EVALUATION OF REACTIVE OXYGEN SPECIES GENERATION AND DNA METHYLATION CHANGE IN LINE-1 AND ALU INDUCED BY NANOPARTICLES IN HEK293 AND HACAT CELLS

Miss Siwapron Nilyai

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2014

การศึกษาผลของอนุมูลอิสระจากการกระตุ้นด้วยอนุภาคระดับนาโนเมตร ต่อการเปลี่ยนแปลงระดับเมทิลเลชันของ LINE-1 และ Alu ในเซลล์ไต Hek293 และเซลล์ผิวหนัง HaCaT

นางสาวศิวาพร นิลใย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	EVALUATION OF REACTIVE OXYGEN SPECIES GENERATION AND DNA METHYLATION CHANGE IN LINE-1 AND ALU INDUCED BY NANOPARTICLES IN HEK293 AND HACAT CELLS
By	Miss Siwapron Nilyai
Field of Study	Medical Science
Thesis Advisor	Assistant Professor Amornpun Sereemaspun, M.D., Ph.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Medicine

(Associate Professor Sophon Napathorn, M.D.)

THESIS COMMITTEE

.....Chairman

(Professor Apiwat Mutirangura, M.D., Ph.D)

.....Thesis Advisor

(Assistant Professor Amornpun Sereemaspun, M.D., Ph.D.)

.....Examiner

(Associate Professor Chintana Chirathaworn, Ph.D.)

.....Examiner

(Associate Professor Kanya Suphapeetiporn, M.D., Ph.D)

.....External Examiner

(Associate Professor Parin Chaivisuthangkura, Ph.D.)

ศิวาพร นิลใย : การศึกษาผลของอนุมูลอิสระจากการกระตุ้นด้วยอนุภาคระดับนาโนเมตรต่อการเปลี่ยนแปลงระดับเมทิลเลชันของ LINE-1 และ Alu ในเซลล์ไต Hek293 และเซลล์ผิวหนัง HaCaT (EVALUATION OF REACTIVE OXYGEN SPECIES GENERATION AND DNA METHYLATION CHANGE IN LINE-1 AND ALU INDUCED BY NANOPARTICLES IN HEK293 AND HACAT CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ.อมรพันธุ์ เสรีมาศพันธุ์, หน้า.

ปัจจุบันวัสดุระดับนาโนเมตรโดยเฉพาะอนุภาคระดับนาโนเมตรถูกนำมาใช้อย่างแพร่หลายในหลายด้าน รวมไปถึงการนำมาประยุกต์ใช้ทางการแพทย์ อย่างไรก็ตามยังจำเป็นต้องมีการศึกษาถึงความเป็นพิษของอนุภาคระดับนาโนเมตรก่อนที่จะถูกนำมาใช้กับสิ่งมีชีวิต การศึกษาที่ผ่านมาพบว่าอนุภาคระดับนาโนเมตรสามารถสร้างความเป็นพิษต่อเซลล์ได้ทั้งในทางตรงและทางอ้อม และมักพบว่ามีการกระตุ้นการสารอนุมูลอิสระเพิ่มมากขึ้น ซึ่งอาจเป็นสาเหตุทำให้เกิดความเสียหายต่อสารชีวโมเลกุลภายในเซลล์และยังอาจมีผลเปลี่ยนแปลงดีเอ็นเอเมทิลเลชัน โดยในงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของสารอนุมูลอิสระที่เป็นผลมาจากอนุภาคระดับนาโนเมตรต่อการเปลี่ยนแปลงของดีเอ็นเอเมทิลเลชันทั่วจีโนมและดีเอ็นเอเมทิลเลชันของชิ้นส่วนดีเอ็นเอที่สามารถเคลื่อนที่ได้ชนิดไลน์วันและอลู โดยทำการศึกษาในเซลล์ไตและผิวหนังที่ได้รับอนุภาคทองคำระดับนาโนเมตร, อนุภาคซิลิการะดับนาโนเมตร และอนุภาคไคโตซานระดับนาโนเมตร ผลการศึกษาพบว่ามีเพียงอนุภาคซิลิการะดับนาโนเมตรที่ทำให้สารอนุมูลอิสระเพิ่มมากขึ้นอย่างมีนัยสำคัญ และเกิดขึ้นเฉพาะในเซลล์ผิวหนังเท่านั้น การศึกษาระดับเมทิลเลชันทั่วจีโนมพบว่ามีระดับเมทิลเลชันลดลงในเซลล์ผิวหนังที่ได้รับอนุภาคซิลิกาและอนุภาคไคโตซานระดับนาโนเมตร และยังพบการเปลี่ยนแปลงเดียวกันนี้ในดีเอ็นเอเมทิลเลชันของชิ้นส่วนดีเอ็นเอที่สามารถเคลื่อนที่ได้ชนิดอลูในเซลล์ผิวหนัง ทั้งนี้ไม่พบการเพิ่มขึ้นของสารอนุมูลอิสระและการเปลี่ยนแปลงระดับเมทิลเลชันในเซลล์ไตที่ได้รับอนุภาคระดับนาโนเมตรทุกชนิด นอกจากนี้ยังพบการผันกลับของดีเอ็นเอเมทิลเลชันของชิ้นส่วนดีเอ็นเอที่เคลื่อนที่ได้ชนิดอลู ในเซลล์ผิวหนังที่ได้รับสารต้านอนุมูลอิสระก่อนได้รับอนุภาคระดับนาโนเมตร ผลการศึกษาแสดงให้เห็นว่าเมทิลเลชันของชิ้นส่วนดีเอ็นเอที่เคลื่อนที่ได้ชนิดอลู สามารถแสดงถึงระดับเมทิลเลชันทั่วจีโนมในเซลล์ที่ได้รับอนุภาคระดับนาโนเมตรได้ และผลของอนุภาคระดับนาโนเมตรต่อการเปลี่ยนแปลงระดับเมทิลเลชันทั่วจีโนมและเมทิลเลชันของชิ้นส่วนดีเอ็นเอที่เคลื่อนที่ได้ในการศึกษาครั้งนี้ พบว่าไม่ขึ้นอยู่กับระดับสารอนมูลอิสระ และเป็นผลที่เกิดขึ้นแบบจำเพาะต่อชนิดของเซลล์

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5474163630 : MAJOR MEDICAL SCIENCE
KEYWORDS: NANOPARTICLES / EPIGENETICS / DNA METHYLATION / LINE-1 / ALU / NANOTOXICITY

SIWAPRON NILYAI: EVALUATION OF REACTIVE OXYGEN SPECIES GENERATION AND DNA METHYLATION CHANGE IN LINE-1 AND ALU INDUCED BY NANOPARTICLES IN HEK293 AND HACAT CELLS. ADVISOR: ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D., pp.

Engineered nanoparticles (ENPs) are one of the most nanomaterials that wildly used in various fields including biomedical applications. However, the adverse effects of ENPs on health risk still need to be concerned. ENPs have been reported to be a matter that cause cellular damage through either direct or indirect, cellular oxidative stress is one of the most nanotoxicity have been found. Reactive oxygen species (ROS) is a cause of cellular oxidative stress that leads to intracellular macro molecules damages and may impact on DNA methylation changes. In this study we aim to investigate the effect of ROS induced by ENPs on DNA methylation that is one important of epigenetic mechanisms. Human embryonic kidney (Hek 293) and human keratinocyte (HaCaT) cells were used as model to expose with three different types of ENPs, gold nanoparticles (AuNPs), Silica nanoparticles (SiNPs) and Chitosan nanoparticles (CSNPs). First, we evaluated cytotoxicity of cells by measuring viability, morphology and ROS levels. Global DNA methylation levels were measured by 5-methylcytosine immunocytochemistry staining, and we also investigated DNA methylation levels of retrotransposable elements, LINE-1 and Alu by using combined bisulfite restriction analysis technique (COBRA). We found ROS level was increased in SiNPs exposed HaCaT cells only. DNA hypomethylation of global and Alu elements was showed in cells were exposed with SiNPs and CSNPs in HaCaT cells only. LINE-1 did not change in both of Hek293 and HaCaT cells. Furthermore, the inversion of Alu DNA methylation level in HaCaT cells exposed with SiNPs and CSNPs was found in antioxidant (N-acetyl cysteine) pretreated cells. Our study demonstrated the new insight that DNA methylation of Alu elements represents the global DNA methylation of cell exposed with ENPs. In this study, the alteration of DNA methylation level in ENPs exposed cells is ROS independent and specific to cell types.

Field of Study: Medical Science Student's Signature .....

Academic Year: 2014 Advisor's Signature .....

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere thanks to my advisor, Assistant Professor Amornpun Sereemaspun for his encouragement, guidance and supervision. I am most grateful for his teaching and advice, not only the research methodologies but also other methodologies in life.

I would like to thank Professor Apiwat Mutirangura and Center of Excellence in Molecular Genetics of Cancer and Human Diseases laboratories for providing the information, protocol and teaching the technique for COBRA experiment.

I would like to thank Mr.Preecha Rengvatchworachai and Ms.Vanida Buasorn for their assistance to success immunocytochemistry staining experiment.

I would like to thank my lab colleagues at Nanobiomedicine Laboratory for their friendship, helping and encouragement throughout my research.

In addition, I am grateful for encouragement from my parent throughout the period of my study.

Finally, I gratefully acknowledge the financial supports from THE 90th ANNIVERSARY OF CHULALONGKORN UNIVERSITY FUND (Ratchadaphiseksomphot Endowment Fund) and The National Research Council of Thailand (NRCT).

CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I
BACKGROUND

Currently, nanotechnology is increasingly received attention since it can be applied across various fields. The definition of materials in nanoscale is at least one dimension should smaller than 100 nm [1]. Engineered nanoparticles (ENPs) are one of the most nanomaterials that wildly used in cosmetics, food additives, etc. and including biomedical applications [2-4]. Although ENPs have more advantages than their bulk size counterparts, adverse effects of ENPs on health risk still need to be concerned. The effects of ENPs on cellular response have been investigated in multifaceted toxicity, and the level of violence often depends on their properties [5-7] such as size, shape, charge and chemical composition. Interestingly, previous studies found that ENPs have ability to create toxicity by increasing level of reactive oxygen species (ROS) [8-11]. ROS have been reported as causes of intracellular macro molecules damages, inflammatory response induced and may impact on epigenetic changes [12].

DNA methylation is an important inheritable epigenetic mechanism that participates in control gene expression without alteration of nucleotide sequences. DNA methylation is adding a methyl group to cytosine base ring in CpG sequences. It plays an essential role in several cellular mechanisms such as gene regulation, differentiation control, chromosome inactivation and mobile elements silencing [13] as well as participates in diseases for instance various types of cancers [14]. In addition, DNA methylation also has been discussed as an important process to maintain the stability of genome [15].

