Proteomics study of the cellular function in response to nanomaterials

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**NANYANG
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UNIVERSITY**

**PROTEOMICS STUDY OF THE CELLULAR FUNCTION
IN RESPONSE TO NANOMATERIALS**

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2012

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Dedicated to my family, especially my parents, who wish the world is a little better than they were born.

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SUMMARY

Because of their attractive chemical and physical properties, graphitic nanomaterials and their derivatives have gained tremendous interest for applications in electronics, materials and biomedical areas. However, few detailed studies have been performed to evaluate the potential cytotoxicity of these nanomaterials on the living systems at the molecular level. In the present study, our group exploited the iTRAQ-coupled 2D LC-MS/MS approach with the purpose of characterizing the cellular functions in response to these nanomaterials at the proteome level. Specifically, single-walled carbon nanotubes (SWCNTs), graphene, as well as their derivatives such as oxidized SWCNTs and graphene oxide (GO) were tested, while the human hepatoma HepG2 cells were used as the *in vitro* model to study the potential cytotoxicity of these nanomaterials on the vital organ of liver. Differentially expressed proteins involved in metabolic pathway, redox regulation, cytoskeleton formation and cell growth were successfully identified through the iTRAQ-coupled 2D LC-MS/MS approach. Based on the protein profile, we found both SWCNTs and oxidized SWCNTs induced oxidative stress and interfered the intracellular metabolic routes, protein synthesis and cytoskeletal systems. However, only moderate variation of protein levels for the cells treated with graphene and GO was observed. Further functional assays such as cell proliferation assay, Western blotting analysis, apoptosis assay and cell cycle analysis were carried out to confirm the data obtained from protein profile. We found oxidized SWCNTs did trigger elevated level of reactive oxygen species (ROS), perturb the cell cycle and result in a significant increase in the proportion of apoptotic cells. In contrast, these functional assays indicated there was no significant increase in the percentage of apoptotic cells and cell cycle was not severely perturbed after exposed to GO.

Specifically, our group successfully identified changes in the levels of key proteins involved in the redox regulation of the cell by applying iTRAQ coupled 2D LC-MS/MS analysis. Peroxiredoxin (Prx) family protein and thioredoxin were found to be significantly up-regulated in the cells treated with pristine and oxidized SWCNTs, which suggested there might be elevated level of intracellular ROS after treated with SWCNTs based nanomaterials. Further measurement of the intracellular ROS level confirmed that oxidized SWCNTs induced significantly higher level of ROS in comparison to the control cells. Interestingly, Prx-5, a thioredoxin peroxidase that inhibits p53-induced apoptosis, was found to be significantly down-regulated for the cells exposure to pristine and oxidized SWCNTs. Since the expression of mangasese superoxide dismutase 2 (SOD2) that could repress p53 expression was also significantly down-regulated after exposure to SWCNTs. These findings suggested that SWCNTs exposure might trigger the activation of the p53 signaling pathway and induce the growth arrest at G₁/S and G₂/M DNA damage recognition point. Futher experiment by Western blotting analysis confirmed the upregulation of tumor suppressor protein p53 after treated with oxidized SWCNTs.

Moreover, we found glutathione S-transferase that modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1 (ASK1) was significantly down-regulated after exposure to pristine and oxidized SWCNTs. Its down-regulation might result in the activation of downstream signaling cascades such as the stress associated protein kinases (SAPKs) and p38 pathway, and finally lead to stress-induced apoptosis. From apoptosis analysis result, we did find a significant increase in the proportion of cells undergoing apoptosis after the treatment of oxidized SWCNTs. Western blotting analysis further confirmed oxidized SWCNTs did induce the phosphorylation of the stress activated MAP kinase p38.

Furthermore, S100-A6 and calmodulin that promote cell growth were found to be down-regulated for the batch treated with oxidized SWCNTs. On the other hand, S100-A11 protein that deters the cell growth was found to be elevated in comparison to the control cells. Since the reciprocal regulation of these proteins decides the cell metabolic rate, our findings suggested metabolic rate of the cells might be reduced after exposure to oxidized SWCNTs. For the graphene treated cells, the similar pattern of these metabolic rate control proteins was also observed and we expected that the metabolic rate might be deterred in a similar manner as oxidized SWCNTs treated cells. Further evidences on reduced metabolic rate could also be obtained from the down-regulation of the metabolic enzymes involved in the ATP generating step and MTT assay result. Unexpectedly, for the cells treated with pristine SWCNTs, these growth rate control proteins and metabolic enzymes involved in ATP generating step showed in the opposite trend. The hyper-metabolic rate for pristine SWCNTs treated cells might be explained by the increasing energy demand caused by elevated protein amount. However, further experiments are needed to confirm such hypothesis.

We also found various molecular chaperones such as heat shock protein, 78 kDa glucose-regulated protein, peptidyl-prolyl cis-trans isomerase and protein disulfide isomerase, were found to be up-regulated for the cells exposed to pristine and oxidized SWCNTs. The significant up-regulation of these chaperone proteins might play an important role in assisting protein folding due to the elevated protein demand and/or SWCNTs retained inside the cells might perturb the protein folding process.

