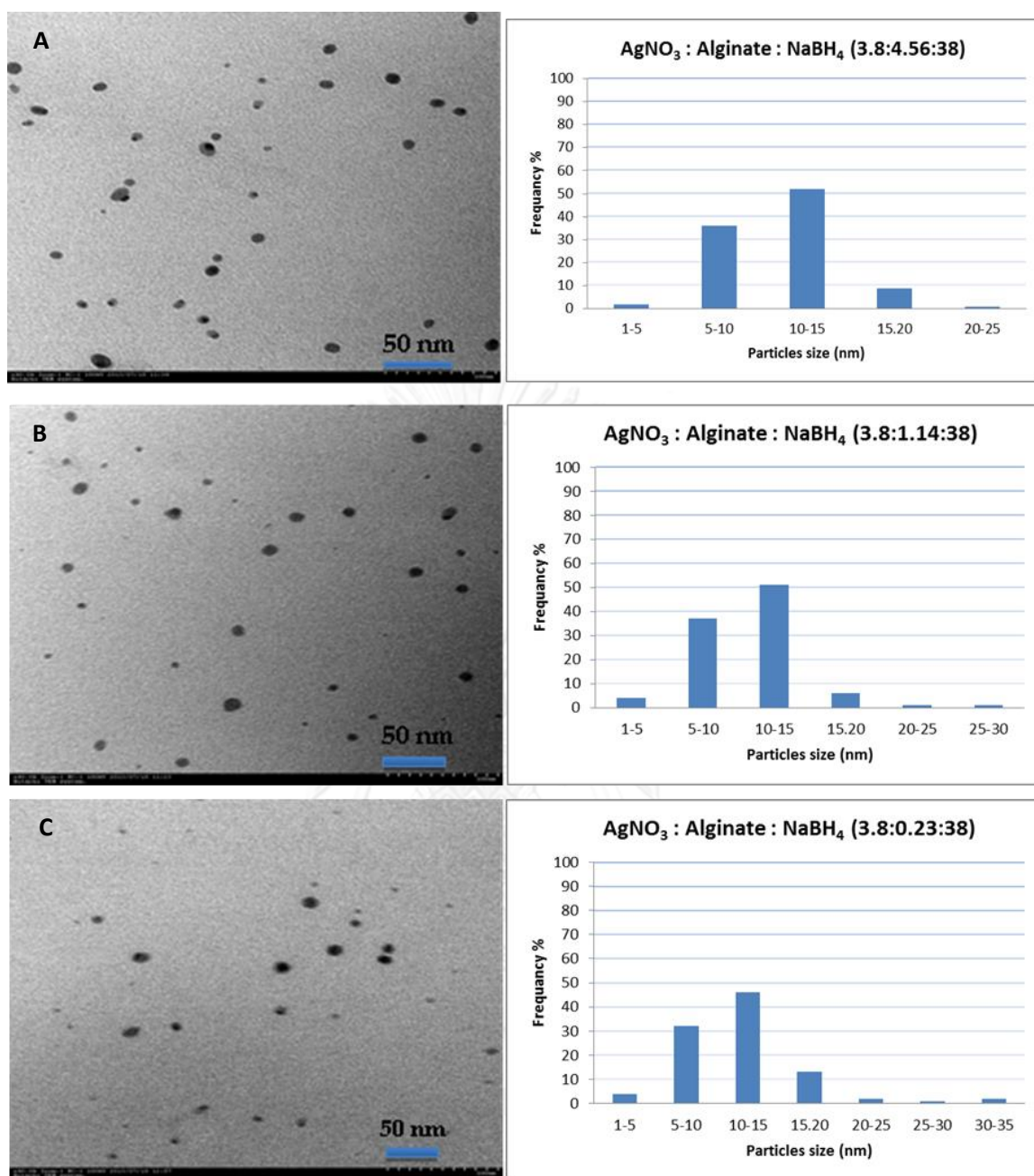
Figure 4.4 TEM images and size distribution of AgNPs prepared by using 3.8 mM AgNO$_3$, 38 mM NaBH$_4$ and alginate: (A) 0.23 mM, (B) 1.14 mM and (C) 4.56 mM
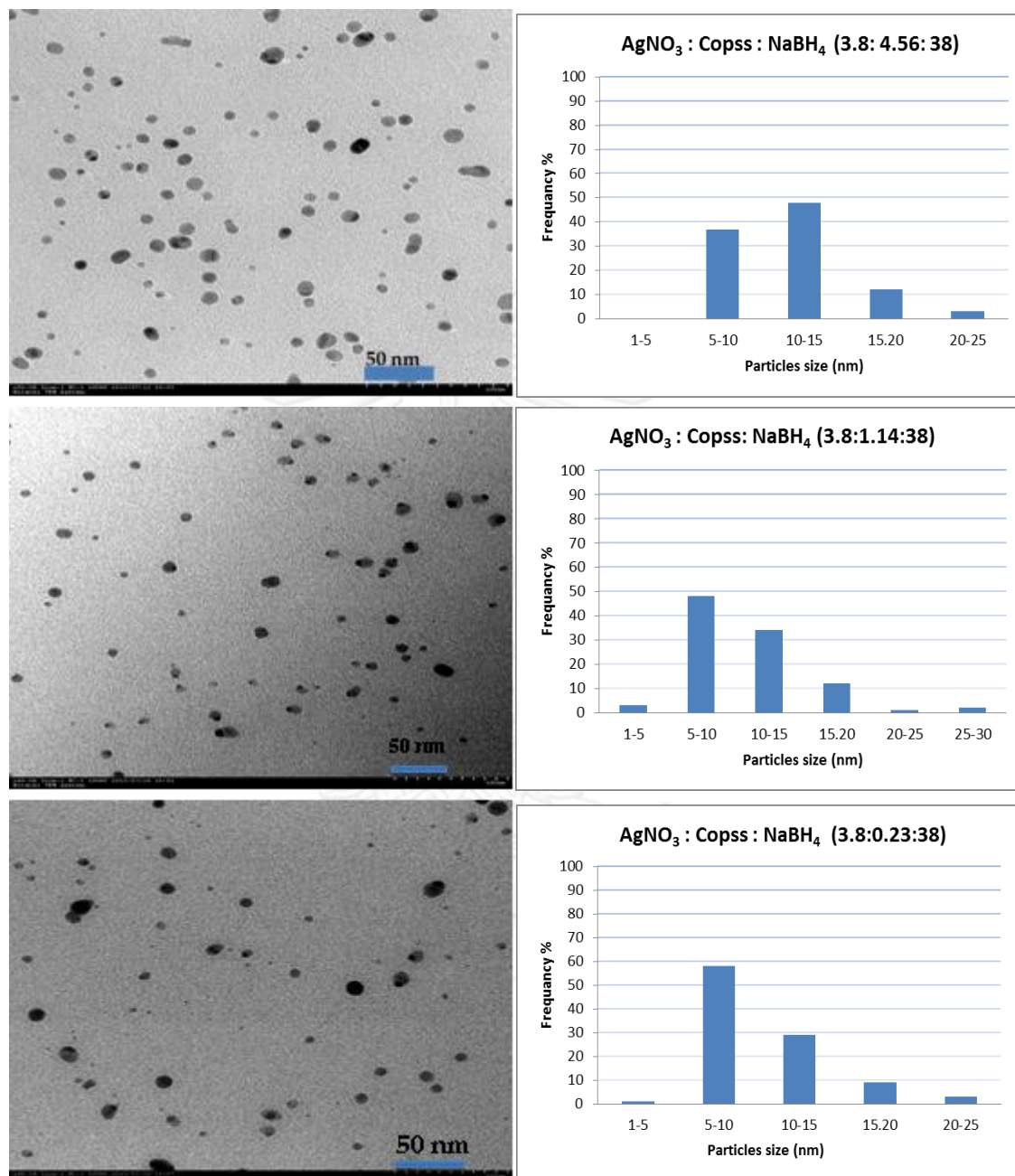
Figure 4.5 TEM images and size distribution of AgNPs prepared by using 3.8 mM AgNO$_3$, 38 mM NaBH$_4$ and Copss: (A) 0.23 mM, (B) 1.14 mM and (C) 4.56 mM

4.1.3 Characterization by zeta potential

In addition, total charge at surface of the particles was measured by zetasizer. The negative charge found on the surface could be used to confirm the presence of anionic of sodium alginate and Copss at the surface of the particles. The result of the zeta potential measurement was shown in Table 4.1

Table 4.1 Zeta potential values of the AgNPs prepared at different ratio of $AgNO_3$ to alginate or Copss to $NaBH_4$

| Silver nanoparticles	Zeta potential (mV)
$AgNO_3$: Alginate : $NaBH_4$ (3.8 : 0.23 : 38)	-31.3±1.8
$AgNO_3$: Alginate : $NaBH_4$ (3.8 : 1.14 : 38)	-33.3±1.2
$AgNO_3$: Alginate : $NaBH_4$ (3.8 : 4.56 : 38)	-36.0±1.5
$AgNO_3$: Copss : $NaBH_4$ (3.8 : 0.23 : 38)	-32.0±2.1
$AgNO_3$: Copss : $NaBH_4$ (3.8 : 1.14 : 38)	-28.7±4.2
$AgNO_3$: Copss : $NaBH_4$ (3.8 : 4.56 : 38)	-26.4±4.0

The results showed that the zeta potential values of the AgNPs capped with alginate and the AgNPs capped with Copss were in the range of -31.3 and -36.0 mV and -26.4 and -32.0 mV, respectively. The zeta potential can be used to indicate the stability of the AgNPs solution depending on the degree of repulsion of the adjacent similarly charged particles. A large zeta potential values, typically less than -25 mV or higher than +25 mV, are preferred since they yield high repulsion force among the particles, thus preventing the aggregation. (Arjmandi, Van Roy, Lagae, & Borghs, 2012). Based on the obtained zeta potential values, the AgNPs prepared by different ratio of $AgNO_3$ to alginate or Copss to $NaBH_4$ in this study were considered stable.