DNA methylation of interspersed nuclear elements, especially LINE-1 and Alu, have been referred into representing global DNA methylation because amount of them account for 30% of the genome [16]. Long interspersed nuclear element-1 (LINE-1) contains about 6,000 base pairs of full length and dispersed around 600,000 copies account for 17% of the genome. On the other hand, Alu is the most abundant of short interspersed nuclear elements (SINEs), they distribute about million copies

approximately 11% of genome. Previous studies reported that the alteration of LINE-1 and Alu DNA methylation level correlated with diseases especially various types of cancers and autoimmune diseases [17]. Moreover, DNA methylation changes of LINE-1 and Alu have been found in models that being exposed by various environment toxicants such as lead, benzene and ultrafine air pollution particles [18-20]. For ENPs, previous study showed that silica nanoparticles (SiNPs) decrease global DNA methylation levels, change the expression of DNA methylation related genes [21] as well as induced promoter hypermetylation of repair gene [22] in human keratinocyte cells (HaCaT) but effects of ENPs on DNA methylation of transposable elements still have not been investigated.

The objective of this study was to determine the changes of LINE-1 and Alu DNA methylation levels induced by ROS from three different types of ENPs. Gold nanoparticles (AuNPs), silica nanoparticles (SiNPs) and chitosan nanoparticles (CSNPs) were selected to use in this study. All of the ENPs exposed to two different cells, human keratinocyte (HaCaT) and human embryonic kidney (Hek293). We assessed the ROS generation level after cells were exposed to ENPs and measured global DNA methylation level by 5-methylacytosine (5-mc) immunocytochemistry. We also evaluated DNA methylation level of retrotransposable elements, LINE-1 and Alu by combined bisulfite restriction analysis (COBRA) methods. To clarify the role of ROS on DNA methylation changes, we compared the results between ENPs exposed cell with and without antioxidant.

CHAPTER II
LITERATURE REVIEW

Engineered nanoparticles (ENPs)

Nanotechnology is increasingly received more attention, so lead to nanomaterials are applied in various field including industry, science and medicine. Nanomaterials are substance range in size between 1 to 100 nanometer [23] and can be engineered in different of types depend on chemical properties or shape such as nanofiber, carbon nanotube, gold nanoparticles, silver nanoparticles, silica nanoparticles etc. The small size characteristic of nanomaterials creates the unique properties that more potential useful than their bulk size counterparts. Engineered nanoparticles (ENPs) are one of the most popular nanomaterials that have been used to apply in various objectives. In medical field, ENPs have been reported to use in drug carrier, gene therapy and imaging [4]. In this study, we selected the ENPs that different in chemical properties including silica nanoparticles (SiNPs), gold nanoparticles (AuNPs) and chitosan nanoparticles (CSNPs) that were popular to apply in medical application.

Silicon dioxide nanoparticles (SiNPs), non-metal oxide, have been mainly used as additive to printer toners, drugs and cosmetics [24] and because of the goods properties such as high stability, biocompatibility and immunogenicity, SiNPs have been chosen to apply in many biomedical applications e.g. biomarkers, cancer therapy, DNA and drug delivery, immunological adjuvant [25-28].

Gold nanoparticle (AuNP) is one of the most metal nanoparticles that more interesting to use in science and technology. AuNPs show a high stability and can be easy synthesized in laboratories. Gold bulk size is gold color but gold in nano-size show in red, blue, brown or green depend on size and shape [29]. Gold in particles

shape and size range in nanoscale (1-100 nm) usually appear in red color. Previously, AuNPs were applied in biomedicine field, preferably for drug delivery, cancer therapy and contrast reagent for molecular imaging [30].

Chitosan (CS) is polysaccharide can obtain from acetylation of chitin. CS found on a structural element in the exoskeleton of crustaceans (eg. crab and shrimp) and also found in yeast fungi cell wall and some insect [31, 32]. However, it is not found in mammalian that have lysozyme and N-acetyl-D-glucosamidase from macrophage for CS degradation [33]. Chitosan nanoparticles (CSNPs) is one of the most nanoparticles that widely developed to use as drug delivery [34, 35] because their biodegradable property.

Toxicity of engineered nanoparticles

Although nanotechnology shows several of advantages and widely to be applied in various fields, adverse effects of nanomaterials on human health and environment risk also need to be concerned. Nanomaterials have more special function than their bulk size counterparts but toxicity of nanomaterial to damage cells is also found in both direct and indirect pathways such as cytotoxicity, oxidative stress and inflammatory response [36].

Interestingly, previous studies that emphasized on toxicity of nanoparticles in various model showed the increasing amount of reactive oxygen species (ROS) [10, 11, 37]. ROS production may indicate to act as the initiate situation of toxicity in nanoparticles exposed cells [5].

Reactive oxygen species (ROS) is one type of free radicals that refer to unstable molecules because they consist of unpaired electrons. Increasing of free radical and decreasing of antioxidant result in cellular oxidative stress. Sources of ROS are not only from endogenous including mitochondrial respiration, inflammation etc. but also from exogenous source such as air pollution and nanomaterials exposure. As mention above, previous studies have been reported that various kinds of nanomaterials showed potential to generate ROS production level.

The following context briefly shows the effect of ROS generation by nanoparticles from the previous studies.

In 2009, Fen wang and colleague reported that silica nanoparticle size 20 and 50 nm can toxic to human embryonic kidney cells (HEK293) and both sizes of nanoparticle can generate reactive oxygen species and they found the toxicity of silica nanoparticle shows by size- and dose-dependent manner [38]. Moreover, in 2010 Youn-Jung Kim and college studied the toxicity of three types of silica dioxide

nanoparticles that difference in size, stabilizer and coating materials. They found two from three types of SiNPs showed inducing of intracellular reactive oxygen species production by dose-dependent manner at 48 h incubation time in human neuronal cell line (SH-SY5Y) [7].

In 2008, Jasmin J. Li and college showed the results of oxidative stress induced by gold nanoparticles in MRC-5 human lung fibroblasts and also found autophagy of cell, they presume that may be a cellular defense mechanism against to oxidative stress [39]. Furthermore, in 2012, Paino and college reported the cytotoxicity and genotoxicity of two types of gold nanoparticles in human hepatocellular carcinoma cells (HepG2) and peripheral blood mononuclear cells (PBMC). The intracellular reactive oxygen species was found significant increasing in both HepG2 and PBMC [40].

Zebrafish embryo exposure to CSNPs has been reported to found hatching rate and increased mortality by concentration-dependent. Moreover, CSNPs is caused of malformations such as a bent spine, pericardial edema, and an opaque yolk in zebrafish embryos. Furthermore, embryos that exposed to chitosan nanoparticles showed increasing of rate of cell death, reactive oxygen species, and overexpression of heat shock protein 70, indicating that CSNPs can cause physiological stress in zebrafish [41].

Epigenetics and DNA methylation of transposable elements

Epigenetics is the heritable mechanism of cell that changes gene expression by without alter DNA sequence [42]. The fundamental key mechanisms include DNA methylations, Histone tail modifications and RNA-mediated mechanisms. However, the complicate role of epigenetic mechanism not yet completely understood. Among epigenetic mechanisms, DNA methylation is one of the most studied mechanisms, because it can be detected easily than the other mechanisms of epigenetics and has been known basic information more than remaining mechanisms. DNA methylation, furthermore, participates in control of gene expression and also plays a key role in transposable element control as well as maintaining stability of genome [43]. DNA methylation has been reported to use as biomarkers in several diseases, especially cancers [44]. Particularly, DNA methylation of transposable elements has been found to be used as markers for cancers [17], aging [45], environmental toxic exposure [18-20] and represent stability of the genomes [43]. Previous reports demonstrated the association between hypomethylation of transposable elements in many cancer cells and their counterpart normal cell types.

The component of human genome excluding transcribe region also encompasses repetitive sequences that normally not transcribe but contain nearly half of the DNA content, some of them were known as transposable elements. Transposable element can be divided into two main classes [46], DNA transposons are move by cut from one site to paste into new genome site, while retrotransposons are move by copy into RNA and transcribe into DNA to insert in new location of genome. DNA transposons in genome are found only 3% and in the present, they not mobile in human genome. In contrast with DNA transposon, retrotransposon consists approximately 45% of the human genome and they are distinguished by LTR (long terminal repeat) existence, LTR retrotransposons are

mainly represented by families of human endogenous retroviruses (HERVs) that currently inactivity in human, as non-LTR retrotransposons are the most abundant transposon element contain in human genome [47]. Long interspersed nuclear element-1 (LINE-1) is notable autonomous retrotransposon, have ability to moving by itself, that contains around 17% of human genome. Full length of LINE-1 sequences contains about 6,000 base pairs and they spread approximately 600,000 copies in genome [48]. However, most of LINE-1 are inactivated resulting from truncating or by other inactivate mechanism. The DNA methylation level of LINE-1 is the most studied and has been found the correlation with cancer. Most of cancer types have been found hypomethylation of LINE-1 compared to normal cell while some cancer have not been found changing in LINE-1 methylation level [49]. Therefore, LINE-1 hypomethylation may be a potential marker for several cancer types but not specific to cancer.

Alu, predominantly short interspersed nuclear elements (SINEs), is another one of the most studied retrotransposon contains approximately 300 base pairs in length [50] and spreads about million copies, account for approximately 11% [51], in human genome. Contrast to LINE-1, Alu is non-autonomous retrotransposon that lack of efficacy to move by itself. Alu element requires the essential enzymes encoded by autonomous retrotransposon or host in order to activate mobility [43]. Previously, DNA methylation level of Alu element has been observed and demonstrated hypomethylation in some cancer [52-54].

The studies in some cancer types have been found hypomethylation of both LINE-1 and Alu, beside cancer, hypomethylation level of retrotransposon also associated with other conditions such as aging [45] and expose to environment toxicant [18, 19]. However, methylation change of retrotransposon is type-specific because previous studies shown Alu and HERV-K express hypomethylation but not

LINE-1 in aging[17]. Moreover, DNA methylation level of LINE-1 and Alu are increasing being represented the global DNA methylation because they spread largest proportion of DNA methylation in genome [16].

Table 1 Classes of mobile elements in human genome. (Modified from Wilson et al., 2007) [51]

Classes of Mobile elements	DNA transposons	Retrotransposons and endogenous retroviruses		
		LTR	Non-LTR	
			Autonomous	Non-autonomous
Length	80-3,000 bp	Range = 1.5-11 kb	4-6 kb (full length)	100-300 bp (full length)
Fraction of human genome	3%	8%	21%	SINEs = 11-13%
Number in human genome	300,000	Autonomous LTR = 450,000 Nonautonomous LTR = 40,000-100,000	850,000	SINEs = 1,500,000
Examples	Mariners, Tcl elements and mariner-like elements	Autonomous=HERVs; Non-autonomous=MaLR	LINEs	SINEs=Alu

Effect of nanoparticles on epigenetic mechanisms

The effects of nano-sized materials on epigenetic mechanisms have been reported a few in last decade years. Majority of studies focus on epigenetic changes induced by pollution particles such as particle matter (PM), black carbon, and diesel exhaust particles (DEP) and previous studies show in Table 2. The effects of ultrafine particles on retrotransposon DNA methylation changes have been found especially LINE-1 and Alu.