Based on the information obtained from protein profile, graphene and GO appeared to be more biocompatible and less cytotoxic as these proteins remained almost unchanged in comparison to the control cells. Such hypothesis was further supported

by functional assays as we did not observe noticeable cell cycle perturbation and there was no significant increase in the proportion of apoptotic cells after exposure to GO.

Taken together, our results therefore provide molecular evidence on cellular functions in response to these nanomaterials. By the close look at cellular functions at proteome level, we clearly identified the distinct pattern of cellular responses between these nanomaterials, which suggested different modes of action to the living systems. We envision that the systematic characterization through comparative proteomics technique coupled with functional assays would provide us a more detailed understanding of the interactions between living systems and these nanomaterials.

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LIST OF ABBREVIATIONS

2D	two-dimensional
2-DE	two-dimensional gel electrophoresis
Ach	Acetylcholine
ADP	adenosine diphosphate
AFM	atomic force microscopy
ALG	sodium alginate
AmB	amphotericin B
ASK	apoptosis signal-regulating kinase
ASOND	antisense oligodeoxynucleotide
ATP	adenosine triphosphate
iTRAQ	isobaric tag for relative and absolute quantitation
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
CAR	carvedilol
CEA	carcinoembryonic antigen
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CHI	chitosan
CID	collision-induced dissociation
CNTs	carbon nanotubes
CV	coefficient of variance
CVD	chemical vapor deposition
DEX	dexamethasone
DFDA	dihydrofluorescein diacetate

DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DOX	doxorubicin
ECs	endothelial cells
EDTA	ethylenediaminetetraacetic acid
EF	error factor
EPI	epirubicin
ER	endoplasmic reticulum
ESCs	embryonic stem cell
ESI	electrospray ionization
FA	folic acid
FITC	fluorescein isothiocyanate
FTIR	Fourier transform infrared
FT-MS	Fourier transform ion cyclotron
GAPs	GTPase-activating proteins
GDP	guanosine diphosphate
GFP	green fluorescent protein
GO	graphene oxide
GRP	glucose-regulated protein
GST	glutathione S-transferase
HMDA	hexamethylenediamine
HPLC	high performance liquid chromatography
IL	interleukin
LC	liquid chromatography

MALDI	matrix-assisted laser desorption/ionization
MAPK	mitogen activated protein kinases
MDR	multidrug resistance
MMTS	methylmethanethiosulfonate
MOA	modes of action
MS	mass spectrometry
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAD ⁺	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide plus hydrogen
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
PABA	poly aminobezene sulphonic acid
PABS	poly amino benzene sulfonic acid
PBS	phosphate buffered saline
PDDA	poly(diallyldimethylammonium) chloride
PEG	polyethylene glycol
PEI	polyethylenimine
PL	phospholipid
PMMA	polymethyl methacrylate
Prx	peroxiredoxin
PTMs	post-translational modifications
RGD	cyclic arginine–glycine–aspartic acid
ROS	reactive oxygen species
RP	reversed-phase

SCX	strong cation exchange
SD	standard deviation
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SILAC	amino acids in cell culture
SKP	S-phase kinase-associated protein
SOD	superoxide dismutase
ssDNA	single-stranded DNA
SWCNTs	Single-walled carbon nanotubes
TBHP	tert-Butyl hydroperoxide
TCEP	tris(2-carboxyethyl)phosphine (TCEP)
TOF	time-of-flight
TPGS	tocopheryl polyethylene glycol succinate

PUBLICATIONS

1. **Jifeng Yuan**, Hongcai Gao, Jianjun Sui, Wei Ning Chen, Chi Bun Ching. Cytotoxicity of single-walled carbon nanotubes on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology In Vitro*. 2011; 25(8): 1820-7.
2. **Jifeng Yuan**, Hongcai Gao, Chi Bun Ching. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011; 15; 207(3); 213-21.
3. **Jifeng Yuan**, Hongcai Gao, Jianjun Sui, Hongwei Duan, Wei Ning Chen, Chi Bun Ching. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.
4. **Jifeng Yuan**, Chi Bun Ching. Combinatorial engineering of the ribosome entry sequences for rapid tuning expression of multiple genes in the synthetic operon. 2011; Submitted for publication.
5. **Jifeng Yuan**, Chi Bun Ching. Macromolecular scaffold based artificial multienzyme complexes for modular control over metabolic flux. 2011; In preparation.
6. **Jifeng Yuan**, Chi Bun Ching. Improved catalytic efficiency for the conversion of 1,3-butanediol to 4-hydroxyl-2-butanone through scaffolding glycerol dehydrogenase with NAD⁺ cofactor regeneration system. In preparation.