In addition, there was a tendency that the zeta potential value depends conversely on the concentration of the capping agent. The zeta potential of the AgNPs capped with alginate decreased as the concentration of alginate increased. On

the opposite way for capping with Copss, this study has been found that, the zeta potential of the AgNPs capped with Copss increase as the concentration of Copss increase. Since both alginate and Copss are negatively charged, higher concentration of capping agent should give lower zeta potential values. In case of alginate, the result was as expected. However, in case of Copss, it was opposite. This could be explained by the difference in the conformation structure of alginate and Copss that covers the surface of the particles. At high concentration of Copss, malic acid and sulfuric acid within the molecule can cause repulsive force to each other, resulting in the high stretching of the molecule when dissolved in water (Cai, Wang, Hu, Qian, & Chen, 2011). As a result, the total number of the negative charge on the surface of AgNPs did not increase as the concentration of Copss increased. This explanation could be depicted in Figure 4.6

A)
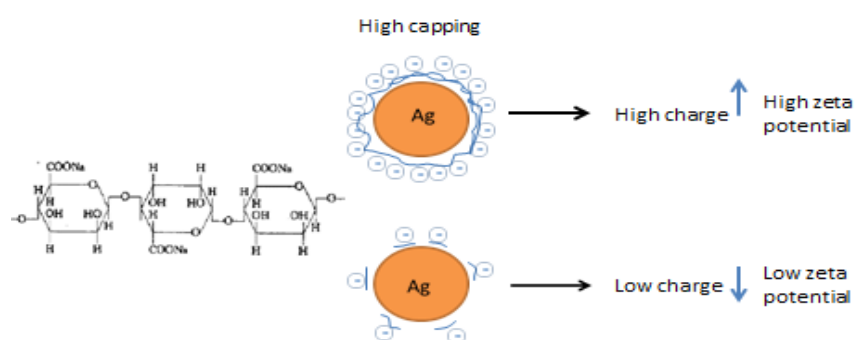
B)
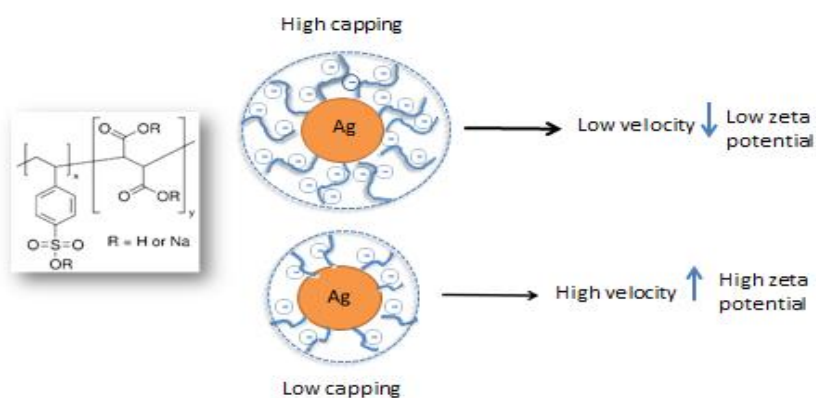
Figure 4.6 Diagrams of silver nanoparticles capped with A) alginate and B) Copss

4.2 Effect of AgNPs on morphology of human skin cells

Effect of $AgNO_3$, alginate, Copss and $NaBH_4$ which were used to synthesize the AgNPs on the morphology of human skin normal cells (CCD-986SK) was investigated. Cells were treated separately with each chemical for 72 h and were observed under an inverted fluorescent microscope as shown in Figure 4.6.
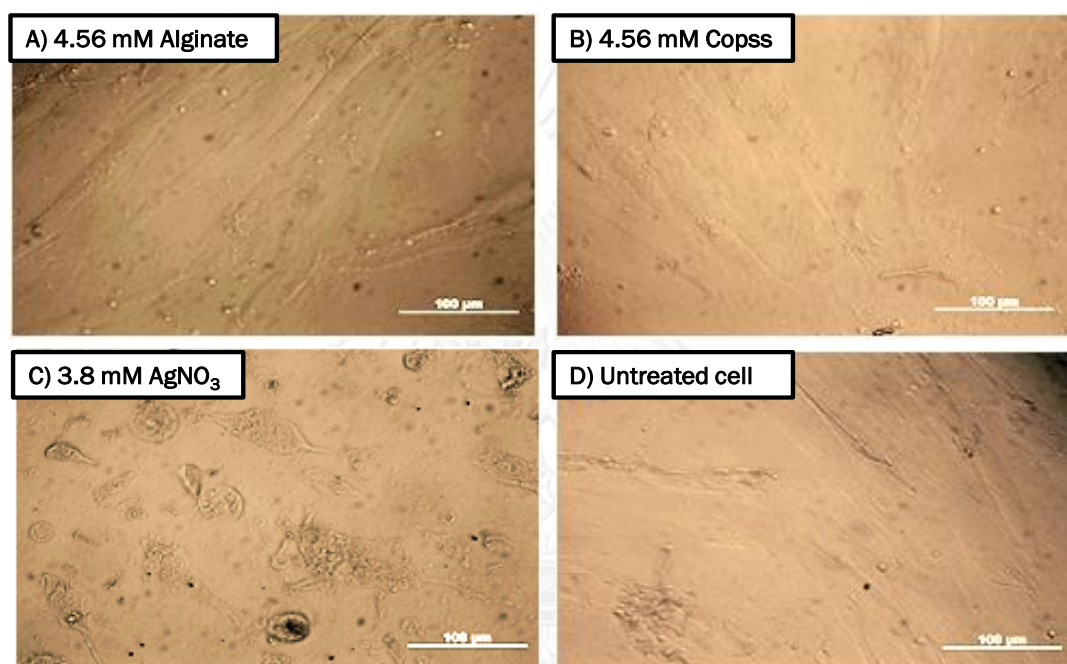
Figure 4.7 Morphology of human skin normal cells CCD-986SK treated with (A) 4.56 mM alginate, (B) 4.56 mM Copss, (C) 3.8 mM $AgNO_3$ for 72 h and (D) untreated cells. All images were magnified 40X and the scale bar was 100 µm. A red arrow indicated unhealthy cell.

It could be seen that the untreated cells (D) have fibroblast-like morphology cells spread and healthy cell. Like the untreated cells, the cells treated with alginate and Copss were healthy and showed the same growth characteristic. On the contrary, cells treated with AgNO3 were drying-out and did not have fibroblast-like morphology.

These indicated that $AgNO_3$ alone was toxic to CCD-986SK cells where as both alginate and Copss did not have obvious effect on the cells.

The effect of the AgNPs precursors on human skin cancer cells (A375) was also studied as shown in Figure 4.7. Like those found in the normal cells, only $AgNO_3$ was toxic to the cancer cells.
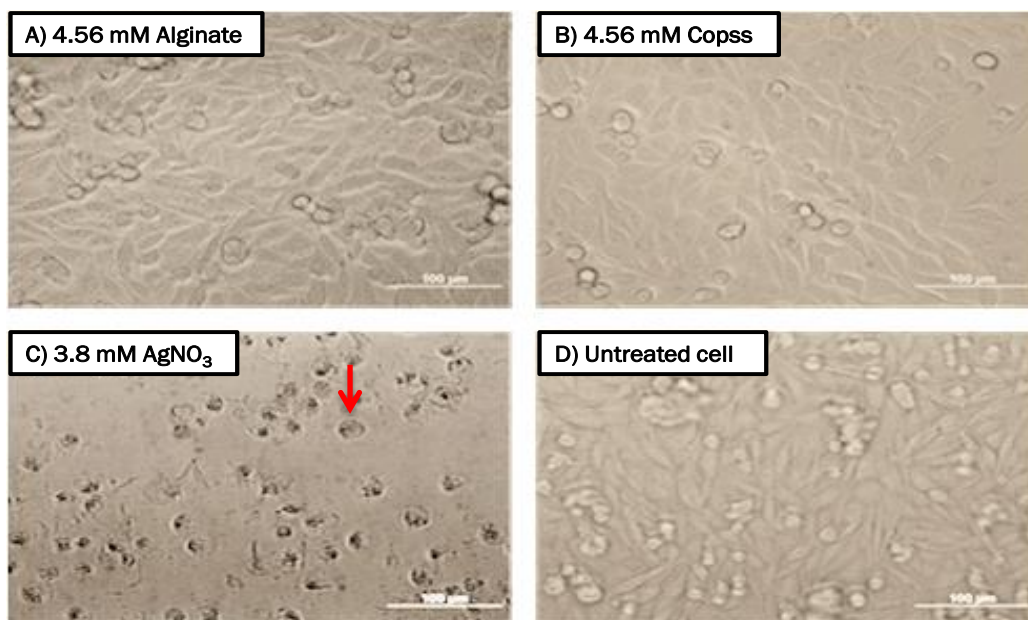
Figure 4.8 Morphology of human skin cancer cells A375 treated with (A) 4.56 mM alginate, (B) 4.56 mM Copss, (C) 3.8 mM $AgNO_3$ for 72 h and (D) untreated cells. All images were magnified 40X and the scale bar was 100 µm. A red arrow indicated unhealthy cell.

These results suggested that alginate and Copss could be used to coat on the AgNPs so that the particles would not toxic to the cells. Therefore, both cells were treated with 600 µg/ml of AgNPs prepared by using either alginate or Copss as the coating agent for 72 h and were observed under the inverted fluorescent microscope as shown in Figure 4.9 - 4.12. Surprisingly, the AgNPs were toxic to the A375 cells in all conditions tested while the particles coated with alginate at all three concentrations or Copss at 4.56 mM did not affect the CCD-986SK cells.