A few studies of ENPs effect on epigenetic mechanisms have been reported and are summarized in Table 3. Interestingly, the only one study reported the effect of ENPs on global DNA methylation that showed SiO_2 NPs cause global DNA hypomethylation in HaCaT cells [21].

Table 2 Effect of ultrafine particles on epigenetic mechanisms. (Modified from Stoccoro et al., 2013) [42]

Study model	Particle	Epigenetic effect	Reference
DNA methylation			
Blood cells	$PM_{2.5}$, black carbon	PM2.5 and black carbon associated with hypomethylation of LINE1	Baccarelli et al. (2009) [18]
Blood cells	$PM_{2.5}$, black carbon	Prolonged exposure to black carbon associated with hypomethylation of LINE1 and Alu	Madrigano et al. (2011) [19]
Blood cells	$PM_{2.5}$, particle number, black carbon	Effect from air pollution (inflammation, coagulation, etc.) was stronger among subjects having higher Alu, but lower LINE-1, tissue factor (F3), or Toll-like receptor 2 (TLR-2) methylation status	Bind et al. (2012) [55]
Blood cells	PM_{10}, metals	PM10 associated with lower LINE1 and Alu methylation. iNOS methylation was significantly lower in postexposure	Tarantini et al. (2009) [20]

		blood samples (after 3 working days) compared with baseline	
Buccal cells	$PM_{2.5}$	Increased 7-day average PM2.5 exposure was associated with lower iNOS methylation	Salam et al. (2012) [56]
Blood cells	Air pollution, $PM_{2.5,}$ PM_{10}	Increased exposure to ambient air pollution was associated with hypermethylation of the Foxp3 locus	Nadeau et al. (2010) [57]
Blood cells	PM_{10}, PM_{1}, various metals	Promoter DNA methylation levels of APC and p16 were higher in post-exposure samples compared to the levels in baseline samples. Mean levels of p53 or RASSF1A promoter methylation was decreased	Hou et al. (2011) [58]
C57BL/CBA mice (Sperm)	Air pollution particles near steel mill and highway	Sperm DNA was hypermethylated in mice breathing air particles when compared to HEPA-filtered air, and this change persisted following removal from the environmental exposure	Yauk et al. (2008)
BALB/c mice (CD4+ cells)	DEP	Diesel particle exposure resulted in hypermethylation of the IFNG promotor and hypomethylation of IL4 promoter in CD+ cells	Liu et al. (2008)
Mice and cultured lung cells	$PM_{2.5}$	PM 2.5 led to increase expression of the DNA methyltransferase 1 (DNMT1), and methylation of the p16 promoter in mice and cells.	Soberanes et al. (2012)
Histone modification			
Histone modifications Blood cells (Steel plant workers)	PM_{10}, PM_{1}, various metals	H3K4me2 and H3K9ac increased in association with years of employment in the steel plant. No clear relation to exposure to total mass of PM10 or PM1 but to inhalable nickel and arsenic.	Cantone et al. (2011)
A549 cell line	PM_{10}	PM10 induced histone H4 acetylation	Gilmour et al.

		at the IL8 promoter as well as increased IL8 expression.	(2003) [59]
BEAS-2B cells	DEP	Diesel particle exposure led to increased histone H4 acetylation at the COX2 promoter as well as increased COX2 expression.	Cao et al. (2007) [60]
mRNA expression			
Human primary bronchial epithelial cells	DEP	Diesel particle exposure led to changes in miRNA expression; miR-513, miR-494 and miR-923 were up-regulated whereas miR-96 was down-regulated	Jardim et al. (2009) [61]

Table 3 Effect of engineered nanoparticles (ENPs) on epigenetic effects. (Modified from Stoccoro et al., 2013) [42]

Study model	ENPs	Epigenetic effect	Referenece
DNA methylation			
HaCaT cell line	SiO2 NPs	Global DNA hypomethylation and DNMT1, DNMT3a and MBD2 mRNA repression	Gong et al. (2010) [21]
HaCaT cell line	SiO2 NPs	PARP-1 hypermethylation and PARP-1 mRNA repression	Gong et al. (2012) [22]
Histone modifications			
MCF-7	CdTe QDs	Global hypoacetylation	Choi et al. (2008) [62]
C57BL/6BomTac mice	TiO2NPs	Upregulation of miR-449a (6 fold), miR-1 (2.6 fold), and miR-135b (60 fold)	Halappanavar et al. (2011) [63]
miRNA expression			
MRC5 cell line	AuNPs	Upregulation of miR155 with concomitant down-regulation of PROS1 gene; chromatin condensation	Ng et al. (2011) [64]
NIH/3T3 cells	MW-CNTs	Deregulation of miRNA expression	Li et al. (2011) [65]
NIH/3T3 cells	CdTe QDs	Global alteration miRNAs expression patterns	Li et al. (2011) [66]
Nicotiana tabacum	Al2O3NPs	Increased expression of many miRNA such as miR395, miR397, miR398, and miR399	Burklew et al. (2012) [67]

CHAPTER III
MATERIALS AND MEDTHODS

Cell culture

Human embryonic kidney cells (Hek293) and human keratinocyte cells (HaCaT) were growth in Dulbecco's Modified Eagle Medium (DMEM; Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, USA) and 1% (v/v) penicillin/streptomycin (Gibco, USA). The cells were grown in 37 °C and 5% carbon dioxide (CO_2) incubator (Esco, Singapore).

Engineered nanoparticles (ENPs) preparation and Characterization

Gold nanoparticles (AuNPs) were synthesized by reaction of 1 ml of 1% Gold (III) chloride trihydrate ($HAuCl_4.3H_2O$), 12 ml of 0.0202 mM sodium acetate and 37 ml of Milli-Q water. All components were stirred at constant temperature 75 – 95 °C for 2h. The AuNPs solution will appear in red-wine color. Silica nanoparticles (SiNPs) LUDOX® AM (aqueous suspensions in H_2O) were purchased from Sigma-Aldrich (Sigma, USA). Chitosan nanoparticles (CSNPs) were synthesized and provided from the Chitin Research Center, Chulalongkorn University, Thailand. All ENPs were characterized by Zetasizer Nano Series (Malvern instruments Ltd, UK) in order to measure total electric charge on surface and stability of particles. The size and shape of ENPs were observed by transmission electron microscopy (TEM; Hitachi, Japan).

Cell Viability

Viability of cell was assessed by PrestoBlue® (Invitrogen, USA) which resazurin bases reagent, non-fluorescent form of PretoBlue® will turn into strong fluorescent form after accepting electrons from molecule in cellular respiration that contains in living cells. Briefly, the cells were plated in 96-well plates and challenged with ENPs

for 72 h. After complete the exposure time, PrestoBlue® were added and then, the plate was incubated at 37°C for 30 min. Fluorescence intensity was detected at emission 495 nm and excitation 530 nm using Varioskan flash microplate reader (Thermo scientific, USA). Percentage of cell viability was calculated by compared to control group (non-treated cells).

Cell morphology

Hek 293 and HaCaT cells were seeded in 24-well plate. After the cells attached to the bottom, ENPs were treated for 72 h. The cell morphology was observed by using phase contrast inverted microscope (Nikon, Eclipse TS 100, Japan).

Intracellular reactive oxygen species generation measurement

The formation of intracellular reactive oxygen species (ROS) induced by ENPs was determined using 2',7'- dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, USA) which non-fluorescent and will be removed acetate groups by intracellular esterase into DCFH. DCFH will turn into DCF that express fluorescent signal after challenge to reactive oxygen species. Stock of H_2DCFDA (10 mM) was prepared in DMSO and diluted with DMEM without serum 1000-fold to use as working concentration .Briefly, cells in 96-well plate were washed with PBS twice and added working H_2DCFDA to each well. After incubate at 37°C for 30 min in dark place, the cells were washed with PBS twice and treated with nanoparticles. Fluorescence intensity was detected after 3h and 6h at emission 485 nm and excitation 528 nm using Varioskan Flash microplate reader (Thermo, UK). Percentage of ROS generation was calculated by normalize to control group.

Immunocytochemical staining of 5-methylcytosine

Cells were exposed with ENPs under indicated condition, then low melting points agarose gel was used to forming gel blogs. Briefly, gel blocks that contain cells were fixed by formalin. Then, blocks of gel were paraffin-embed and sections (5μM). Sections were dewaxed in xylene and rehydrated using ethanol. Sections were antigen retrieval in a microwave oven (full power) for 10 min in citric acid (pH6). Then the slides were immersed in hydrochloric acid at room temperature for 15 minutes in order to expose the CpG dinucleotides. To quench endogenous peroxidase activity, the sections were then treated with 3.0% hydrogen peroxide for 4 min. Then, the sections were incubated for 60 min at room temperature with a commercial antibody against 5-methylcytosine (5-mc) to assess global DNA methylation (Abcam, UK) and were incubated for 60 min with secondary antibody (HRP with polymer). Then, the sections were incubated with 3,3'-diaminobenzidine (DAB) and counterstained with haematoxilin. The sections were scanned by Axio Scan.Z1 (Zeizz, Germany) and random fields were selected in order to count by Adobe Photoshop program (CS6). Global methylated cells showed in brown color and global unmethylated cells showed in blue color. Cells were directly counted from 10 random-fields of each slide. The percentage of methylation was calculated from methylated cells out of total cell counted.

Combined Bisulfite Restriction Analysis (COBRA)

COBRA is a standard technique and simple method for detection methylation level of interspersed repetitive nuclear elements [45, 68]. Cells were collected after time completed incubation with under indicated nanoparticles condition and DNA was extracted by using the QIAamp® DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Bisulfite modification of genomic DNA was performed by using EZ DNA methylation Gold kit (Zymo Research, Orange, CA) as

described by the manufacturer. DNA treated bisulfite was amplified by polymerase chain reaction (PCR) of LINE-1 and Alu. Primers were specific to LINE-1 (F: 5'-CCG-TAA-GGG-GTT-AGG-GAG-TTT-TT-3' and R: 5'-RTA-AAA-CCC-TCC-RAA-CCA-AAT-ATA-AA-3') [49] and Alu (F: 5'- GGCGCGGTGGTTTACGTTTGTAA-3' and R: 5'-CAC-CAT-ATT-AAC-CAA-ACT-AAT-CCC-GA-3') sequences, PCR condition consist of pre-denaturation 95°C 15 minutes, 35 cycles of denaturation 95°C 1 minute, annealing 55°C for LINE-1 and 53°C for Alu 1 minutes, extension 72°C 1 minute and then final extension 72°C 7 minutes. The PCR products of LINE-1 (160 bp containing 2 CpG dinucleotides) were cut by 1U of TaqI and TasI restriction enzyme at 65 °C overnight and separated on 8% non-denaturating polyacrylaminde gels, stained with SYBR Green and visualized under STORM scanner. The PCR products of Alu (133 bp containing 2 CpG dinucleotides) was performed the same as LINE-1 that described above. Total DNA methylation level of LINE-1 and Alu were calculated as previously described [69].