Chapter 1 Introduction

1.1 Carbon nanotube and its applications

Carbon nanotubes (CNTs) are cylindrical graphite tubes with diameter in nanoscale and length in nano or micro meter ranges. Based on the number of concentric cylinders of graphite sheets, CNTs can be categorized into single- or multi-walled CNTs (SWCNTs and MWCNTs) (Fig. 1.1). Currently, there are mainly three methods that have been utilized for synthesizing CNTs: arc-discharge (Ebbesen and Ajayan, 1992; Thess et al., 1996), laser ablation (Kuo et al., 2001), and chemical vapor deposition (CVD) (Varadan and Xie, 2002). For the arc-discharge and laser ablation methods, graphite rods are used as a source of carbon. While in CVD method, hydrocarbons like methane and ethylene are utilized as the carbon source. The pristine CNTs are intrinsically hydrophobic and lack of solubility in biological media. Hence, various approaches have been developed to facilitate the solubilization of CNTs through surface modification, with the purpose of integrating them into biological systems for biomedical applications (Banerjee et al., 2003; Wang et al., 2003; Wang et al., 2005).

Since its discovery (Iijima, 1991), both SWCNTs and MWCNTs have been widely exploited for bio-related applications due to their attractive chemical, electrical, and mechanical properties. CNTs have been integrated with a number of biomolecules and used as biosensor for ultrasensitive disease diagnosis. Of late, considerable efforts have been made to utilize CNTs as biomaterials for tissue regeneration and biocompatible carriers for *in vivo* gene and drug delivery (Fig.1.2).

Figure 1.1 Schematic illustration of single-walled carbon nanotube and multi-walled carbon nanotube. (Adapted from: Iijima S. Carbon nanotubes: past, present, and future. *Phys B*. 2002; 323: 1–5.)

Figure 1.2 Schematic diagram showing potential applications of CNTs in bio-related area. (Adapted from: Jithesh et al. Tailored carbon nanotubes for tissue engineering applications. *Biotechnol Prog*. 2009; 25(3): 709-21.)

1.1.1 CNT-mediated scaffold for tissue engineering

CNTs for bone regeneration

Bone tissues are made up of collagen fibers and hydroxylapatite crystals. Osteoblasts can proliferate on the bone surface, secrete matrix proteins and mineralize the matrix by hydroxylapatite production. Currently, polymers and peptide fibers are among the mostly used artificial bone scaffolds in bone graft. However, these materials have relatively low mechanical strength and tend to trigger immune rejection. Because of its high mechanical strength, low density and elasticity, CNTs can be a promising material to develop biocompatible scaffold with high mechanical strength that can support the growth and proliferation of osteoblast.

The idea of injecting CNTs into a bone fracture for supporting the growth of new tissues has been examined by Haddon's group (Zanello et al., 2006; Zhao et al., 2005). To be used as a scaffold for bone graft, the scaffold must be able to attract calcium ions and initiate the crystallization process of hydroxylapatite. In their experiment, SWCNTs were functionalized with phosphonates to introduce negatively-charged functional groups on the CNT surface. They found that the functionalization of SWCNTs resulted in the nucleation and crystallization of hydroxyapatite crystals on the surface of SWCNTs. Calcium ions were successfully attracted to the nanotubes because of the negatively charged functional groups introduced on the SWCNTs. The same group also tested the bone formation on CNTs obtained from various methods of functionalization. Specifically, pristine SWCNTs and MWCNTs, acid-treated SWCNTs, polyethylene glycol (PEG) coated SWCNTs and polyamino benzene

sulfonic acid (PABS) decorated SWCNTs were deposited on cover slips and bone-forming cells were cultivated on the CNT modified cover slips. Their study revealed that the growth and proliferation of osteoblast bone-forming cells were inhibited when cultivated on the surface-charged CNTs. In comparison, surface-neutralized CNTs promoted the cell growth and the formation of bone structure. Interestingly, the presence of CNTs in the scaffold was found to increase the cell adhesion, which is crucial for osteoblast cell proliferation and differentiation. Moreover, another group also demonstrated that the presence of vertically aligned CNTs in the scaffold significantly enhanced cell adhesion and proliferation of CAL-72 osteoblast-like cells (Giannona et al., 2007).

Extensive studies related to osteoblast growth and mineralization on CNT-strengthened porous polyurethane nanocomposite was also performed by Jell et al (Stevens et al., 2008). Their study revealed that the CNT-reinforced matrix showed good biocompatibility and did not cause any cytotoxicity on SaOS-2 osteoblast cells. Moreover, there was an elevated level of a potent angiogenic factor (vascular endothelial growth factor) in proportion to the amount of CNT loaded on the scaffold, which suggested CNT modified scaffold could also influence the cellular behaviors. However, detailed study on the effect of nanoscale architectures and surface chemistries introduced by CNTs on the cellular functions still need to be performed.

Beside the aforementioned studies on bone regeneration carried out *in vitro* cell culture models, *in vivo* studies have also been performed to explore the effect of CNTs on bone formation and the bone-tissue compatibility (Abarrategi et al., 2008; Usui et al., 2008; Wang et al., 2007b). These studies showed that the incorporation of MWCNTs

did not introduce any major inflammatory reactions and newly formed bone was successfully remodeled around the CNTs.