Figure 4.9 Microscopic examination of CCD-986SK cells treated with 600 µg/ml AgNPs prepared by using 3.8 mM $AgNO_3$, 38 mM $NaBH_4$ and alginate at (A) 0.23 mM, (B) 1.14 mM, (C) 4.56 mM , and (D) untreated cells. All images were magnified 40X and the scale bar was 100 µm.
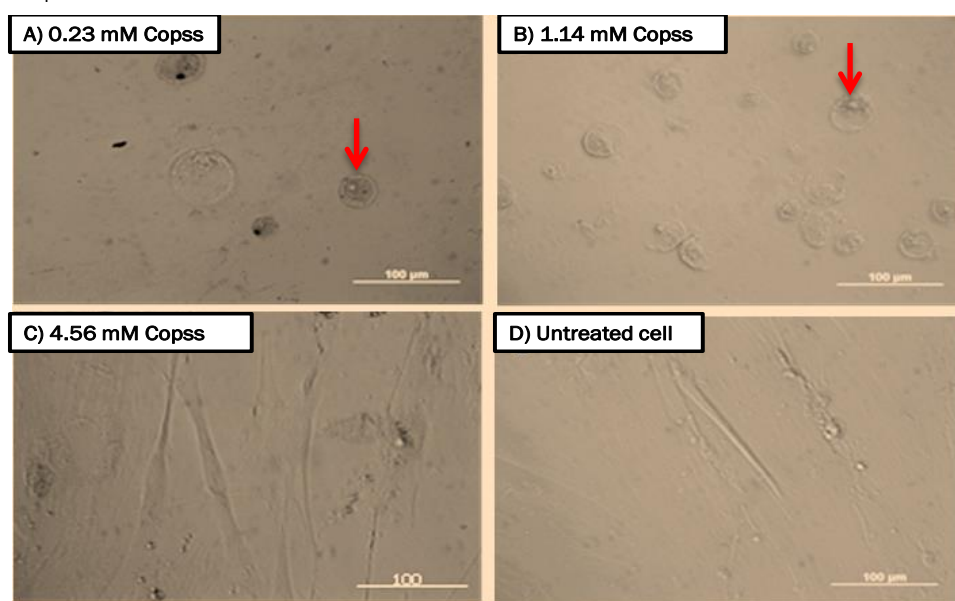
Figure 4.10 Microscopic examination of CCD-986SK cells treated with 600 µg/ml AgNPs prepared by using 3.8 mM $AgNO_3$, 38 mM $NaBH_4$ and Copss at (A) 0.23 mM, (B) 1.14 mM, (C) 4.56 mM , and (D) untreated cells. All images were magnified 40X and the scale bar was 100 µm. A red arrow indicated unhealthy cell.
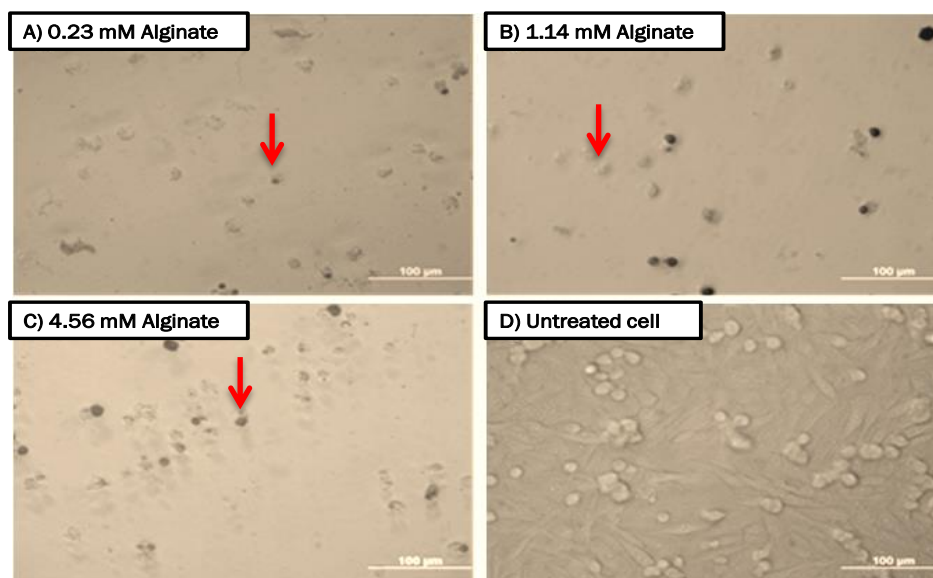
Figure 4.11 Microscopic examination of A375 cells treated with 600 µg/ml AgNPs prepared by using 3.8 mM AgNO$_3$, 38 mM NaBH$_4$ and alginate at (A) 0.23 mM, (B) 1.14 mM, (C) 4.56 mM , and (D) untreated cells. All images were magnified 40X and the scale bar was 100 µm. A red arrow indicated

Figure 4.12 Microscopic examination of A375 cells treated with 600 µg/ml AgNPs prepared by using 3.8 mM AgNO$_3$, 38 mM NaBH$_4$ and Copss at (A) 0.23 mM, (B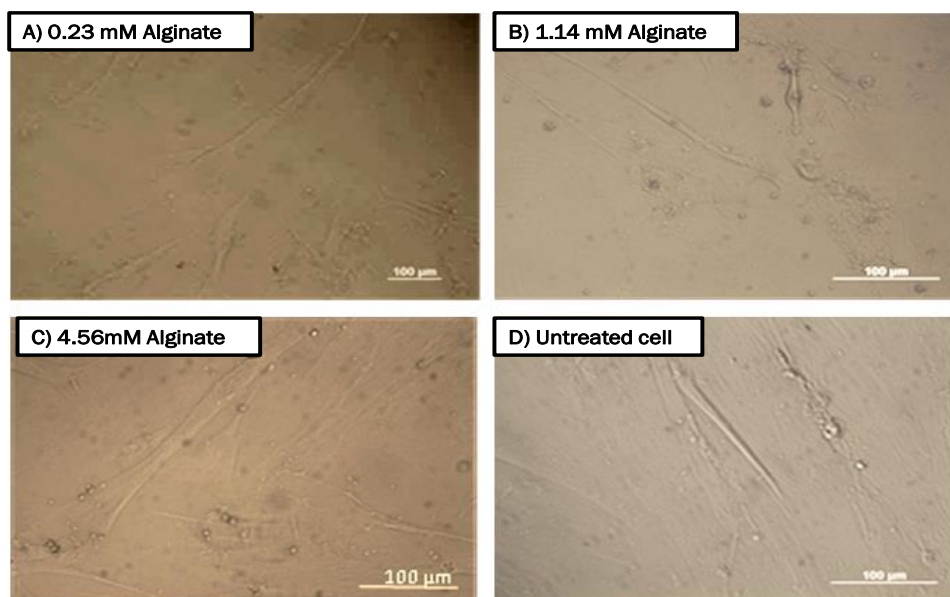) 1.14 mM, (C) 4.56 mM, and (D) untreated cells. All images were magnified 40X and the scale bar was 100 µm. A red arrow indicated unhealthy cell.
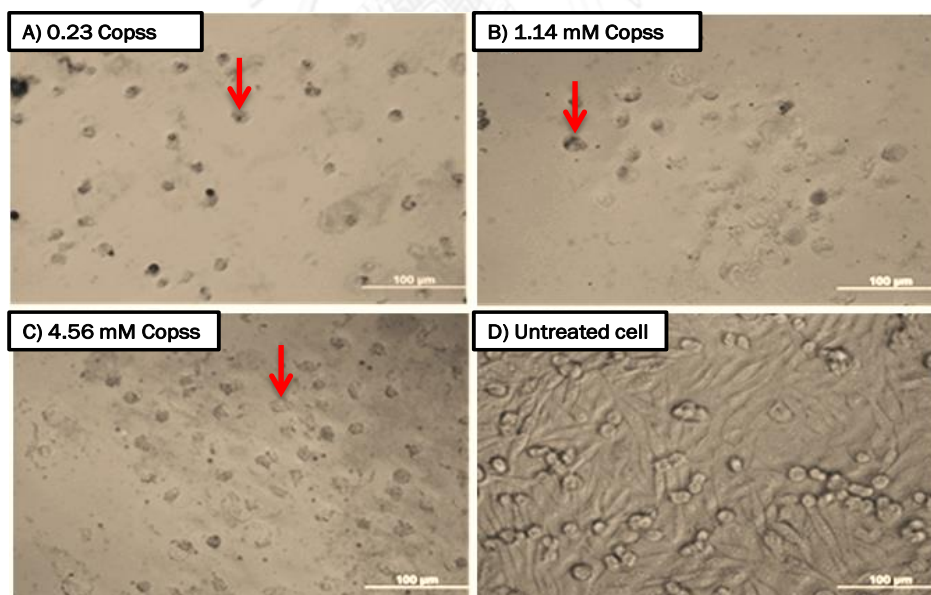
Morphological observation of the skin normal and cancer cells displayed distinct cellular extension in $AgNO_3$ and AgNPs treated cells as compared with the untreated cells. These findings were in accordance with a previous report showing potentially disturbance of cytoskeletal functions caused by the AgNPs (AshaRani et al., 2008). The cytoskeleton injury in most instances blocks chromosome segregation and cytokinesis. Similar patterns of cytoskeletal injury were reported in melanoma cells lacking filamin, a dimeric actin cross-linking protein. The absence of filamin in cells produces unstable pseudopods (filapodia) around the cells so inhibiting their spreading (Cunningham et al., 1992). Morphological degeneracy in cells exposed to AgNPs is possibly due to interpolation with structure and functions of actin cytoskeleton, which might be one of the reasons for inhibition of cell division. The cytoskeleton damage could result from calcium fluctuations and gene dysregulation.

The suggested that alginate can prevent adverse effect of the AgNPs on the normal cells but not on the cancer cells. This could be due to the fact that both cell types have different proteins on the surface of cell membrane (A. B. Lansdown, 2010). Therefore, the ability of the AgNPs to penetrate the cell membrane of both cell types is different. In case of Copss, only high concentration can prevent the toxic effect of the particles. This could be preliminarily explained by the reason that Copss at high concentration does not have enough negative charge (-26.4 mV of zeta potential values) to repel the particles from the cells.