Statistical analysis

The data of all experiments were performed as mean ± SD using GraphPad Prism 5 and comparisons between groups were analyzed by using *ANOVA* (Analysis of variance). The differences among groups were analyzed by Tukey's multiple comparison tests. The comparison between two groups was analyzed by unpaired *t-test*. *P value < 0.05* was considered as significant.

CHAPTER IV
RESULTS AND DISCUSSION

Characterization of engineered nanoparticles

To estimate size and dispensation of nanoparticles before using in the experiments, gold, silica and chitosan nanoparticles (AuNPs, SiNPs and CSNPs) were measured morphology and primary diameter by transmission electron microscope (TEM). Morphology of all the ENPs used in this study was spherical (Figure 1 A-C) and approximate size of AuNPs, SiNPs and CSNPs showed 23, 17 and 54 nm, respectively. The zeta potential was measured by Zetasizer was shown in Table 4. AuNPs and SiNPs showed negatively charge whereas CSNPs showed positively charge and SiNPs that commercial nanoparticles showed the greatest stability among three nanoparticles used in this study.

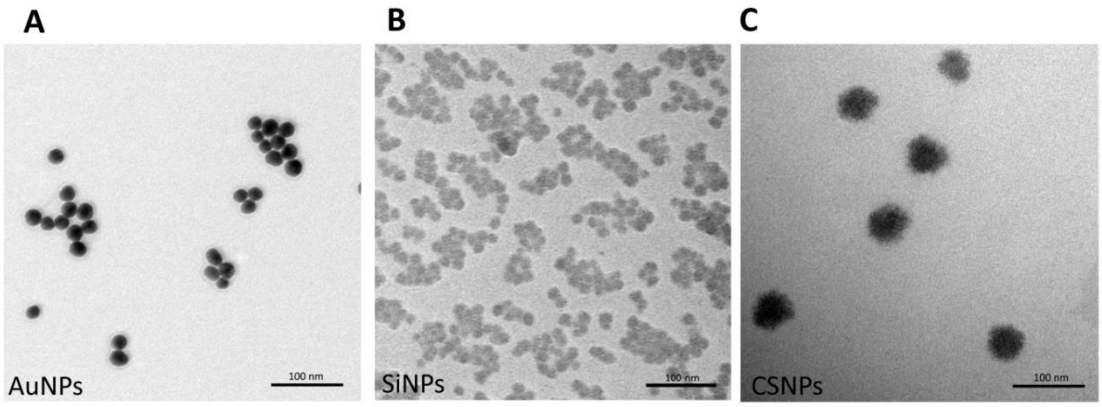

Figure 1.Transmission Electron Microscopic (TEM) images of engineered nanoparticles. TEM images of gold nanoparticles (AuNPs), silicon nanoparticles (SiNPs) and chitosan nanoparticles (CSNPs) were shown in A, B and C, respectively. Scale bars: 100 nm.

Table 4 Size distribution and zeta potential value of engineered nanoparticles.

Nanoparticles	Average size (nm)	Zetapotential (mV)
Gold nanoparticles (AuNPs)	23.53	-8.14
Silicon nanoparticles (SiNPs)	17.21	-29
Chitosan nanoparticles (CSNPs)	54.67	0.073

The Effect of ENPs on cellular viability

In this study, we used human embryonic kidney (Hek293) and human keratinocyte (HaCaT) cells as model because they have been used in nanotoxicity investigation. Kidney was mentioned as a secondary target organ of nanoparticles while keratinocyte is skin cells that act as a barrier area regularly expose with environment.

The viability of cells was performed to assess the cytotoxicity of ENPs in different concentrations and chemical compositions on Hek293 and HaCaT cells. Cells were exposed with AuNPs and SiNPs for 24 and 72 h (CSNPs exposed only 72h) and were measured the viability by PrestoBlue reagent that reduced metabolically active cells.

The data of all nanoparticles treated groups were compared into percentage of control (only media treated cells). Viability of Hek293 and HaCaT cells is shown in figure 2A - C and 2D - F, respectively. The viability of Hek 293 cells was significantly decreased after exposure with AuNPs at 80 and 100 ug/ml (both 24 and 72 h) showed in figure 2A, whereas exposure with SiNPs was significantly decreased at 50, 80 and 100 ug/ml only at 72h, figure 2B. In HaCaT, viability was significantly decreased only cells treated with AuNPs at 50, 80 and 100 ug/ml (both 24 and 72h), figure 2D. Viability results of Hek293 and HaCaT cells exposed with CSNPs showed no significant at all concentrations, figure 2C and 2F.

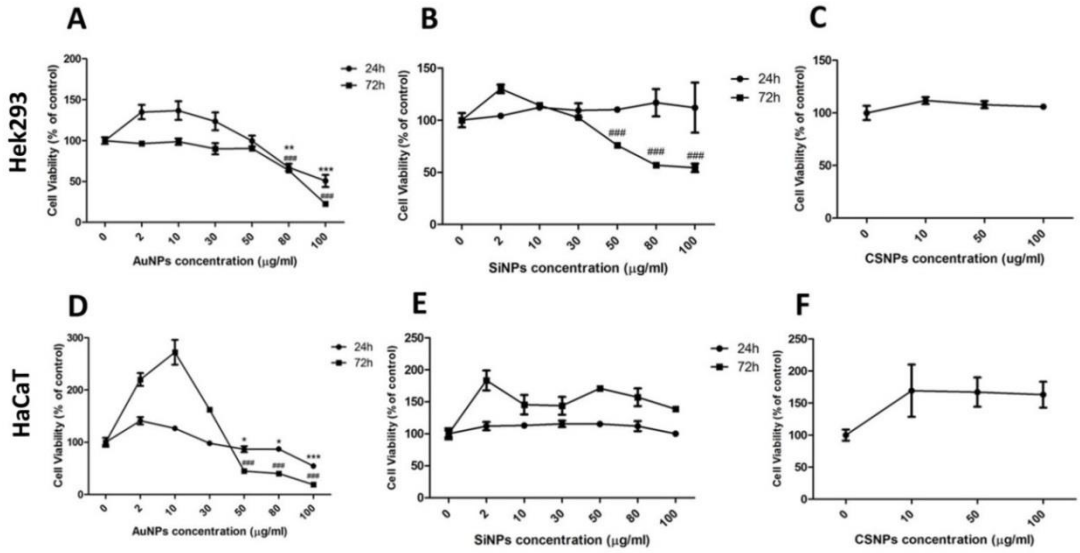

Figure 2. Cellular viability of Hek293 and HaCaT cells after exposure to nanoparticles for 24 and 72h. Cells were treated with nanoparticles at concentration 0 ug/ml to 100 ug/ml. Viability of Hek293 cells after exposure to (A) AuNPs, (B) SiNPs and (C) CSNPs, Cell viability of HaCaT cells after exposure to (D) AuNPs, (E) SiNPs and (F) CSNPs. The percentage cellular viability was calculated and compared to control (media without nanoparticles). Values are mean ± SD significance indicated by: $*,^{\#}$ $p<0.05$, $**,^{\#\#}$ $p<0.01$ and $***,^{\#\#\#}$ $p<0.001$ versus control group (* in 24h and $^{\#}$ in 72h.)

Effect of ENPs on cellular morphology

To monitor the effect of ENPs on morphological changes of Hek293 and HaCaT cells, the cellular morphology was observed by phase-contrast microscope after exposure for 72 h that are shown in Figure 3. In Hek 293, the exposure to AuNPs 10 ug/ml and CSNPs 100 ug/ml showed the same morphology as control whereas high dose of AuNPs (100ug/ml) caused to decrease amount of cells. Hek 293 cells were exposed to SiNPs in both 10 and 100 ug/ml found the morphological changes, especially at high doses the cells appeared impaired and deformed conformation and also showed shrinkage feature. HaCaT cell treated with AuNPs 10 ug/ml and CSNPs 100 ug/ml showed normal morphology same as control, whereas the cells exposure to AuNPs 100 ug/ml showed amount decreased and the cells exposure to SiNPs showed to decrease amount by dose-dependent manner.

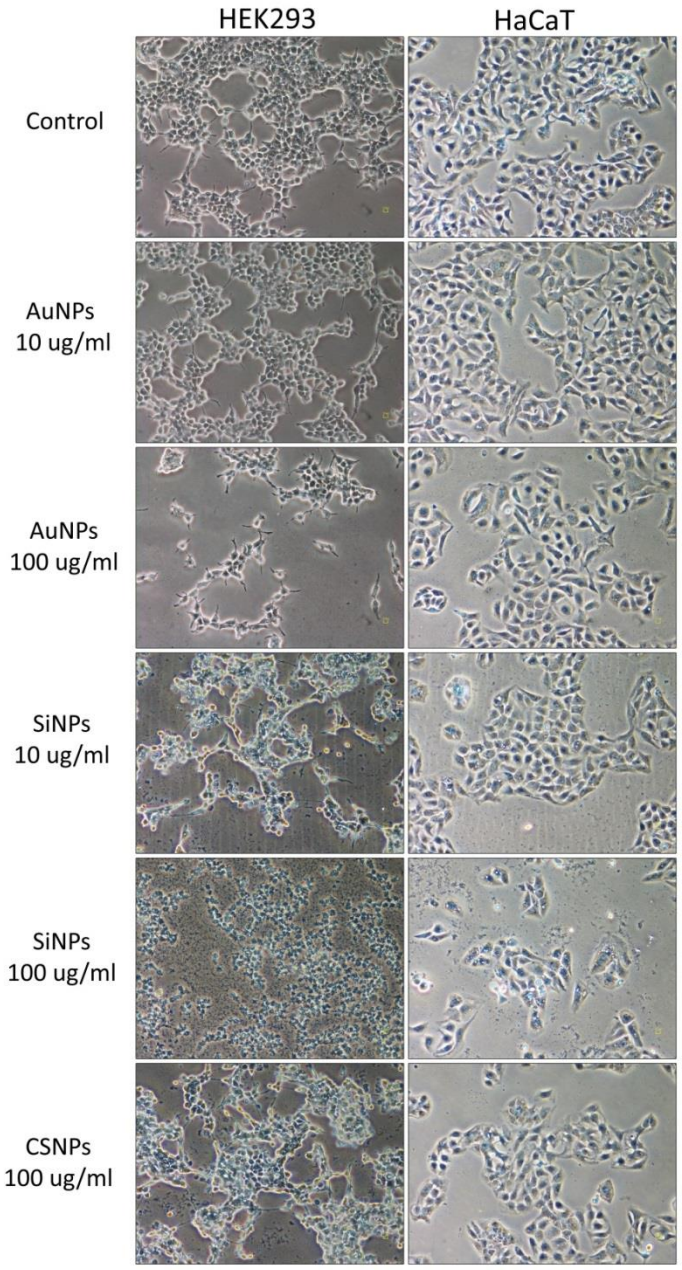

Figure 3. Cellular morphology of nanoparticles treated cells at 72h. Cells were visualized under phase contrast microscope (magnification 200x). Morphology of Hek 293 and HaCaT cells was shown in left and right column, respectively. Control group was treated with media without nanoparticles.