Overall, results from the aforementioned studies are very encouraging, which open the door towards CNT-based materials for bone regeneration. However, detailed studies of biomineralization with CNTs and strategies for the design of biocompatible CNT-based materials are still needed before CNT-reinforced materials for bone replacement are commercialized.

CNTs for neuron regeneration

Neuron regeneration for reestablishing intricate connection between neurons after brain injury has been considered to be notoriously difficult. Considering that CNTs are electrically conductive, possess similar diameters and aspect ratio to nerve fibers, they might be promising candidates for neuron regrowth and remodeling.

The first report regarding the growth of neurons on cover slips deposited with functionalized MWCNTs was demonstrated by Mattson et al (Mattson et al., 2000). They found that embryonic rat hippocampal neurons successfully attached to the nanotube's surface during the growth and the presence of 4-hydroxynonenal on MWCNTs promoted the neurite branching when compared to the unmodified nanotubes. Their findings suggested that the incorporation of functional groups such as bioactive molecules on the surface of CNTs could be used to direct the neurite branching for the development of a regulated neuron network on selected matrices. In another study, the effect of CNT's surface charge on neurite outgrowth and branching was also carried out (Hu et al., 2005). When hippocampal neuron cells were cultured

on the positively-charged SWCNT-PEI coated glass cover slips, neuron cells showed more branched neurites in comparison to the control substrates, which suggested that the surface charge did have profound effects on neuron cell branching and outgrowth.

Moreover, the effect of directly adding CNTs to the cell culture medium on the formation of neuron network was also investigated by a number of groups. Ni et al. demonstrated that neuron cells grew well with the presence of carboxyl functionalized SWCNTs modified with poly aminobezene sulphonic acid (PABA) or polyethylene glycol (PEG) in the hippocampal neuronal cultures (Ni et al., 2005). They also discovered that the SWCNT graft copolymers could facilitate the neurite outgrowth. These findings suggested that CNTs might be used for reestablishing the neural network at the injury sites as they were able to promote the neurite outgrowth and branching *in vivo*. Another study on the differentiation and survival of neurons in CNT incorporated cell culture was reported by Matsumoto et al (Matsumoto et al., 2007). With the purpose of enhancing the neuron cell growth and formation of neurites, they covalently immobilized neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) on amine functionalized MWCNTs. As expected, the neurite outgrowth was found to be significantly promoted by the incorporation of modified nanotubes in the cell culture medium.

These results for CNT mediated neuron regeneration are very promising, and it may pave the way to achieve the goal of regenerating the neural network in a certain pattern under *in vivo* conditions. However, since CNTs will be in constant interaction with brain tissues, the potential impact on the human health still needs to be carefully studied and evaluated.

Figure 1.3 Number of articles published in the last decade related to CNT-based drug delivery application. (Adapted from: Sandeep et al. Delivery of drugs and biomolecules using carbon nanotubes. *Carbon*. 2011; 49: 4077-97.)

1.1.2 CNTs as a delivery system

Since the efficacy during the traditional administration relies entirely on the physico-chemical properties of the drug, drug delivery systems that improve the pharmacological profile of bioactive molecules while preserving them from breakdown before reaching the targeted sites are urgently needed. Considering that most anti-cancer drugs are toxic and harmful to normal tissues, it is highly desirable to develop a targeted drug delivery system that can distinguish a cancer cell from healthy

cells and deliver the drugs to specific cancer sites. CNTs could be promising nanotransporters for the efficient delivery of drugs and biomolecules because of their large surface area and their ability to cross the cellular barriers. They can be non-covalently or covalently conjugated with drugs, biomolecules and other chemicals towards the development of a new-generation delivery system for drugs and biomolecules. Figure 1.3 shows the continuously increasing research efforts for applying CNTs as a drug delivery system during the last decade.

CNTs for drug delivery

Doxorubicin (DOX), an anthracycline antibiotic that functions as a DNA intercalating agent, has been widely used in treating various kinds of cancers. For traditional administration of DOX, it suffers from the poor distribution, low selectivity towards cancer cells and inability to cross cellular barriers. However, these limitations regarding to the traditional administration of DOX can be addressed by using CNTs as a novel drug carrier.

For example, the development of an anticancer drug delivery system by combining DOX, monoclonal antibody and fluorescein on the carboxylated SWCNT sidewall was reported (Fig. 1.4) (Heister et al., 2009). In their study, the monoclonal antibody that recognizes the tumor marker such as carcinoembryonic antigen (CEA) was incorporated to assist in the effective binding of DOX to the desired target sites on cancer cells. Through monitoring the fluorescence signals, their study confirmed that the drug-SWCNT complexes could selectively bind to cancer cells, transport and release DOX into WiDr colon cancer.