4.3 *In vitro* cytotoxicity assay

Effect of $AgNO_3$, alginate, Copss and $NaBH_4$ which were used to synthesize the AgNPs on the cell viability of human skin normal cells (CCD-986SK) and cancer cells (A375) was studied. Both cells were treated separately with each chemical for 72 h. Then, MTT assay was performed to assess the percentage of cell viability which was shown in Figure 4.13. It was found that the viabilities of the cells treated with either alginate or Copss approximately were in the range of 80 - 100%. Whereas those of the cells treated with $AgNO_3$ were below 10% for A375 cells and about 35 – 40% for CCD-986SK cells. These results were in an agreement with the results obtained in the morphology study that only the $AgNO_3$ was toxic to the cells
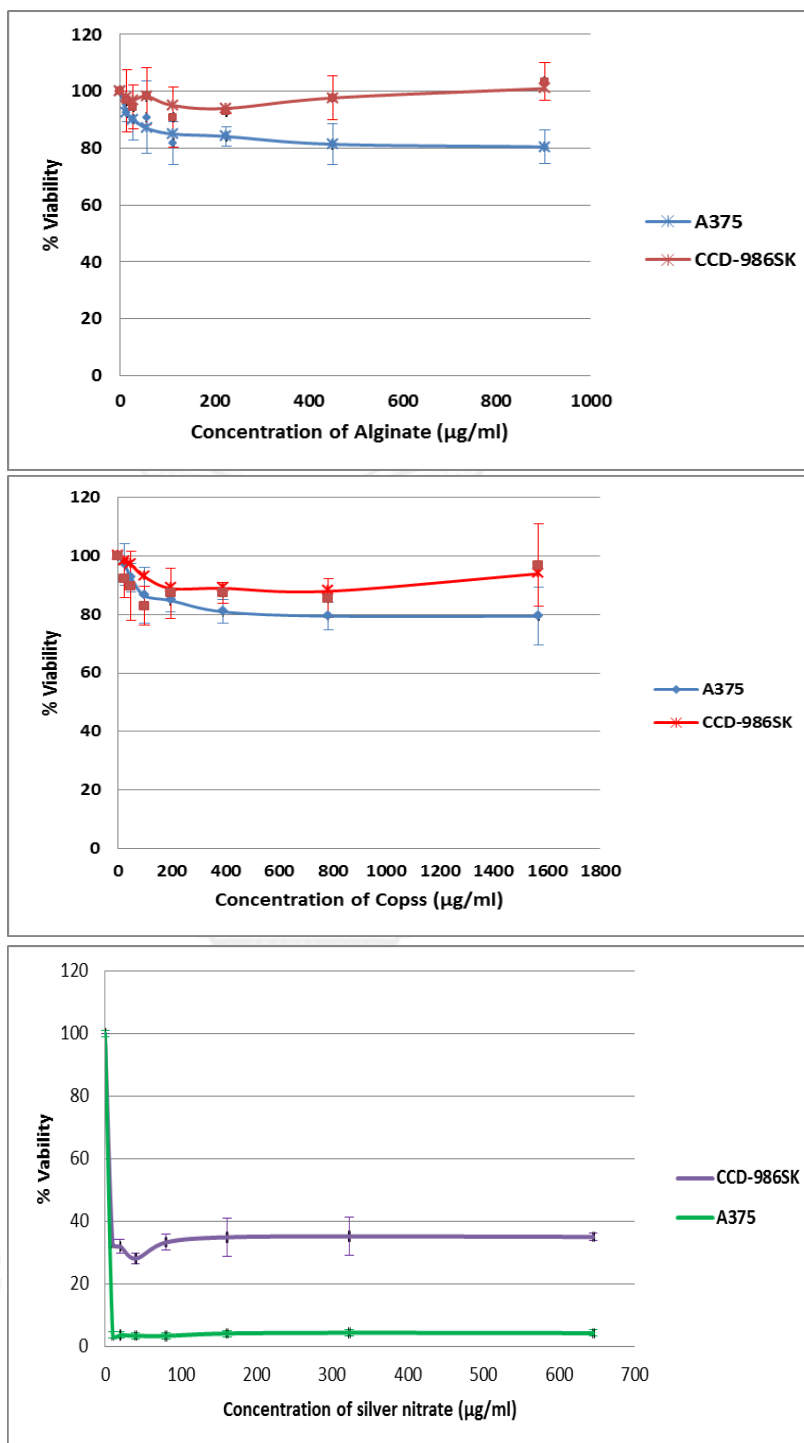
Figure 4.13 Cell viability of human skin normal cell (CCD-986SK) and human skin cancer cell (A375) after treatment with (A) alginate, (B) Copss and (C) AgNO₃ for 72 h

Cytotoxicity of the AgNPs prepared by using either alginate or Copss as the coating agent was measured and shown in Figure 4.13 for normal CCD-986SK cells and Figure 4.14 for cancer A375 cells, respectively. The concentration of the AgNPs was varied up to 600 µg/ml which could be considered as the extremely high concentration found in current applications or other previous studies. The result showed that the % cell viabilities of the normal cells treated with alginate-coated AgNPs were between 70 – 100%. While those treated with Copss-coated AgNPs depended on the concentration of both Copss and AgNPs. The % cell viability of the normal cell varied inversely with the concentration of the AgNPs. In addition, the particles coated with Copss at 4.56 mM were less toxic than those coated at lower concentrations. These results suggested that Copss at high concentration and alginate can make the AgNPs less toxic. In case of the cancer cell, it was very sensitive to the AgNPs. The % cell viability decreased as the AgNPs concentration increased. However, alginate could not reduce the toxicity of the particles as that found in the case of the normal cell.

Figure 4.14 Cell viability of human skin normal cell (CCD-986SK) after 72 h treatment with AgNPs prepared by using (A) alginate and (B) Copss as the coating reagent at different concentrations, 3.8 mM $AgNO_3$ and 38 mM $NaBH_4$

Cytotoxicity of the AgNPs prepared by using either alginate or Copss as the coating agent was measured and shown in Figure 4.14 for normal CCD-986SK cells and Figure 4.15 for cancer A375 cells, respectively. The concentration of the AgNPs

was varied up to 600 μg/ml which could be considered as the extremely high concentration found in current applications or other previous studies. The result showed that the % cell viabilities of the normal cells treated with alginate-coated AgNPs were between 70 – 100%. While those treated with Copss-coated AgNPs depended on the concentration of both Copss and AgNPs. The % cell viability of the normal cell varied inversely with the concentration of the AgNPs. In addition, the particles coated with Copss at 4.56 mM were less toxic than those coated at lower concentrations. These results suggested that Copss at high concentration and alginate can make the AgNPs less toxic. In case of the cancer cell, it was very sensitive to the AgNPs. The % cell viability decreased as the AgNPs concentration increased. However, alginate could not reduce the toxicity of the particles as that found in the case of the normal cell.

These finding could be explained by the reason of the repulsion force. The carboxyl, phosphate and amino groups on the cellular membrane made up the negative charge on the cell surface. Thus, there is a high degree of repulsion between the negatively charged AgNPs capped with alginate and cell membrane. It has been suggested that the toxicity of AgNPs may involve a combination of physical and chemical interaction with the formal one as the limiting step. Once the electrostatic barrier is overcome, the AgNPs can interact with the cell and cause physical damage (El-Badawy, 2010). Since the AgNPs capped with Copss have less negative charge as compared with ones capped with alginate, the repulsive force between the cells and the particles is lower. Consequently, the AgNPs capped with Copss can interact with the cell membrane or diffuse into the cells more easily.

Furthermore, it has been reported that proteins on the cell membrane of normal and cancer cells are different (A. B. Lansdown, 2010). This could lead to the different level of AgNPs toxicity between the two cell types. The difference in toxicity response between normal and cancer cells has been reported. Selenium nanoparticles capped with polysaccharide from *Undaria pinnatifida* extract showed lower cytotoxicity toward skin normal cell (Hs68 human fibroblast) as compared with skin cancer cell (A375) (T. Chen, Wong, Zheng, Bai, & Huang, 2008). The exact toxicity mechanism of the AgNPs is still unclear. However it is believed that metallic silver itself is inert to the human tissue but ionized form in the presence of moisture or body fluid is biologically active with strong affinity for –SH group and other anionic ligands of proteins and cell membrane (R. E. Burrell, 2003). Different surface chemistry and functionalization of the cell affects to different uptake pathways of