The effect of ENPs on ROS generation

The level of intracellular ROS induced by ENPs was evaluated by measuring fluorescent intensity of DCF. The formation of ROS after exposed to ENPs at 3 and 6 h in Hek 293 and HaCaT cell was showed in Figure 4A and 4B, respectively. Hydrogen peroxide (H_2O_2) was used as positive control. Percentage of ROS level in ENPs group treated cells was compared with control group. The results showed no significant change of ROS level in all ENPs treated Hek 293 cell both 3 and 6 h. In HaCaT cells, only the cells exposure to SiNPs showed a significant increase of ROS level at 50 and 100 ug/ml in both 3 and 6h.

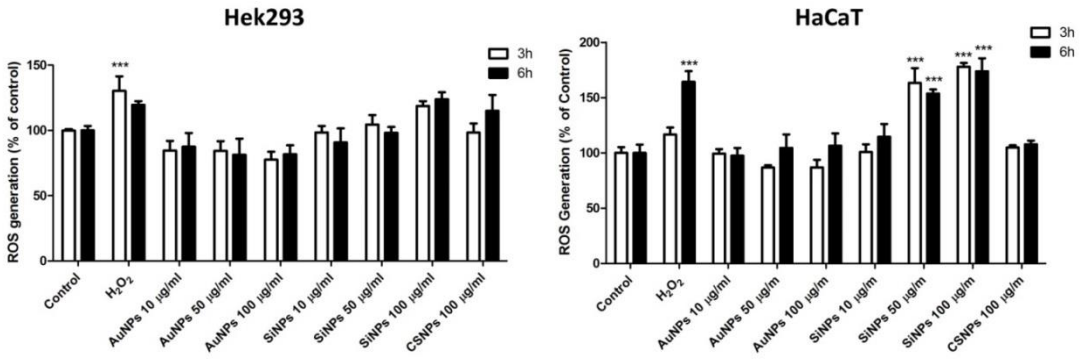

Figure 4. Reactive Oxygen Species (ROS) generation level measurement after expose with engineered nanoparticles for 3 and 6 h. DCF-fluorescence intensity that indicate the level of ROS in Hek 293 and HaCaT cells was shown in A and B, respectively. The percentage of ROS generation was calculating compared to controls (media without nanoparticles). H_2O_2 was used as positive control. Values are mean ± SD, Significance indicated by: *** $p < 0.001$ versus control group.

The effect of ENPs on global DNA methylation

The 5-methylcytosine (5-mc) Immunocytochemical staining in Hek 293 and HaCaT cells was performed to detect the global DNA methylation status after exposure to ENPs (Figure 5). DNA methylated cells were showed in darker intensity of nucleus than DNA demethylated cells. Percentage of DNA methylated was calculated from DNA methylated cells divided by total cells counted (Table 2). 5-azycytidine (Aza), DNA methyltrasferase inhibitor, was used as control of demethylated cells. All of the ENPs treated Hek 293 cells showed heavy methylated indicating that DNA methylation did not change in this cell. In HaCaT, the cells exposure to AuNPs 100 ug/ml showed no significant of DNA methylated cells compared with control, whereas SiNPs 10 ug/ml and CSNPs 100 ug/ml exposed cells clearly showed a significant decrease amount of methylated cells.

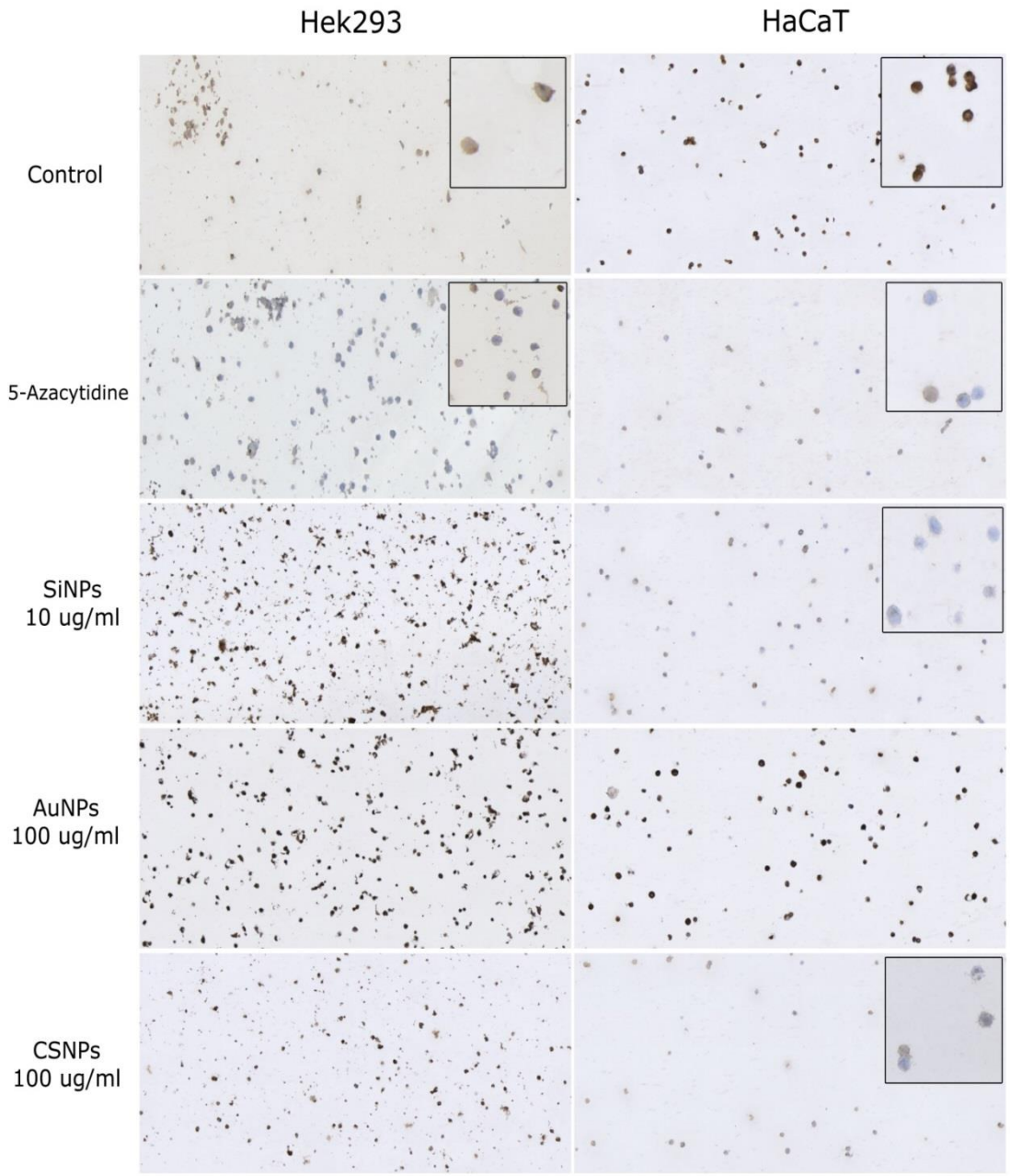

Figure 5. Global DNA methylation of cell exposure to engineered nanoparticles for 72h. Global DNA methylation level was measure by immunocytochemical staining of 5-methylcytosine (5-mc). Immunocytochemical stained of 5-mc in Hek 293 and HaCaT cells was shown in column left and right, respectively. Methylated cells showed in brown color whereas unmethylated cells showed in blue color. 5-Azacytidine was used as control of unmethylated cells.

Table 5 Percentage of methylated cells from immunocytochemical staining of 5-methylcytosine in Hek 293 and HaCaT cell exposed to ENPs for 72 h

Nanoparticles	5-methylcytosine methylated cells (%)	
	Hek 293	HaCaT
Control	100.00	92.39
5-zacytidine	57.00	62.50
SiNPs 10 ug/ml	100.00	92.67
AuNPs 100 ug/ml	100.00	75.10
CSNPs 100 ug/ml	100.00	81.42

The effect of ENPs on DNA methylation level of LINE-1 and Alu

DNA methylation level of retrotransposon, LINE-1 and Alu, in Hek 293 and HaCaT cells was measured after exposure to ENPs for 72 h. The result showed that Hek293 cells were not changed the level of DNA methylation of both of LINE-1 and Alu in all ENPs treatment (Figure 6A and 6B). In HaCaT, DNA methylation level of LINE-1 showed no significant comparted with control (Figure 6C), while DNA methylation of Alu showed to decrease (hypomethylation) by dose dependent of SiNPs treatment in both of low concentration 10 ug/ml (ROS generation not significant increase) and high concentration 100 ug/ml as well as CSNPs that not increase ROS level also showed to decrease Alu methylation level in HaCaT cells (Figure 6D).

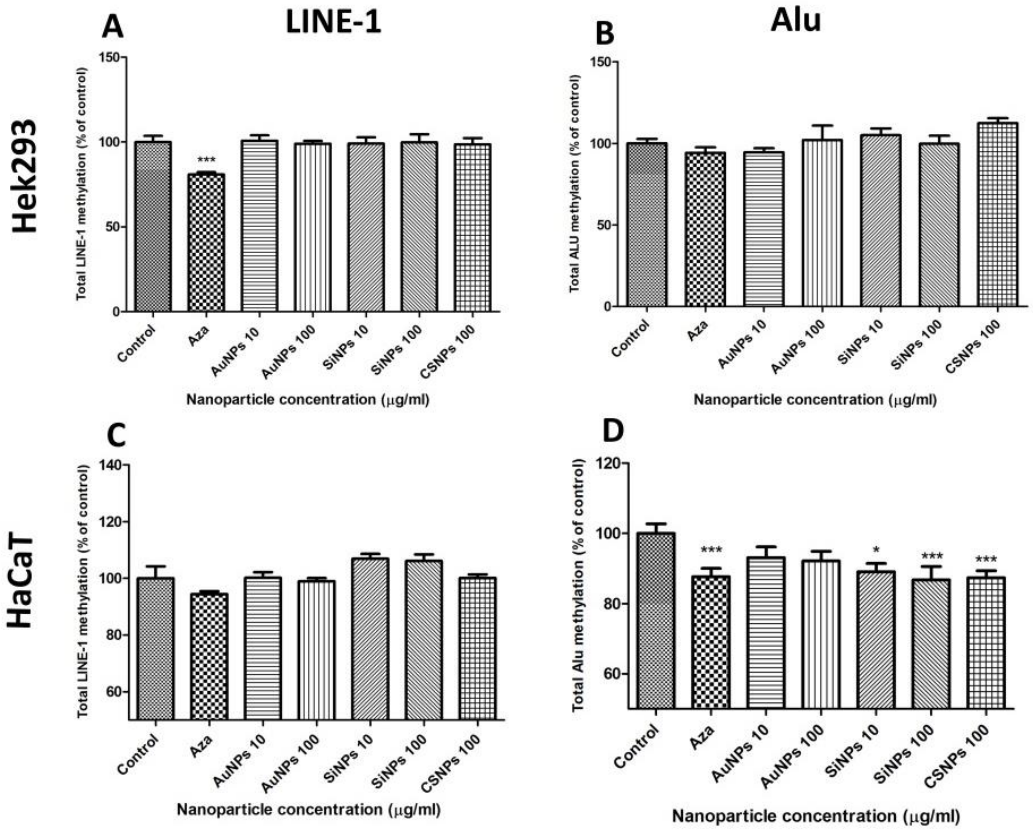

Figure 6. Total Methylation levels of LINE-1 and Alu of cells exposed with ENPs for 72h. Percentage of total LINE-1 and Alu DNA methylation were compared to control. LINE-1 and Alu methylation levels in Hek 293 cells were shown in A and B, respectively and in HaCaT were shown in C and D, respectively. 5-Azacytidine (DNA demethylation agent) was used to represent DNA hypomethylation. Values are mean ± SD, significance indicated by: * $p < 0.05$, *** $p < 0.001$ versus control group.