Figure 1.4 (A) Schematic illustration of DOX-fluorescein-BSA-antibody-SWCNT complexes (red = DOX, green = fluorescein, light blue = BSA, dark blue = antibodies) (B) Confocal image of WiDr cells incubated with DOX-fluorescein-BSA-SWCNT complexes. (Adapted from: Heister et al. Triple functionalisation of single-walled carbon nanotubes with doxorubicin, a monoclonal antibody, and a fluorescent marker for targeted cancer therapy. *Carbon*. 2009; 47(9): 2152-60.)

In another study, noncovalently functionalization by phospholipids-poly(ethylene glycol) (PL-PEG5000-NH₂) or covalently modified by PEGylation of carboxylic groups on SWCNTs were used for ultrahigh capacity loading of DOX through high degrees of π -stacking (Liu et al., 2007a). Specifically, the cyclic arginine–glycine–aspartic acid (RGD) peptide was conjugated to carboxylated SWCNT, which acts as a ligand to introduce recognition moieties for integrin $\alpha_v\beta_3$ receptors and enhances drug delivery to integrin $\alpha_v\beta_3$ -positive U87MG cells. As expected, there was a noticeable improvement in the delivery of RGD–SWCNT–DOX when integrin $\alpha_v\beta_3$ -positive MCF-7 cells were used in comparison to the integrin $\alpha_v\beta_3$ -negative cells.

In another approach, DOX was loaded on the polysaccharide [sodium alginate (ALG) and chitosan (CHI)] coated carboxyl functionalized SWCNTs (Zhang et al., 2009). The incorporation of ALG was aimed to facilitate DOX loading, while the attachment of CHI was used to assist the binding of folic acid (FA), a bioactive molecule that improves the selectivity of DOX release to the HeLa cells as the FA receptor tends to be overexpressed on the surface of these cancer cells. As expected, there was a significant increase in the effectiveness of releasing DOX to the targeted Hela cells and inducing cell death of tumor cells.

The supramolecular stacking of DOX on SWCNT for *in vivo* lymphoma therapy has been studied (Fig. 1.5) (Liu et al., 2009a). Briefly, DOX was loaded on PEGylated SWCNT by supramolecular π – π stacking as mentioned above. The *in vivo* pharmacokinetics profiles, biodistribution, therapeutic efficacy and toxicity of this drug delivery system were then investigated. Mice were treated with either free drug

or drug conjugated with CNT. Their study showed that SWCNT–DOX was more effective for the delivery of DOX to tumor cells and less toxic to normal tissues in comparison to equimolar amounts of DOX. Table 1.1 provides a summary of CNT-based drug delivery systems employed for the delivery of anticancer drugs. Apart from anticancer drugs, CNT-based delivery systems have also been employed for the delivery of other drugs (Table 1.2).

Figure 1.5 (A) Representation (a) and AFM image (b) of the SWCNT-DOX complex. (c) UV/Vis/NIR spectra of plain SWCNTs and SWCNT-DOX. (B) Fluorescence spectroscopy study of the pharmacokinetics and biodistribution of two DOX formulations. (a) SWCNT-DOX showed prolonged blood circulation compared with free DOX. (b) SWCNT-DOX had higher tumor-specific uptake and RES uptake than free DOX. (Adapted from: Liu et al. Supramolecular stacking of doxorubicin on carbon nanotubes for *in vivo* cancer therapy. *Angew Chem Int Ed.* 2009; 48(41): 7668-72.)

Table 1.1 A summary of representative studies regarding to CNT-based anticancer drug delivery system

Drug delivery system	Tumor-targeted modules	Trigger	Drug-CNT conjugate in comparison to drug	Reference
DOX-FA-CHI/ALG-SWCNT	FA	FA-FA receptor interaction, pH triggered release	More cytotoxic and selective	(Zhang et al., 2009)
Fluorescein-PL-PEG-SWCNT-RGD peptide-DOX	RGD	RGD peptide recognition moiety-integrin $\alpha_v\beta_3$ receptor, pH triggered drug release	Less toxic to $\alpha_v\beta_3$ -negative MFC cells	(Liu et al., 2007a)
DOX-PL-PEG-SWCNT	N.M.	pH triggered drug release	More efficient at treating tumors and less toxic to mice	(Liu et al., 2009a)
DOX-amphiphilic polymers-CNT	N.M.	N.M.	More efficient	(Park et al., 2008)
DOX/FA-MWCNT-Fe	FA and Fe	FA-FA receptor interaction (active) and magnetic force (passive)	Prolonged drug release	(Li et al., 2011a)
EPI-c-MWCNT	N.M.	N.M.	Greater EPI release in acidic medium	(Chen et al., 2011)
DOX/PEGylated MWCNT	N.M.	N.M.	Efficient anti-MDR effect	(Cheng et al., 2011)
EGF-CDDP-SWCNT	CDDP and EGF	EGF-EGF receptor interaction	More efficient	(Bhirde et al., 2009)
Biotin-SWCNT-(taxoid-fluorescein)	Biotin	Biotin-biotin receptors mediated endocytosis	More efficient	(Chen et al., 2008)

Table 1.2 A summary of representative studies regarding to CNT-based delivery system for the delivery of other drugs