the particles (AshaRani et al., 2008). Therefore, difference in protein or cell surface compositions between the normal and cancer cell might cause different responses to the AgNPs.
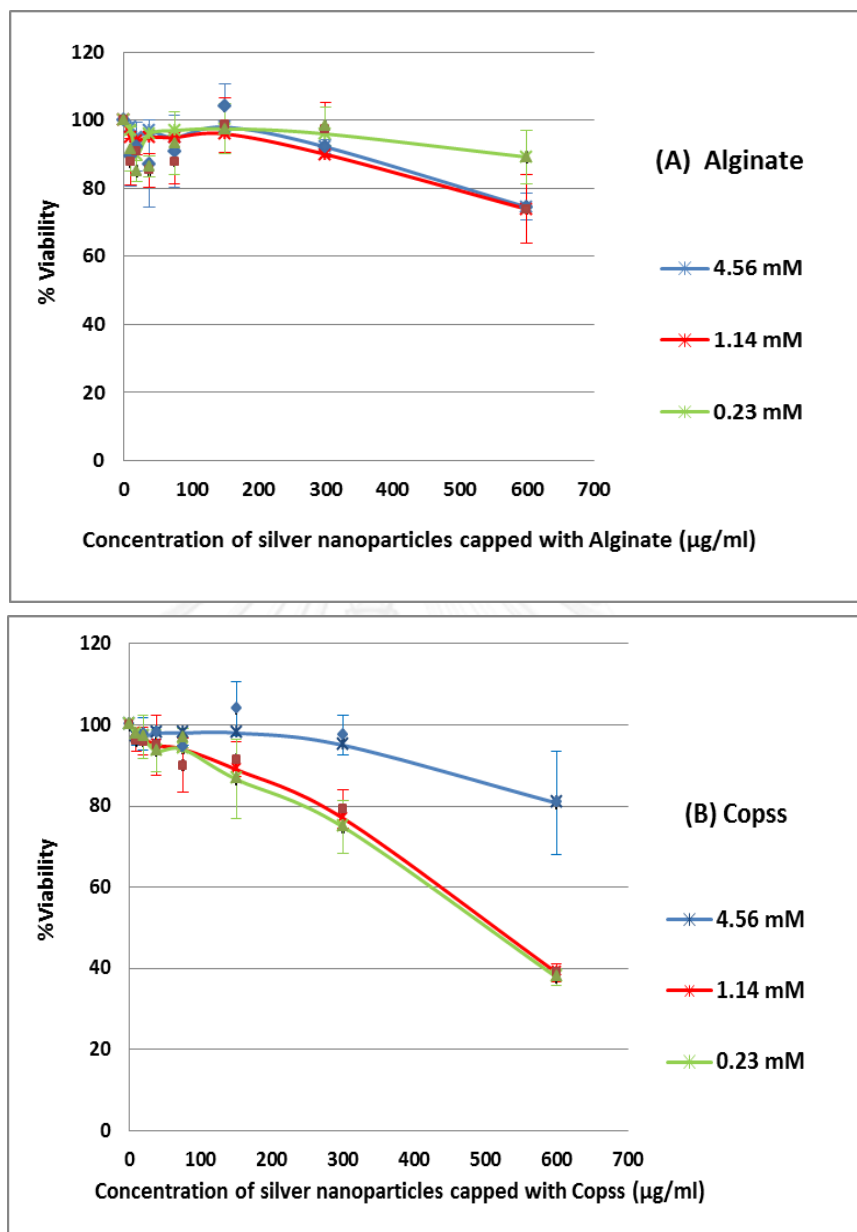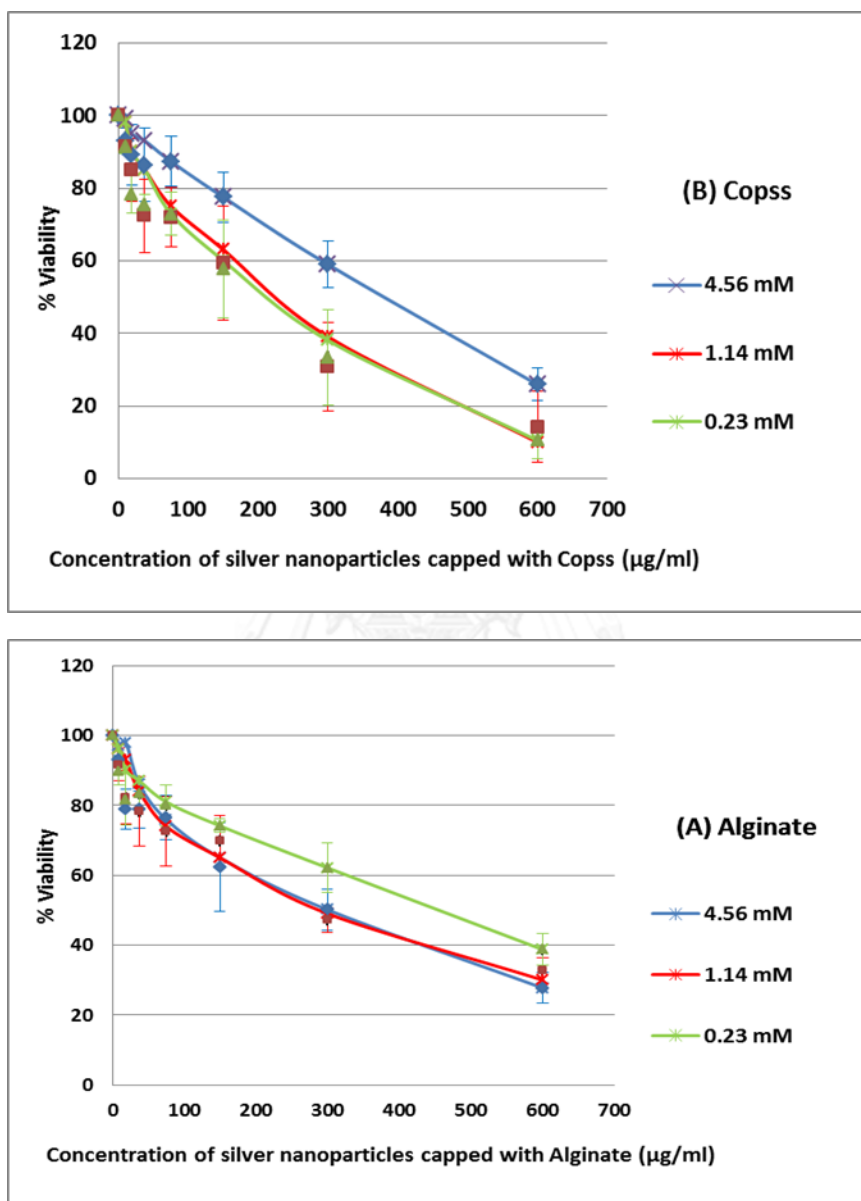

Figure 4.15 Cell viability of human skin cancer cell (A375) after 72 h treatment with AgNPs prepared by using (A) alginate and (B) Copss as the coating reagent at different concentrations, 3.8 mM AgNO$_3$ and 38 mM NaBH$_4$

The degree of toxicity was calculated in term of the 50% inhibition concentration (IC_{50}) which is the least concentration of the AgNPs that causes the 50% cell viability at a specific time. The lower the IC_{50} value, the higher the toxicity. The result shown in Table 4.2 indicated that the cancer cells were more sensitive to the AgNPs than the normal cells. In addition, there was a tendency that Copss was more toxic than alginate, except at the concentration of 4.56 mM. Interestingly, the IC50 values of alginate were higher than 600 µg/ml which is the highest IC_{50} values that has been reported for normal skin cells. The IC_{50} values of the citrate-coated and the carbon-coated AgNPs were 10 µg/ml and 1.7 µg/ml for normal skin cell HaCaT and HEK, respectively (Chambers, Muckherjee, Casey, & O'Claonadh, 2008; Lu et al., 2010). Therefore, alginate might be considered as a coating reagent for non-toxic AgNPs. However, the maximum concentration and the exposure time used in this study were limited at 600 µg/ml and 72 h, respectively. In case of A375, the IC_{50} values were reported at 78 µg/ml when cells were treated with the AgNPs coated with ethanolic extracts from Gelsemium sempervirens (Das et al., 2013).

Table 4.2 Calculated IC_{50} values of AgNPs for human skin normal cell (CCD-986SK) and cancer cell (A375) after exposure for 72 h

Cells / AgNPs	IC_{50} (µg/ml)	
	A375	CCD-986SK
$AgNO_3$: Alginate : $NaBH_4$ (3.8 : 0.23 : 38)	456±57	>600
$AgNO_3$: Alginate : $NaBH_4$ (3.8 : 1.14 : 38)	370±42	>600
$AgNO_3$: Alginate : $NaBH_4$ (3.8 : 4.56 : 38)	347±52	>600
$AgNO_3$: Copss : $NaBH_4$ (3.8 : 0.23 : 38)	264±24 *	536±40
$AgNO_3$: Copss : $NaBH_4$ (3.8 : 1.14 : 38)	281±34 *	507±39
$AgNO_3$: Copss : $NaBH_4$ (3.8 : 4.56 : 38)	389±37 *	>600

Remark: data are expressed as mean ± SD of three independent experiments.

 * denotes a statistically significant ($p < 0.05$) difference between the samples.

Cell viabilities of the human skin cancer cell (A375) after exposure time for 24, 48 and 72 h with the AgNPs coated with alginate and Copss were also shown in Figure 4.16 and 4.17, respectively. The results showed that in most cases. The exposure time between 24 h and 72 h did not affect the toxicity. However, it was found in some studies that the toxicity is varied with the exposure time. For example, cell viability of Caco-2 cell line (human colon adenocarcinoma cell) exposed to peptide-coated AgNPs decreased significantly with respect to exposure time (24 - 48 h) (Böhmert, Niemann, Thünemann, & Lampen, 2012). Like that of Caco-2 cell line, cell viability of HaCaT cell line (non-cancerous human keretinocyes) decreased as the incubation increased from 24 h to 72 h (S. G. Mukherjee, O'Claonadh, Casey, & Chambers, 2012). However, it was suggested that the effect of the exposure time on the cytotoxicity is varied among the AgNPs since their chemistry and structure as well as capping agent are different (Nel, Xia, Mädler, & Li, 2006).