Alu DNA methylation level reversion in antioxidant pretreated HaCaT cells.

To approve the hypothesis that the levels of methylation were changed due to the effect of ROS generation induced by ENPs, HaCaT cells that showed hypomethylation of Alu after exposure to ENPs in previous experiment were incubated with NAC (N-acetyl cysteine, antioxidant agent) for 30 min before exposure with ENPs for 72 h (Figure 7). The result showed that DNA methylation level of Alu in SiNPs (both 10 and 100 ug/ml) and CSNPs treated cells were reversed in NAC treated groups compared with ENPs alone. The cells exposure to AuNPs showed no change of Alu DNA methylation in both NAC-pretreated and AuNPs alone.

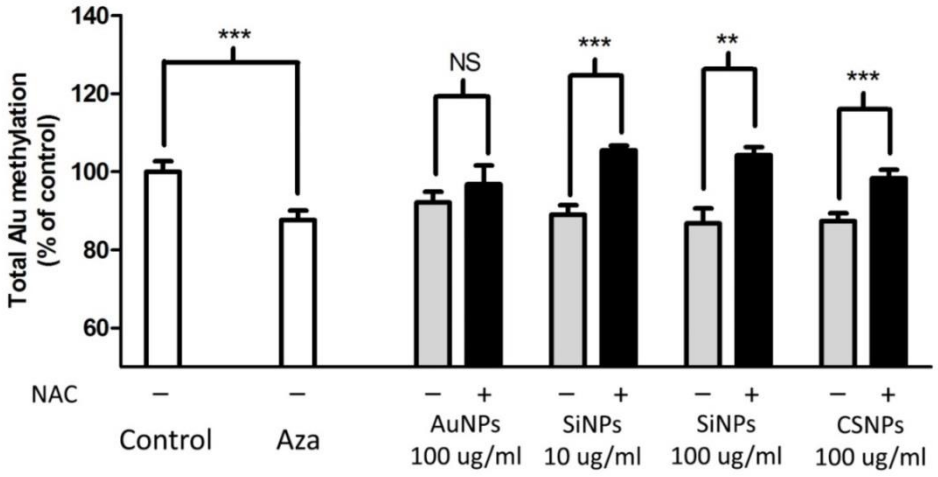

Figure 7. Total DNA methylation level measurement of Alu in HaCaT cells pretreated antioxidant (N-acetyl cysteine) before exposure to ENPs for 72h. Cells were treated with N-acetyl cysteine (NAC) for 30 min before exposure to nanoparticles for 72h. 5-Azacytidine (Aza) was used to represent hypomethylation status. Percentage of total Alu DNA methylation of nanoparticles treated alone was compared by unpaired *t-test* with NAC pretreated group and Aza was compared with control. Values are mean ± SD, significance indicated by: ** $p < 0.01$, *** $p < 0.001$.

Discussion

To our knowledge, our study presented here is the first to report the effect of engineered nanoparticles (ENPs) on DNA methylation level of the major retrotransposable elements, LINE-1 and Alu. The adverse effects of ENPs have been reported in various aspects but a few studies focused on the impact of ENPs on epigenetic mechanism, especially DNA methylation. In our experiments, we used three different types of ENPs; AuNPs, SiNPs, and CSNPs that have been used in many biomedical medical applications. The results of this study showed that various physiochemical properties of ENPs lead to different toxicity levels. Among all ENPs used, toxicity results indicated that the biocompatibilities of CSNPs are more than the remaining in both Hek 293 and HaCaT cells. AuNPs and SiNPs showed toxicity to Hek 293 and HaCaT cells in dose-dependent manner, but SiNPs caused the most harmful effect observed by cell morphology. We also measured the potential of ENPs to induce cellular oxidative stress, the findings showed that only SiNPs can increase ROS production in HaCaT cells. Interestingly, we found that global DNA methylation and Alu elements' methylation levels were decreased in HaCaT cells after being treated with SiNPs and CSNPs, but not found in AuNP counterpart. There were no changes of LINE-1 methylation levels in both of HaCaT and Hek 293 cells. Although retrotransposon is non-coding DNA sequences, several studies have indicated several physiological and pathological roles of global DNA methylation especially in LINE-1 and Alu elements [47]. LINE-1 is linked to many types of cancer, whereas Alu element is associated with aging [45] and some types of cancer [52-54]. Based on these considerations, using HaCaT human keratinocyte and Hek 293 human kidney cell lines as models, DNA methylation of Alu elements is an epigenetic target to be concerned after exposure to nanoparticles.

ENPs have been reported to be a matter that cause cellular damage through either direct or indirect mechanisms, leading to oxidative stress, mitochondrial aberration, DNA damage, and cell death [1]. Our results found that AuNPs increased mortality of cells by dose-dependent manner and decreased amount of cells. SiNPs clearly showed ability to cause malformation of cell morphology especially in Hek 293 cells, but viability in HaCaT cells did not decreases in all of ENPs exposure. Viability of HaCaT cells exposed to ENPs was found higher the percentage of viability than control up to 300% (in AuNPs 10 ug/ml), this result might cause from sensitive cell stress response of HaCaT cells. PretoBlue® reagent that a resazurin bases will turn into fluorescent form after accepting free electron from a molecule in cellular respiration such as NADPH [70] that also presenting in cellular stress response status [71] , so signal of fluoresce dye also occur mainly from cell stress, not majority from living cells. On the other hand, CSNPs clearly showed biocompatible in both Hek 293 and HaCaT cells. Additionally, Oxidative stress is one of the most nanotoxicity that is found inducing by various types of nanomaterials. In this study, we found only SiNPs increases ROS generation significantly in HacaT cells only. The ROS toxicity of SiNPs on keratinocyte cells was reported and found the same results as our study [72]. However, no change of ROS production in Hek 293 cells exposed to all three ENPs was found. This result was disagreeable with previous study of Fen wang and coworkers that they showed silica nanoparticles size 20 and 50 nm can induce ROS by dose-dependent at 24 h in Hek 293 cells [38]. The contrary results can be explained because sources of nanoparticle used in each research group are different and also differ in physical and chemical properties such as size, charge and stabilizer. It is one problem of nanotoxicity study because we cannot compare result with other groups and found the different results in same model.

A few studies focused on the effect of ENP-inducing global epigenetic changes. Alteration DNA methylation induced by SiNPs was reported previously, the authors found consequently global DNA hypomethylation and the decrease of expression level of genes regulating DNA methylation in HaCaT cells [21]. Our data revealed corresponding results that SiNPs influence to decrease 5-mc level in HaCaT cells

even at low dose. Remarkably, we also found genomic hypomethylation in HaCaT cells treated with CSNPs. The CSNPs are one of the most commonly used drug/gene delivery vectors that are considered the safe in skin and other organs. However, increasing numbers of both *in vitro* and *in vivo* toxicity of CSNPs have been reported [41, 73, 74]. Here, we address a new additional toxicity of CSNPs induction of global DNA methylation change. Furthermore, not only the occurrence of alteration of global DNA methylation, we also evaluated DNA methylation of LINE-1 and Alu retrotransposable elements because they have been discussed to represent global DNA methylation and found correlation with various types of cancers as well as environmental exposures [16]. These two non-coding elements comprise of high intensity of CpG sequences and abundantly contained throughout in human genome. Our results represented hypomethyaltion of Alu in HaCaT but not in Hek293 cells after being exposed with SiNPs and CSNPs. These findings of Alu methylation are in accordance with global DNA methylation results. Contrary, DNA methylation of LINE-1 did not change in all cells after three-nanoparticle treatment. These results indicate that effect of the ENPs on DNA methylation of retrotransposable elements depends on DNA sequence-specific. The possible explanations for our findings are that LINE-1 and Alu are regulated by different mechanisms [75], and the difference of transcription patterns in responses to cellular stressors [76]. Previous studies of other pollution exposure found a relationship between hypomethylation of Alu but not LINE-1 and persistent organic pollutant exposure [77, 78]. Another study report that Alu element has been showed a strong correlation to cell stress responses [79]. These reports, together with our results, suggest the possible use of Alu methylation change as a genetic responsive element to nanoparticle-induced cellular toxicity. However, the underlying mechanism of different DNA methylation change in LINE-1 and Alu has not been clearly elucidated. To our standpoint, the concordance of global DNA methylation and Alu elements methylation may suggest that DNA methylation level of Alu is capable representing the global genome methylation changes after exposure of the ENPs.

From the results of the present study, two out of three nanoparticles alter Alu methylation status as well as global DNA methylation level by unclarified mechanism. Various ENPs have potential to produce reactive oxygen species (ROS). The ROS has been mentioned as a factor that involves in alteration of DNA methylation [80, 81]. According to our findings, even though DNA methylation changes were not directly correlated with cellular ROS production level, we found that Alu hypomethylation can be significantly reverse in cells pretreated with N-acetyl cysteine (NAC). NAC is a substrate for glutathione (GSH) synthesis that plays a major role as intracellular antioxidant. ENPs have been though to create toxicity by increasing ROS production and/or depleting cellular antioxidant capacity such as glutathione [24]. Interestingly, the production of GSH directly influences DNA methylation by altering S-adenosyl methionine (SAM), methyl donor, pools [82]. Some reports have indicated that ENPs cause the depletion of GSH level; the exposed cells will require more production of GSH to defense with oxidative stress [24]. This result in the lack of methyl groups to add to cytosine bases. These imply that ENPs indirectly induce DNA methylation changes by disturbing cellular oxidative defense process.

There are the conflicting results in this study regarding to SiNPs negative-influence on cell viability and morphology, but no changes of ROS level and DNA methylation pattern in Hek293 kidney cell. On the other hand, lesser pathological changes were observed in cell viability and morphology of HaCaT cell, whereas there were significant ROS production and alteration of DNA methylation. The effects of SiNPs are considered to be results of cell-type specific [1] Moreover, high concentration of AuNPs showed no change in ROS production and DNA methylation pattern while the viability of cells was decreased. Surprisingly, CSNPs that are commonly founded as biocompatible nanomaterials showed DNA hypomethylation. It is believed that various properties of cell such as expression of receptors, metabolic activities, xenobiotic clearance systems and oxidative stress defense mechanism result in different of toxicity responses [1]. The discrepancy of the data is possibly governed by physical and chemical characteristics of each ENPs. Further

studies are needed to investigate relationship among nanoparticle internalization into the cell, DNA methylation status and other epigenetic control to confirm these results.