Drug delivery system	Drug effect	Drug delivery methods	Drug-CNT conjugate in comparison to drug	Reference
Dopsone-O-(7-azabenzotriazol-1-yl)-N, N,N',N'-tetramethyluronium hexafluorophosphate/N,N-diisopropylethylamine-f-MWCNT	Anti-microbial and anti-inflammatory	N.M.	More effecient	(Vuković et al., 2010)
Polyethylene oxide-pentaerythritol triacrylate-[(S)-(+)]ketoprofen-MWCNT	Anti-inflammatory	Electrospinning	More effecient	(Im et al., 2010)
DEX-CHI-SWCNT	Anti-inflammatory	Electrical stimulation	More effecient	(Naficy et al., 2009)
Amb-fluorescein-MWCNT	Antibiotic	N.M.	More efficient	(Wu et al., 2005)
TPGS-MWCNT	Vitamin E delivery	N.M.	N.M.	(Yan et al., 2007)
CAR-MWCNT	Anti-hypertensive	N.M.	More efficient	(Li et al., 2011b)
Theophylline-AL/CNT	N.M.	N.M.	More efficient	(Zhang et al., 2010b)
Ach-SWCNT	Alzheimer's disease therapeutic agent	pH stimulation	More efficient	(Yang et al., 2010b)

CNTs for DNA/RNA delivery

Virus mediated gene delivery, which utilizes the virus to inject DNA inside a host cell, is the currently mostly adopted method for gene delivery. However, this approach can sometimes induce undesirable immune responses, inflammation and even oncogenic effects (Thomas et al., 2003). Hence, a variety of nonviral vectors such as liposomes, polymers and cationic lipids have been developed to substitute the virus-mediated gene delivery approach (Davis, 2002). More recently, CNTs have attracted tremendous interests for gene delivery applications and extensive investigations have been carried out to exploit CNTs as the biological cargos for gene delivery. To use as a cargo for gene delivery, nucleotides might be able to load on CNTs. Interestingly, it has been reported that CNTs could interact directly with DNA through Van der Waals and hydrophobic forces (Gao and Kong, 2004), which facilitates the loading process of plasmid DNAs on the CNTs. In addition, a number of other approaches have been reported to covalently attach either single or double stranded DNA to the CNTs (Ramanathan et al., 2005; Yang et al., 2007). Table 1.3 provides a summary of different approaches that have been attempted in CNT-based gene delivery.

Of interest, Singh et al. demonstrated that both SWCNTs and MWCNTs functionalized with positively-charged ammonium or lysine on the surface of the nanotubes were able to load negatively-charged plasmid DNA (Singh et al., 2005). By simply mixing of the positively-charged CNTs with negatively-charged plasmid DNA, it can lead to the formation of plasmid-CNT complexes through the static electronic forces formed between them. Once the plasmid-CNT complexes enter the cell, the change in ion strength inside the cells could result in the release of DNAs from the

nanotubes and its translocation into nuclei for gene expression.

On the other hand, CNT-based RNA delivery has also been extensively explored (Table 1.3). In this direction, Dai's group performed gene silencing studies with CNT covalently conjugated siRNA (Liu et al., 2007b). To load siRNA onto CNTs, the nanotubes were first functionalized by adsorbing phospholipids-polyethylene glycol (PL-PEG2000-NH₂). Next, the siRNA was modified with thiol groups. Subsequently, the CNT-siRNA conjugates could be formed by conjugating the thiol-modified siRNA to the amine groups of PEG on the sidewalls of CNTs through disulfide linkages. It has shown that the delivery of siRNA into cells could be achieved by simply incubating the cells with the CNT-siRNA complexes. The siRNA transfection efficiency of CNT-mediated delivery system was found to be comparable with those of liposome-based RNA transfection approaches. With 3 days of incubation T-cells with CNTs-siRNA conjugates, 90% of gene appeared to be silenced in these T-cells. Moreover, the application of these CNT-siRNA conjugates in human primary cells also resulted in a 60% of knock down.

The aforementioned results are very encouraging, and it might pave the way to achieve the goal of CNT mediated gene delivery system. However, most gene delivery studies with CNT reported so far are based *in vitro* cell cultures. For the success of DNA/RNA delivery *in vivo* system, a systematic study of targeting, delivery, release and biological functions of the CNT-nucleic acid conjugate is needed.