Figure 4.16 Cytotoxicity of alginate-coated AgNPs in human skin cancer cell (A375) after exposure for 24, 48, and 72 h determined by MTT assay: (A) alginate 0.23 mM, (B) alginate 1.14 mM, (C) alginate 4.56 mM. * denotes a statistically significant (p<0.05) difference from the exposure time.
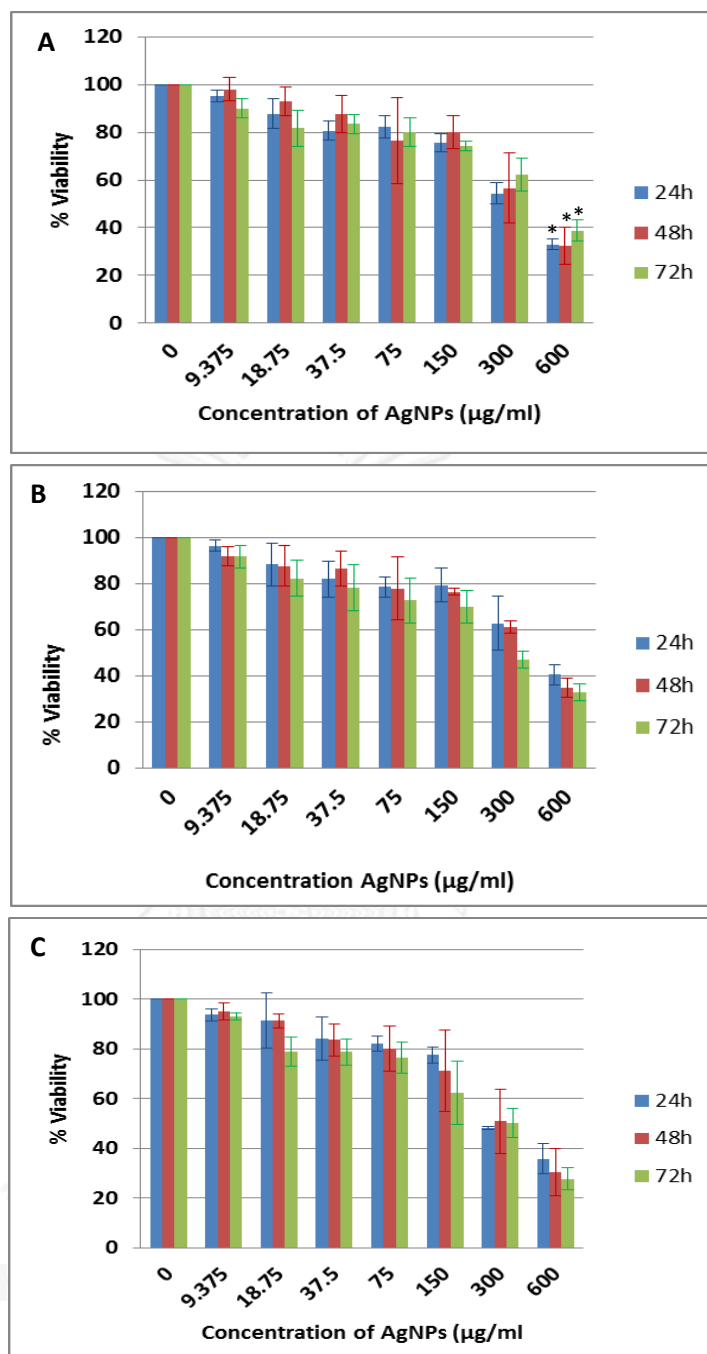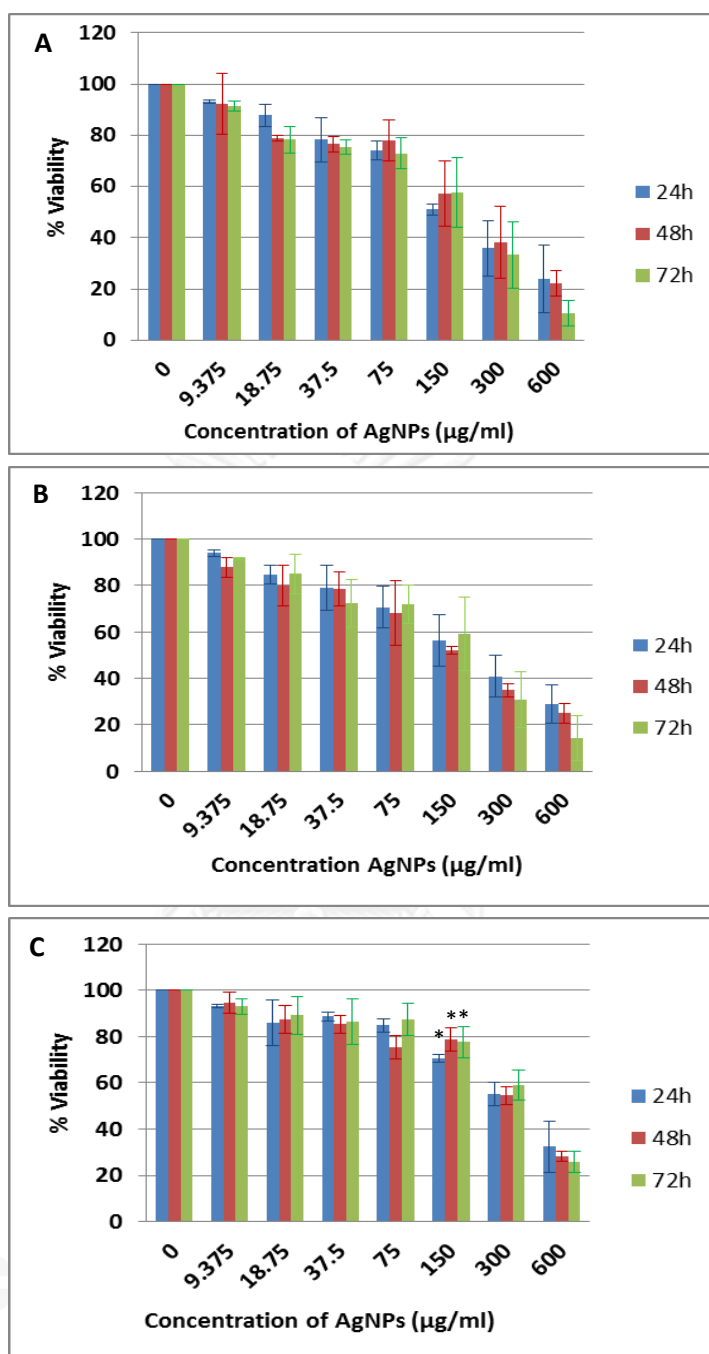
Figure 4.17 Cytotoxicity of Copss-coated AgNPs in human skin cancer cell (A375) after exposure for 24, 48, and 72 h determined by MTT assay: (A) Copss 0.23 mM, (B) Copss 1.14 mM, (C) Copss 4.56 mM. * denotes a statistically significant (p<0.05) difference from the exposure time.

4.4 Apoptosis and necrosis test

4.4.1 Human skin normal cell (CCD-986SK)

In the previous section, it was found that the alginate-coated AgNPs were not toxic to human skin normal cell (CCD-986SK) and the Copss-coated AgNPs were toxic to both human skin normal cell and cancer cell (A375). Consequently, cells were checked with apoptosis analysis using Annexin V and propidium iodide staining method. Flow cytometry profile of the CCD-986SK cell was shown in Figure 4.17. It can be seen that the untreated cells (A and B) were in the quadrant 3 of the profile, indicating that cells were not stained with both dyes. This suggested that cells were still intact. Cells treated with the alginate coated AgNPs (C) were also in the quadrant 3 as expected since the particles coated with alginate were not toxic to the cells. In case of cells treated with the Copss coated AgNPs (D), they were mostly in the quadrant 3. However, some cells were in the quadrant 1 due to the cell death by necrosis. In this experiment, cells treated with doxorubicin were used as the control of cell death. Furthermore, the percentage of cells in each quadrant was calculated as shown in Figure 4.18. It suggests that about 80% of the cell were lived while less than 20% of cells were died. Doxorubicin (drug) causes cell death in the late apoptosis about 60%.

4.4.2 Human skin cancer cell (A375)

The cytotoxicity test in the section 4.3 showed that both alginate coated and Copss coated AgNPs were toxic to the A375 cells. Flow cytometry profile of the cells shown in Figure 4.19 indicated that most of the cells died in late apoptosis (C and D). In addition, the percentage of cell death was shown in Figure 4.20. It suggested that more than 80% of the cells were died in the late apoptosis while about 5% – 10% of the cells were died due to necrosis. Doxorubicin (drug) causes cell death late apoptosis about 80%.

Recently several studies have been reported that AgNPs induced cell death. AgNPs enter to the cell may cause dissolution of AgNPs and release Ag^+ ion, Ag^+ ion induced mitochondrial dysfunction and cause reactive oxygen species. It has been reported to major role in the toxicity of AgNPs (Xia T, 2008). AgNPs induced cell damage and cause DNA fragmentation by reactive oxygen species (ROS) production and induction apoptosis. ROS such as hydroxyl radicles invade cellular constitutive including DNA and protein to cause several of oxidative damage (Denisova, Cantuti-