CHAPTER V
CONCLUSION

In conclusion, the present study shows global DNA hypomethylation in SiNPs and CSNPs exposed cells as well as hypomethylation of Alu element but not LINE-1 by cell-types specific. We found Hek 293 cells did not show changing of ROS level, global DNA methylation and DNA methylation of LINE-1 and Alu retrotransposable elements. In contrast, HaCaT cells showed increasing of ROS level after exposure to SiNPs. Furthermore, the cells showed both global and Alu but not LINE-1 DNA hypomethylation. Our results also verified that the alteration of DNA methylation of Alu transposable elements in HaCaT cells exposure to SiNPs and CSNPs is ROS production-independent. AuNPs that were used in this study show lack of ability to induce alteration of ROS level and DNA methylation in both of Hek 293 and HaCaT cells. Finally, our results demonstrated the new insight that DNA methylation of Alu elements represents the global DNA methylation of cell exposed with ENPs.

REFERENCES

1. Magdolenova, Z., et al., *Mechanisms of genotoxicity. A review of in vitro and in vivo studies with engineered nanoparticles.* Nanotoxicology, 2014. **8**(3): p. 233-78.
2. Slowing, I.I., et al., *Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers.* Adv Drug Deliv Rev, 2008. **60**(11): p. 1278-1288.
3. Wang, J.J., et al., *Recent advances of chitosan nanoparticles as drug carriers.* Int J Nanomedicine, 2011. **6**: p. 765-74.
4. Kim, B.Y.S., J.T. Rutka, and W.C.W. Chan, *Nanomedicine.* New England Journal of Medicine, 2010. **363**(25): p. 2434-2443.
5. Sohaebuddin, S.K., et al., *Nanomaterial cytotoxicity is composition, size, and cell type dependent.* Part Fibre Toxicol, 2010. **7**: p. 22.
6. Yang, H., et al., *Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition.* J Appl Toxicol, 2009. **29**(1): p. 69-78.
7. Kim, Y.-J., et al., *Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by silica nanomaterials in human neuronal cell line.* Molecular & Cellular Toxicology, 2010. **6**(4): p. 336-343.
8. Nabeshi, H., et al., *Amorphous nanosilica induce endocytosis-dependent ROS generation and DNA damage in human keratinocytes.* Part Fibre Toxicol, 2011. **8**: p. 1.
9. Li, J.J., et al., *Autophagy and oxidative stress associated with gold nanoparticles.* Biomaterials, 2010. **31**(23): p. 5996-6003.
10. Sarkar, A., et al., *Nano-copper induces oxidative stress and apoptosis in kidney via both extrinsic and intrinsic pathways.* Toxicology, 2011. **290**(2-3): p. 208-217.

11. Jaeger, A., et al., *Oxidative stress-induced cytotoxic and genotoxic effects of nano-sized titanium dioxide particles in human HaCaT keratinocytes.* Toxicology, 2012. **296**(1-3): p. 27-36.
12. Ziech, D., et al., *Reactive Oxygen Species (ROS)-Induced genetic and epigenetic alterations in human carcinogenesis.* Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis, 2011. **711**(1-2): p. 167-173.
13. Baylin, S.B., *DNA methylation and gene silencing in cancer.* Nat Clin Pract Oncol, 2005. **2 Suppl 1**: p. S4-11.
14. Das, P.M. and R. Singal, *DNA methylation and cancer.* J Clin Oncol, 2004. **22**(22): p. 4632-42.
15. Hou, L., et al., *Environmental chemical exposures and human epigenetics.* Int J Epidemiol, 2012. **41**(1): p. 79-105.
16. Nelson, H.H., C.J. Marsit, and K.T. Kelsey, *Global methylation in exposure biology and translational medical science.* Environ Health Perspect, 2011. **119**(11): p. 1528-33.
17. Kitkumthorn, N. and A. Mutirangura, *Long interspersed nuclear element-1 hypomethylation in cancer: biology and clinical applications.* Clin Epigenetics, 2011. **2**(2): p. 315-30.
18. Baccarelli, A., et al., *Rapid DNA methylation changes after exposure to traffic particles.* Am J Respir Crit Care Med, 2009. **179**(7): p. 572-8.
19. Madrigano, J., et al., *Prolonged exposure to particulate pollution, genes associated with glutathione pathways, and DNA methylation in a cohort of older men.* Environ Health Perspect, 2011. **119**(7): p. 977-82.
20. Tarantini, L., et al., *Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation.* Environ Health Perspect, 2009. **117**(2): p. 217-22.
21. Gong, C., et al., *SiO(2) nanoparticles induce global genomic hypomethylation in HaCaT cells.* Biochem Biophys Res Commun, 2010. **397**(3): p. 397-400.

22. Gong, C., et al., *Methylation of PARP-1 promoter involved in the regulation of nano-SiO2-induced decrease of PARP-1 mRNA expression.* Toxicol Lett, 2012. **209**(3): p. 264-9.

23. Johnston, H.J., et al., *A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity.* Crit Rev Toxicol, 2010. **40**(4): p. 328-46.

24. Akhtar, M.J., et al., *Nanotoxicity of pure silica mediated through oxidant generation rather than glutathione depletion in human lung epithelial cells.* Toxicology, 2010. **276**(2): p. 95-102.

25. Skrastina, D., et al., *Silica Nanoparticles as the Adjuvant for the Immunisation of Mice Using Hepatitis B Core Virus-Like Particles.* PLoS One, 2014. **9**(12): p. e114006.

26. Santra, S., et al., *Conjugation of biomolecules with luminophore-doped silica nanoparticles for photostable biomarkers.* Anal Chem, 2001. **73**(20): p. 4988-93.

27. Hirsch, L.R., et al., *Nanoshell-mediated near-infrared thermal therapy of tumors under magnetic resonance guidance.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13549-54.

28. Bharali, D.J., et al., *Organically modified silica nanoparticles: a nonviral vector for in vivo gene delivery and expression in the brain.* Proc Natl Acad Sci U S A, 2005. **102**(32): p. 11539-44.

29. Alkilany, A.M. and C.J. Murphy, *Toxicity and cellular uptake of gold nanoparticles: what we have learned so far?* Journal of Nanoparticle Research, 2010. **12**(7): p. 2313-2333.

30. Jain, S., D.G. Hirst, and J.M. O'Sullivan, *Gold nanoparticles as novel agents for cancer therapy.* The British Journal of Radiology, 2012. **85**(1010): p. 101-113.

31. Scholl, I., G. Boltz-Nitulescu, and E. Jensen-Jarolim, *Review of novel particulate antigen delivery systems with special focus on treatment of type I allergy.* J Control Release, 2005. **104**(1): p. 1-27.

32. Ilium, L., *Chitosan and Its Use as a Pharmaceutical Excipient.* Pharmaceutical Research, 1998. **15**(9): p. 1326-1331.

33. Lee, C.G., *Chitin, chitinases and chitinase-like proteins in allergic inflammation and tissue remodeling.* Yonsei Med J, 2009. **50**(1): p. 22-30.

34. Janes, K.A., et al., *Chitosan nanoparticles as delivery systems for doxorubicin.* Journal of Controlled Release, 2001. **73**(2–3): p. 255-267.

35. Pan, Y., et al., *Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo.* International Journal of Pharmaceutics, 2002. **249**(1–2): p. 139-147.

36. Singh, N., et al., *NanoGenotoxicology: the DNA damaging potential of engineered nanomaterials.* Biomaterials, 2009. **30**(23-24): p. 3891-914.

37. AshaRani, P.V., et al., *Cytotoxicity and genotoxicity of silver nanoparticles in human cells.* ACS Nano, 2009. **3**(2): p. 279-90.

38. Wang, F., et al., *Oxidative stress contributes to silica nanoparticle-induced cytotoxicity in human embryonic kidney cells.* Toxicology in Vitro, 2009. **23**(5): p. 808-15.

39. Li, J.J., et al., *Gold nanoparticles induce oxidative damage in lung fibroblasts in vitro.* Advanced Materials, 2008. **20**(1): p. 138-+.

40. Paino, I.M., et al., *Cyto and genotoxicity of gold nanoparticles in human hepatocellular carcinoma and peripheral blood mononuclear cells.* Toxicol Lett, 2012. **215**(2): p. 119-25.

41. Hu, Y.L., et al., *Toxicity evaluation of biodegradable chitosan nanoparticles using a zebrafish embryo model.* Int J Nanomedicine, 2011. **6**: p. 3351-9.

42. Stoccoro, A., et al., *Epigenetic effects of nano-sized materials.* Toxicology, 2013. **313**(1): p. 3-14.

43. Carnell, A.N. and J.I. Goodman, *The long (LINEs) and the short (SINEs) of it: altered methylation as a precursor to toxicity.* Toxicol Sci, 2003. **75**(2): p. 229-35.

44. Levenson, V.V., *DNA methylation as a universal biomarker.* Expert Review of Molecular Diagnostics, 2010. **10**(4): p. 481-488.

45. Jintaridth, P. and A. Mutirangura, *Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences.* Physiol Genomics, 2010. **41**(2): p. 194-200.

46. Druker, R. and E. Whitelaw, *Retrotransposon-derived elements in the mammalian genome: a potential source of disease.* J Inherit Metab Dis, 2004. **27**(3): p. 319-30.

47. Schulz, W.A., C. Steinhoff, and A.R. Florl, *Methylation of endogenous human retroelements in health and disease.* Curr Top Microbiol Immunol, 2006. **310**: p. 211-50.

48. Cordaux, R. and M.A. Batzer, *The impact of retrotransposons on human genome evolution.* Nat Rev Genet, 2009. **10**(10): p. 691-703.

49. Kitkumthorn, N., et al., *LINE-1 methylation in the peripheral blood mononuclear cells of cancer patients.* Clin Chim Acta, 2012. **413**(9-10): p. 869-74.

50. Batzer, M.A. and P.L. Deininger, *Alu repeats and human genomic diversity.* Nat Rev Genet, 2002. **3**(5): p. 370-9.

51. Wilson, A.S., B.E. Power, and P.L. Molloy, *DNA hypomethylation and human diseases.* Biochim Biophys Acta, 2007. **1775**(1): p. 138-62.

52. Cho, Y.H., et al., *Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients.* Anticancer Res, 2010. **30**(7): p. 2489-96.

53. Kwon, H.J., et al., *DNA methylation changes in ex-adenoma carcinoma of the large intestine.* Virchows Arch, 2010. **457**(4): p. 433-41.

54. Xie, H., et al., *Epigenomic analysis of Alu repeats in human ependymomas.* Proc Natl Acad Sci U S A, 2010. **107**(15): p. 6952-7.