Table 1.3 A summary of representative studies performed with CNT-based DNA/RNA delivery

Delivery system	Biological system	Results	Reference
BSA/DNA-amino-MWCNT	Hela and HL cells	BSA and DNA were covalently bound to amino-MWCNT	(Awasthi et al., 2009)
DNA-PEI-MWCNT	293 cells, COS7 and HepG2 cells	PEI served as anchor points for DNA immobilization; good transfection efficiency for the delivery of DNA	(Liu et al., 2005)
ASODNs-PEI-MWCNT	Hela cells	ASODN interacted with positively charged amine groups on PEI-MWCNTs	(Jia et al., 2007)
Plasmid-carboxylic f-MWCNT with embedded Ni	B-lymphoma, B-cells and primary neurons	DNA-MWCNT entered these cells driven by magnetic field and cells remained high viable after transduction	(Cai et al., 2005)
GFP gene-amnio-MWCNT	Umbilical vein endothelial cells	Amino group functionalized MWCNT effectively delivered the pEGFPN1 plasmid into cells	(Gao et al., 2006)
BSA-SA-DNA-carboxyl-SWCNT	N.M.	Protein-SWCNT entered into living cells as carrier-cargo complexes	(Kam et al., 2006)
ssDNA-Au-SWCNT	N.M.	Target DNA hybridization to ssDNA probes	(Jung et al., 2010)
ssDNA-pristine SWCNT	N.M.	ssDNA bound to SWCNT got released by desorption potential	(Zheng et al., 2009)
ssDNA-carboxyl-SWCNT	Breast carcinoma cells	CNT-modified DNA probe binds to a specific target mRNA inside living cells.	(Wu et al., 2008)
NF- κ B decoy-SWCNT	Hela cells	Covalent binding of NF- κ B decoy on SWCNT greatly reduced the NF- κ B dependent gene expression	(Crinelli et al., 2010)
siRNA-PEI/pyridinium-f-MWCNT	Human lung cancer cell line	Both types of f-MWCNTs showed increasing silencing activity and cytotoxicity	(Varkouhi et al., 2011)
siRNA-PDDA-HMDA-SWCNT	Isolated rat heart cells	PDDA-HMDA-SWCNT bound negatively charged siRNA by electrostatic interactions	(Krajcik et al., 2008)
siRNA-PL-SWCNT	Human T cells and primary cells	CNTs were capable of siRNA delivery to human T Cells and primary cells	(Liu et al., 2007b)
siRNA/DNA-PL-PEG-SWCNT	Hela cells	PL-PEG-SWCNT could bind to various biomolecules	(Kam et al., 2005)

1.2 Graphene and its applications

Graphene, one-atom-thick planar sheets of sp^2 -bonded carbon atoms that are packed in a honeycomb crystal lattice, has recently gained tremendous interests in the fields of materials science, physics, chemistry, and biotechnology (Geim, 2009). Although graphene is the fundamental basis of different carbon forms, it was only first prepared in 2004, by peeling a single layer of graphene using sticky tape and pencil. During the past several years, various other approaches for producing graphene have been developed, such as micromechanical exfoliation, epitaxial growth, chemical vapour deposition and chemically synthesis (Lotya et al., 2009; Obraztsov, 2009; Park and Ruoff, 2009; Shao et al., 2010; Sutter et al., 2008; Williams et al., 2008; Zhu et al., 2010).

Because of the unique chemical and physical properties, graphene and its derivatives have also attracted strong interest in biological studies (Akhavan and Ghaderi, 2010; Choi et al., 2010; Hong et al., 2010; Hu et al., 2010; Liu et al., 2010; Mohanty and Berry, 2008; Sun et al., 2008a; Yang et al., 2010a). A number of graphene-based nanomaterials have been exploited to fabricate functionalized biosystems integrated with nucleic acids, peptides, proteins and even cells. Moreover, the discovery of graphene to quench electron donors, the ability to protect biomolecules from enzymatic cleavage, as well as transportation capability in living cells and *in vivo* systems, open the door towards applications in biological studies such as biosensor and drug delivery.

For example, modified graphene oxide (GO) has been investigated as a transporter for

the delivery of cancer drugs, which the purpose of improving the delivery efficiency to targeted positions (Liu et al., 2008b). To load these cancer drugs onto graphene based carrier, GO was first functionalized with polyethylene glycol (PEG) to facilitate the loading of anti-cancer drug, as well as render high solubility in physiological conditions. A water-insoluble aromatic molecule, SN38, was next attached to PEGylated GO (GO-PEG) to yield GO-PEG-SN38 conjugates. Interestingly, the GO-PEG-SN38 complex was found to have high potency with IC₅₀ values of 6 nM for HCT-116 human colon cancer cells, which was comparable to that of free SN38 drugs. To enhance the selectivity of anticancer drugs to specific cancer cells, GO can be also modified with tumor targeting moieties such as folic acid (FA) (Zhang et al., 2010a). In this direction, two anticancer drugs, doxorubicin (DOX) and CPT-11 were both loaded onto the FA-conjugated GO (FA-GO) to yield FA-GO-drug complex. It was found that FA-GO-drug conjugates showed specific selectivity to MCF-7 human breast cancer cells which express FA receptors on their surface. As expected, FA-GO-drug complexes with targeting ability led significantly higher cytotoxicity in the tumor cells when compared to the FA free GO-drug complexes.

With the success of the above studies, it is likely that graphene-based nanocarriers will find widespread application in biomedicine in the future. However, cytotoxicity, the cellular uptake mechanism, and the intracellular metabolic pathway of graphene and its derivatives remain almost unknown, which are crucial for exploiting graphene in living cell studies and for *in vivo* applications.