Castelvetri, Hassan, Paulson, & Joseph, 2001; Halliwell & Aruoma, 1991). The previous study showed the AgNPs was more ROS production in adenocarcinoma cell (HeLa) as compared to the non-cancerous keratinocytes cell (HaCaT) (Chambers et al., 2008). The incapacity of mitochondria is important in the level of regulator of apoptosis; the loss of mitochondria can be induced or inhibited of regulators of apoptosis (Piao et al., 2011). Better capping efficiency and stability indicated a more proficient entry into the cell (Chambers et al., 2008). AgNPs were entering the cell by endocytosis rather than diffusion. AgNPs were deposition in the nuclear. The nuclear enwrap has nuclear pore complex (diameter 9-10 nm) which transport by protein takes place. AgNPs was readily diffused into the nucleus and their release Ag^+ ion inside the cell nucleus. Ag^+ may bind to DNA and ROS production which cause DNA damage (AshaRani et al., 2008). Surface chemistry and different surface functionalization of the cell affect to different uptake pathways of particles (AshaRani et al., 2008).
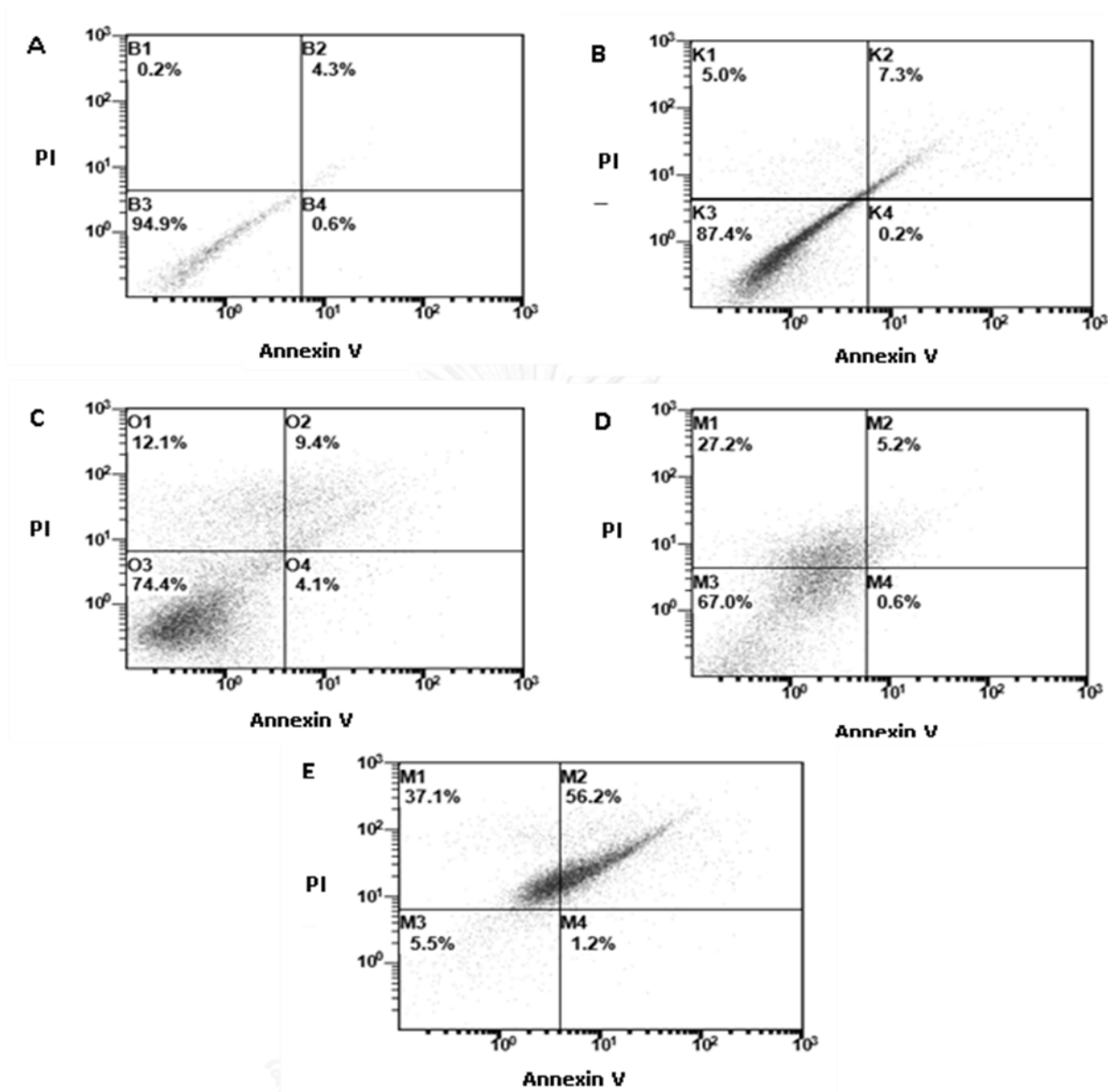
Figure 4.18 Annexin V-PI staining of CCD-986SK cells treated with 600 µg/ml AgNPs for 72 h. A: untreated and unstained cells, B: untreated cells stained with Annexin V and PI, C: cells treated with AgNPs capped with alginate and stained with Annexin V and PI, D: cells treated with AgNPs capped with Copss and stained with Annexin V and PI, E: cells treated with 1 µg/ml doxorubicin and stained with Annexin V and PI (positive control). Quadrant 1: necrosis, quadrant 2: late apoptosis, quadrant 3: live cell, quadrant 4: early apoptosis
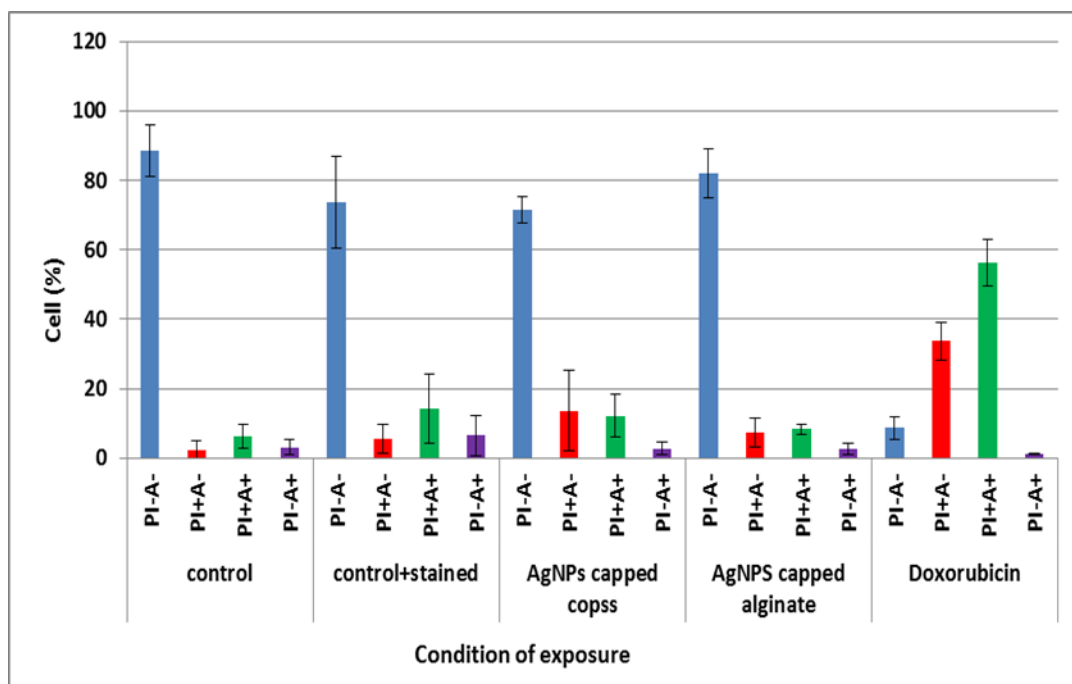
Figure 4.19 Percentage of CCD-986SK cells stained with Annexin V-PI after treatment with 600 µg/ml AgNPs or 1 µg/ml doxorubicin where PI- A- : live cells, PI+ A-: necrotic cells, PI+ A+: late apoptotic cells and PI- A+: early apoptotic cells.
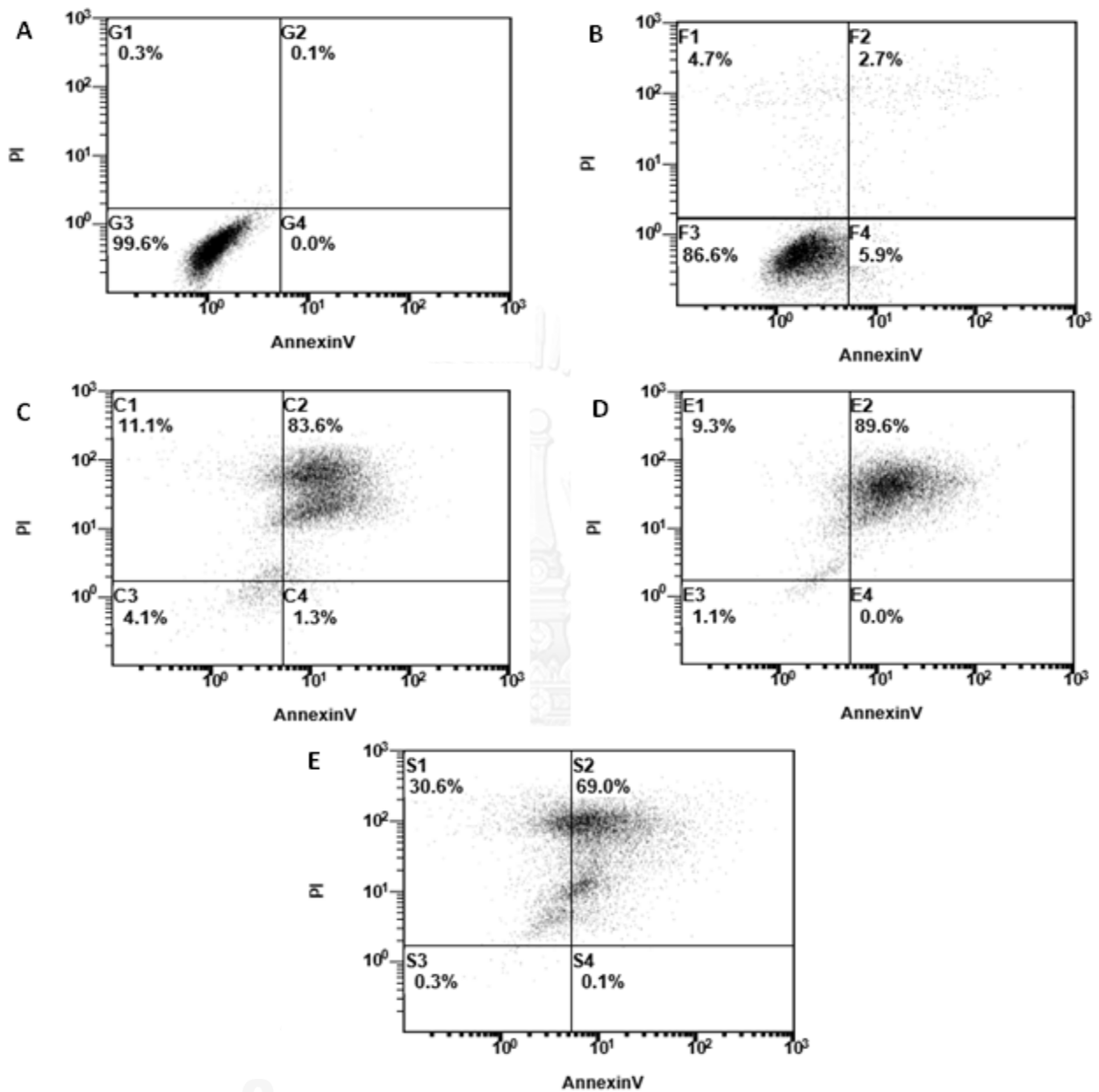
Figure 4.20 Annexin V-PI staining of A375 cells treated with 600 µg/ml AgNPs for 72 h. A: untreated and unstained cells, B: untreated cells stained with Annexin V and PI, C: cells treated with AbNPs capped with alginate and stained with Annexin V and PI, D: cells treated with AgNPs capped with Copss and stained with Annexin V and PI, E: cells treated with 1 µg/ml doxorubicin and stained with Annexin V and PI (positive control). Quadrant 1: necrosis, quadrant 2: late apoptosis, quadrant 3: live cell, quadrant 4: early apoptosis
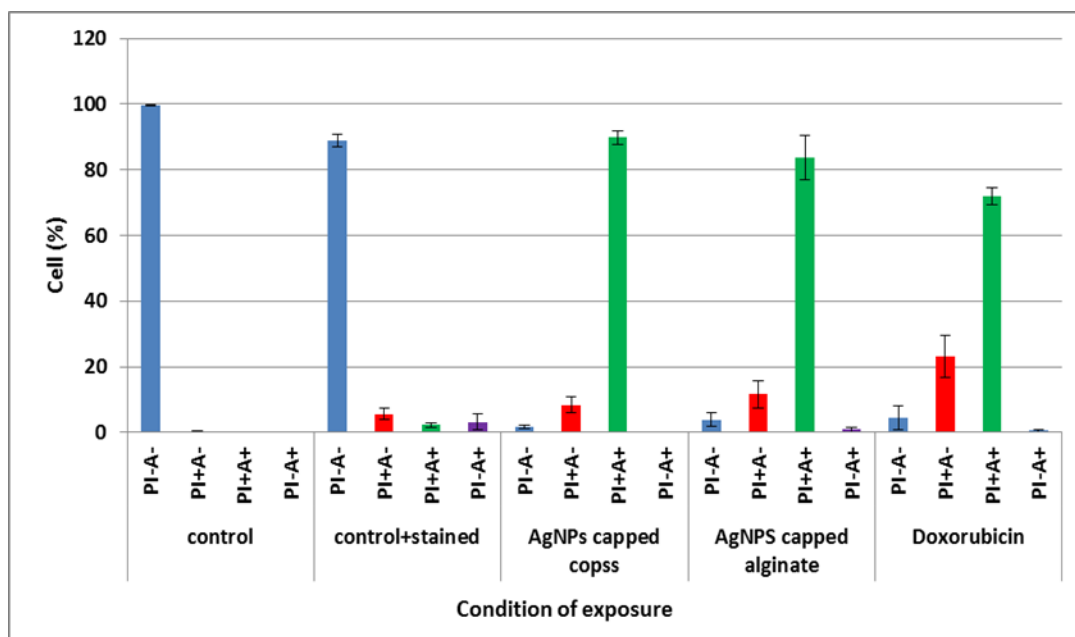
Figure 4. 21 Percentage of A375 cells stained with Annexin V-PI after treatment with 600 µg/ml AgNPs or 1 µg/ml doxorubicin. Where PI- A- : live cells, PI+ A-: necrotic cells, PI+ A+: late apoptotic cells and PI- A+: early apoptotic cells