55. Bind, M.A., et al., *Air pollution and markers of coagulation, inflammation, and endothelial function: associations and epigene-environment interactions in an elderly cohort.* Epidemiology, 2012. **23**(2): p. 332-40.

56. Salam, M.T., et al., *Genetic and epigenetic variations in inducible nitric oxide synthase promoter, particulate pollution, and exhaled nitric oxide levels in children.* J Allergy Clin Immunol, 2012. **129**(1): p. 232-9 e1-7.

57. Nadeau, K., et al., *Ambient air pollution impairs regulatory T-cell function in asthma.* J Allergy Clin Immunol, 2010. **126**(4): p. 845-852 e10.

58. Hou, L., et al., *Ambient PM exposure and DNA methylation in tumor suppressor genes: a cross-sectional study.* Part Fibre Toxicol, 2011. **8**: p. 25.

59. Gilmour, P.S., et al., *Histone acetylation regulates epithelial IL-8 release mediated by oxidative stress from environmental particles.* Am J Physiol Lung Cell Mol Physiol, 2003. **284**(3): p. L533-40.

60. Cao, D., P.A. Bromberg, and J.M. Samet, *COX-2 expression induced by diesel particles involves chromatin modification and degradation of HDAC1.* Am J Respir Cell Mol Biol, 2007. **37**(2): p. 232-9.

61. Jardim, M.J., et al., *Disruption of microRNA expression in human airway cells by diesel exhaust particles is linked to tumorigenesis-associated pathways.* Environ Health Perspect, 2009. **117**(11): p. 1745-51.

62. Choi, A.O., et al., *Quantum dot-induced epigenetic and genotoxic changes in human breast cancer cells.* J Mol Med (Berl), 2008. **86**(3): p. 291-302.

63. Halappanavar, S., et al., *Pulmonary response to surface-coated nanotitanium dioxide particles includes induction of acute phase response genes, inflammatory cascades, and changes in microRNAs: a toxicogenomic study.* Environ Mol Mutagen, 2011. **52**(6): p. 425-39.

64. Ng, C.T., et al., *The induction of epigenetic regulation of PROS1 gene in lung fibroblasts by gold nanoparticles and implications for potential lung injury.* Biomaterials, 2011. **32**(30): p. 7609-15.

65. Li, S., et al., *Assessment of nanomaterial cytotoxicity with SOLiD sequencing-based microRNA expression profiling.* Biomaterials, 2011. **32**(34): p. 9021-30.

66. Li, S., et al., *MicroRNAs as participants in cytotoxicity of CdTe quantum dots in NIH/3T3 cells.* Biomaterials, 2011. **32**(15): p. 3807-3814.

67. Di Virgilio, A.L., et al., *Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells.* J Hazard Mater, 2010. **177**(1-3): p. 711-8.

68. Asada, K., et al., *LINE-1 hypomethylation in a choline-deficiency-induced liver cancer in rats: dependence on feeding period.* J Biomed Biotechnol, 2006. **2006**(1): p. 17142.

69. Patchsung, M., et al., *Long interspersed nuclear element-1 hypomethylation and oxidative stress: correlation and bladder cancer diagnostic potential.* PLoS One, 2012. **7**(5): p. e37009.

70. Xu, M., D.J. McCanna, and J.G. Sivak, *Use of the viability reagent PrestoBlue in comparison with alamarBlue and MTT to assess the viability of human corneal epithelial cells.* Journal of Pharmacological and Toxicological Methods, 2015. **71**(0): p. 1-7.

71. Jiang, F., Y. Zhang, and G.J. Dusting, *NADPH oxidase-mediated redox signaling: roles in cellular stress response, stress tolerance, and tissue repair.* Pharmacol Rev, 2011. **63**(1): p. 218-42.

72. Gong, C., et al., *The role of reactive oxygen species in silicon dioxide nanoparticle-induced cytotoxicity and DNA damage in HaCaT cells.* Mol Biol Rep, 2012. **39**(4): p. 4915-25.

73. Qi, L.F., et al., *In vitro effects of chitosan nanoparticles on proliferation of human gastric carcinoma cell line MGC803 cells.* World J Gastroenterol, 2005. **11**(33): p. 5136-41.

74. Park, M.R., et al., *Chitosan nanoparticles cause pre- and postimplantation embryo complications in mice.* Biol Reprod, 2013. **88**(4): p. 88.

75. Gonzalgo, M.L. and P.A. Jones, *Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE).* Nucleic Acids Res, 1997. **25**(12): p. 2529-31.

76. Li, T.H. and C.W. Schmid, *Differential stress induction of individual Alu loci: implications for transcription and retrotransposition.* Gene, 2001. **276**(1-2): p. 135-41.

77. Kim, K.Y., et al., *Association of low-dose exposure to persistent organic pollutants with global DNA hypomethylation in healthy Koreans.* Environ Health Perspect, 2010. **118**(3): p. 370-4.

78. Rusiecki, J.A., et al., *Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit.* Environ Health Perspect, 2008. **116**(11): p. 1547-52.

79. Schmid, C.W., *Does SINE evolution preclude Alu function?* Nucleic Acids Res, 1998. **26**(20): p. 4541-4550.

80. Weitzman, S.A., et al., *Free-Radical Adducts Induce Alterations in DNA Cytosine Methylation.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(4): p. 1261-1264.

81. Niu, Y., et al., *Oxidative stress alters global histone modification and DNA methylation.* Free Radic Biol Med, 2015. **82**: p. 22-28.

82. Hitchler, M.J. and F.E. Domann, *An epigenetic perspective on the free radical theory of development.* Free Radic Biol Med, 2007. **43**(7): p. 1023-36.

APPENDIX

LIST OF ABBREVIATIONS

AuNPs	Gold nanoparticles
Aza	5-Azacitidine
ANOVA	Analysis of variance
COBRA	Combined bisulfite restriction analysis
CSNPs	Chitosan nanoparticles
DMEM	Dulbecco's Modified Eagle Medium
ENPs	Engineered nanoparticles
FBS	Fetal bovine serum
H_2DCFDA	2', 7'-dichlofluorescein diacetate
$HAuCl_4.3H_2O$	Hydrogen tetracholoaurate (III) trihydrate
HaCaT	Immortal human keratinocyte cell line
HEK293	Immortal human embryonic kidney cell line
LINE-1	Long interspersed nuclear element-1
5-mc	5-methylcytosine
NAC	N-acetyl cysteine
PBS	Phosphate buffered saline
ROS	Reactive oxygen species
SINEs	Short interspersed nuclear elements
SiNPs	Silicon dioxide nanoparticles
TBE	Tris-borate-EDTA
TEM	Transmission electron microscope

EQUIPMENT AND CHEMICALS

1. Autoclave (Hirayama, Japan)
2. Biohazard Laminar Flow (Gibco, USA)
3. CO_2 incubator (Esco, Singapore)
4. Fluorescent microscope (Nikon, Japan)
5. Laboratory balance (Denver instrument, Germany)
6. Malvern Zetasizer Nano Series (Malvern Instrument, England)
7. Microcentrifuge (Hettich, Germany)
8. pH meter (Denver instrument, Germany)
9. Phase contrast inverted microscope (Nikon, Japan
10. Transmission Electron Microscope (Hitachi, Japan)
11. Varioskan Flash microplate reader (Thermo, England)
12. Vortex mixer (Scientific industries, USA)
13. Water bath (Memmert, Germany)
14. 24-well plate (Corning, USA)
15. 96-well plate (Corning, USA)
16. Cell culture flask (SPL, Korea)
17. Centrifuge tube 1.5 mL (Corning, USA)
18. Centrifuge tube 15 mL (Corning, USA)
19. Centrifuge tube 50 mL (Corning, USA)
20. Dulbecco's Modified Eagle's Medium (Sigma, USA)
21. Fetal Bovine Serum (Gibco, USA)
22. Filter Tip (Corning, USA)
23. H2DCFDA (Molecular probes, USA)
24. Penicillin/Streptomycin (Gibco, USA)

25. PrestoBlue™ Cell viability Reagent (Invitrogen, USA)
26. Go Taq Flexi DNA polymerase (Promega, USA)
27. 25 bp DNA Ladder (Invitrogen, USA)
28. NEBuffer 3 (New England Biolabs, UK)
29. BSA (New England Biolabs, UK)
30. Gel star Nucleic Acid Gel Stain (Lonza, USA)
31. Gold (III) Chloride trihydrate (Sigma-Aldrich, USA)
32. ACRYL/BIS 19:1 (ameresco, USA)
33. Ammonium persulfate (ameresco, USA)
34. TMET (Sigma-Aldrich, USA)

CHEMICAL PREPARATIONS

1. Phosphate buffer saline

KCl	0.2	g
KH_2PO_4	0.2	g
NaCl	8.0	g
Na_2HPO_4	1.15	g

Mix all of chemical components and add DI water to 1,000 mL, then adjust pH to 7.4 with HCl

2. Dulbecco's Modified Eagle's Medium (DMEM)

DMEM	13.4	g	(1 bottle)
Na_2HCO_3	3.7	g	
Fetal Bovine Serum	10%		
Pen/strep	1%		

Dissolve 13.4 g of DMEM and 3.7 g of Na_2HCO_3 with 800 mL DI water, then adjust pH to 7.2 with HCl and add DI water to 1,000 mL. Filtrate by 0.2 μM filter and keep as a stock medium. For working medium preparation, add 100 mL of Fetal Bovine Serum and 10 mL of antibiotic (Pen-Strep) into 890 mL of stock medium.

3. 10X TBE

Boric acid	27.5	g
Trist-base	54	g
0.5M EDTA (pH 8.0)	20	ml

4. Chitosan nanoparticles (CSNPs) synthesis

HOBt (Hydroxybenzotriazole)

Phenylalanine	0.1	g
mPEG-COOH	0.9	g

EDC	0.35	g
(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride)		
Deionized water	20	ml

CS was mixed with HOBt in deionized water and stirred to obtain a clear solution. Next, 0.1 g Phe (Fluka Chemika, Switzerland), 0.9 g mPEG-COOH (Sigma-Aldrich, USA), and 0.35 g EDC was added to 20 mL deionized water, mixed with the CS solution, and incubated overnight at room temperature. The product was dialyzed, lyophilized, washed with methanol, and dried using a vacuum.

5. Gold nanoparticles (AuNP) synthesis

1% hydrogen tetracholoaurate (III) trihydrate ($HAuCl_4.3H_2O$)	1 ml
0.0202 mM sodium acetate	12 ml
Ultrapure water (Milli-Q)	37 ml

Stir all components in flask on hot-plate at 75-95 °C for 2 hours.

VITA

Siwaporn Nilyai was born in Samutprakan, Thailand on 8 February, 1989. She graduated with Bachelor Degree of Science, from Chulalongkorn University, Faculty of Science, Department of Botany (major in genetic) in 2010. She enrolled in Master Degree of Medical Science, Faculty of Medicine at Chulalongkorn University in 2011.