It has been proposed that after the AgNPs enter to the cell, Ag^+ is formed, thus inducing mitochondrial dysfunction and generating reactive oxygen species (ROS) (Xia T, 2008). The incapability of mitochondria function affects the level of regulator of apoptosis (Piao et al., 2011). ROS such as hydroxyl radicals invade cellular constitutive including DNA and protein to cause several oxidative damages (Halliwell & Aruoma, 1991).

In addition, it has been found that Ag^+ ion could inhibit Ca^{2+} release from the intracellular stores (Moutin MJ, 1989). Disruption of calcium homeostasis plays an important role in pathological and toxicological conditions and is a signal to start cell injury. Calcium ions have the potential to activate catabolic enzymes such as phospholipase, proteases and endonuclease that further augment the toxicity (Orrenius, McCabe Jr, & Nicotera, 1992). Moreover, Ca^{2+} excess in mitochondria could release apoptogenic factors such as cytochrome C, endonuclease G and other apoptosis inducing factors to the cytosol to initiate apoptosis (Belizario, Alves, Occhiucci, Garay-Malpartida, & Sesso, 2007)

CHAPTER V

CONCLUSION

In summary, silver nanoparticles (AgNPs) were prepared by chemical reduction of 3.8 mM silver nitrate (AgNO$_3$) with varying concentration of reducing agent (NaBH$_4$) and capping agent, either sodium alginate or poly (4-styrenesulfonic acid-co-maleic acid) sodium salt (Copss). It was found that to synthesize the stable AgNPs, the optimum concentration of AgNO$_3$ and NaBH$_4$ was 3.8 mM and 38 mM, respectively, while that of capping agent was between 0.23 - 4.56 mM. The size of the obtained AgNPs was estimated to be 5 -15 nm by transmission electron microscopy. The UV-visible spectrum showed the maximum absorbance at the wavelength between 390 and 420 nm. The zeta potential values of the synthesized AgNPs were negative, indicating the present of the anionic sodium alginate or Copss at the surface of the particles. The morphology observation under the inverted fluorescence microscope and the cytotoxicity test determined by MTT assay demonstrated that AgNO$_3$ was toxic while alginate and Copss alone were not toxic. In addition, the human skin cancer cells (A375) were sensitive to the AgNPs than the normal cells (CCD-986SK). Alginate could make the AgNPs less toxic to the normal cell only while the Copss coated AgNPs were toxic to both cells. The cell death of the CCD-986SK cell was due to necrosis while that of the A375 cell was in the late apoptosis (about 80%) and necrosis (about 10%).

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APPENDIX

1. Preparation of silver nanoparticles

   Start prepared silver nanoparticles (AgNPs) 400 ppm = 400 µg/ml= 0.0004 g/ml= 0.4 mg/ml

   This want get Ag 0.4 mg/ml weight $AgNO_3$ = 0.4 mg/ml x1.59 =0.636 g/l

   $AgNO_3$ /Ag = 170/107 = 1.59

$$mol = g/MW$$

$$mol = 0.636(g/l)/169.87(g/l) = 3.8 \text{ mM}$$

   Ratio of $AgNO_3$: capping agent = 1: 0.01- 1: 1.2 mM

   Ratio of $AgNO_3$: capping agent = 1: 1- 1: 10 mM

   Prepared $AgNO_3$ $\longrightarrow$ g = MW x V x M (molecular weight x volume x molarity)

   Prepared stock solution of $AgNO_3$ 10 mM   g = 170 x 0.2 x 0.01

$$g = 0.338 \text{ in 200 ml DDI water}$$

   Capping agent

   Ratio of $AgNO_3$: Capping agent

   1: 0.01, 1: 0.06, 1: 0.1, 1: 0.3, 1:0.6, 1:1.2 mM

   (3.8: 0.038), (3.8: 0.228), (3.8: 0.38), (3.8: 1.14), (3.8: 4.56) mM

   Ratio of $AgNO_3$: $NaBH_4$

   1: 1, 1:5, 1:10 mM

   (3.8: 3.8), (3.8: 19), (3.8: 38)

   Prepared stock solution of 4.56 mM sodium alginate

$$g = MW \text{ x V x M (molecular weight x volume x molarity)}$$

$$g = 198 \text{ x 0.2 x 0.00456}$$

$$g = 0.180 \text{ in 200 ml DDI water}$$

   Prepared stock solution of 4.56 mM poly (4-styrenesulfonic acid-co-maleic acid) (Copss)

$$g = MW \text{ x V x M (molecular weight x volume x molarity)}$$

g = 344 x 0.2 x 0.00456

g = 0.313 in 200 ml DDI water

Dilution of capping agent by DDI water form 4.56 mM to 0.038, 0.228, 0.38, 1.14 and 2.28 mM

Prepared stock solution of 38 mM $NaBH_4$
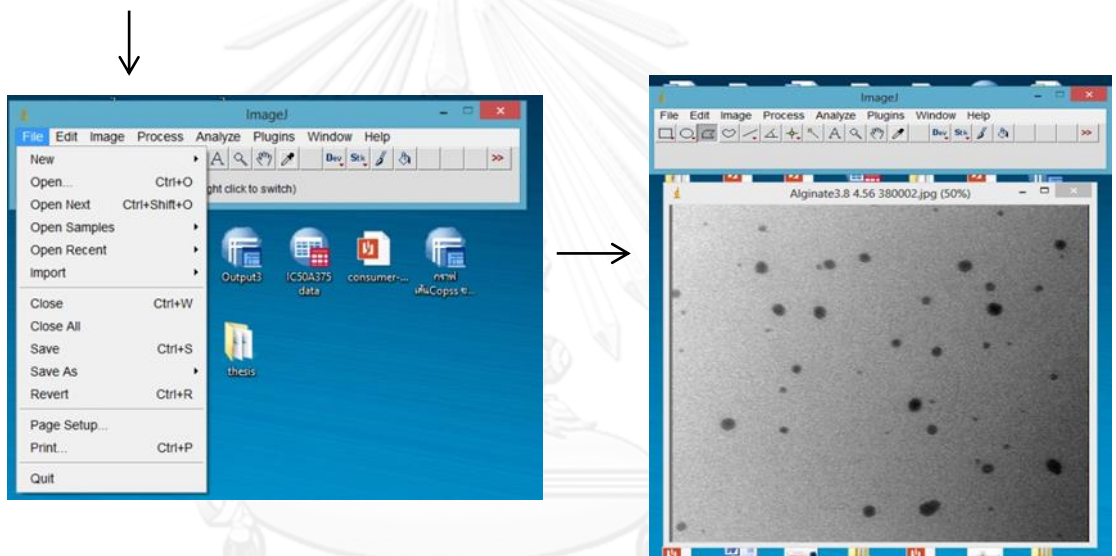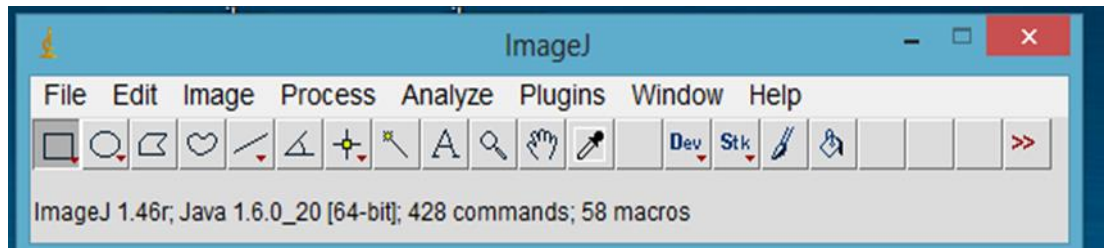
g = MW x V x M (molecular weight x volume x molarity)

g = 37.83 x 0.2 x 0.038

g = 0.287 in 200 ml DDI water

Dilution of $NaBH_4$ by DDI water form 38 mM to 19 and 3.8 mM

2. ImageJ analysis

Analyse ⟶ set scale

Measure ⟵ Analyse
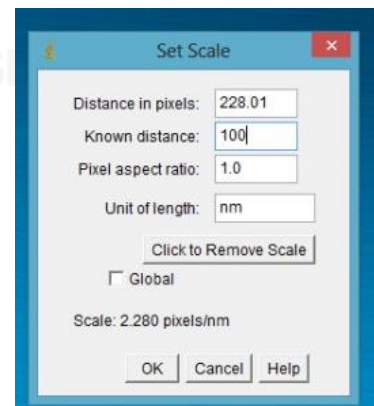
Graph (size distribution)

100 particles (100%)

VITA

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