1.3 Cytotoxicity of CNTs and graphene

Recently, a number of studies either using *in vitro* cell lines or *in vivo* with animal models have been reported on CNT's toxicity. However, the results reported by these studies are contradictory in nature, with some suggesting CNTs are highly cytotoxic and others stating lack of any toxic effects. For example, Cui et al. reported that the treatment of the HEK 293 cells with SWCNTs can lead to cell viability reduction in time and dose dependent manner (Cui et al., 2005). Another study shows that cell exposure to SWCNTs could induce a significant hatching delay of zera fish embryos (Cheng et al., 2007). Whereas, experiments performed on the HL-60 cells revealed that SWCNTs up to 25 µg/mL have no noticeable effect on cell proliferation and viability after a short time period of exposure (Kam and Dai, 2005). *In vivo* and *in vitro* studies by Muller et al. point out that there might be some adverse effects of MWCNTs on epithelial cells (Muller et al., 2008). Some of the recent reviews have extensively discussed on the biocompatibility and toxicity of CNTs (Fiorito et al., 2006; Firme Iii and Bandaru, 2010; Johnston et al., 2010; Tejral et al., 2009). It is true that drawing a genral conclusion about the safe use of CNTs *in vivo* is extremely difficult as most studies are performed on various experimental conditions. The discrepancy in the biocompatibility/cytotoxicity studies of CNTs can be due in part to various factors such as the length and size of nanotubes, the purity of the materials, the dispersion condition of nanotubes and different chemistries for the surface functionalization of nanotubes. Although successful stem cell differentiations have been reported on CNT-based nanomaterials, it should also keep in mind that severe DNA damages were also observed in certain stem cells. For example, studies by Zhu

et al. using MWCNTs on mouse embryonic stem cells (ESCs) revealed that the CNTs could accumulate inside the cells, activate tumor suppressor protein p53 and lead to cell death through apoptosis (Zhu et al., 2007). It was also observed that the MWCNTs could trigger elevated mutation frequency in the mouse ESCs, which was 2-fold in comparison to the control cells. Moreover, Shvedova et al. reported that pharyngeal aspiration of SWCNTs could induce acute inflammatory reaction, lead to the fibrinogenic response and the formation of granulomas in mice. Even after two months of aspiration, the nanotubes were still found to be retained inside the pulmonary granulomatous lesion. Further studies carried out by the same group confirmed that both SWCNTs and MWCNTs could trigger the activation of the human serum complement system via classical pathways (Salvador-Morales et al., 2006). Yet another pulmonary toxicity study of CNTs in mouse model showed that intratracheal administration of SWCNTs into 8 week old male ICR mice resulted in alveolar macrophage activation, various chronic inflammatory responses and severe pulmonary granuloma formation (Chou et al., 2008). In their study, transcription factors like NF- κ b and AP-1 were found to be activated in macrophages after exposure to SWCNTs. Oxidative stress, innate and adaptive immune responses were also noticeable for the mice with intratracheal administration of SWCNTs.

Currently, several cytotoxicity mechanisms have been proposed with some groups claim CNTs could disrupt the intracellular metabolic pathways, and others suggest that CNTs could cause oxidative stress and membrane damage. Among the various cytotoxicity mechanisms proposed, oxidative stress has been considered to be the most acceptable mechanism elucidating the potential cytotoxicity caused by CNTs.

In contrast, there are limited studies regarding the cytotoxicity evaluation of graphene and its derivatives, not to mention elucidating the potential mechanisms at the molecular level (Chang et al., 2011; Ryoo et al., 2010; Zhang et al., 2011; Zhang et al., 2010c). For the application in the biotechnology field such as CNT/graphene based cellular probing, diagnostics, drug delivery and therapy, the cytotoxicity and long-term impact of these nanomaterials on human health need to be systematically characterized before applying them *in vivo*.

1.4 Comparative proteomics techniques

The term 'proteome' was first introduced by Wilkins et al. decades ago (Wilkins et al., 1996), which refers to the entire set of proteins expressed in a given type of cell, tissue or organism. Since proteins are the vital parts of living systems, proteomics always provides more useful information about an organism than genomics as it directly addresses the level of genome products and their interactions.

In the rapidly growing field of proteomics, mass spectrometry has become a basic technology to address the increasing bioanalytical demands encountered in proteomic studies. Currently, there are various MS interfaces that could facilitate the ionization of the samples and introduce them into the high-vacuum mass spectrometer for analysis. Based on their individual mechanisms, MS interface can be classified into the following four groups: atmospheric-pressure chemical ionization spray interface, ion spray (IS) interface, electrospray ionization (ESI) interface and matrix-assisted laser desorption/ionization (MALDI). In these days, ESI and MALDI are among the most widely used MS interfaces for proteomics study. For ESI interface, the analytes

dissolved in polar solvent are sprayed into the ionization source where a high electrical potential is applied. And highly charged droplets are formed and then vaporized with the aid of a neutral gas. For MALDI interface, a dry crystalline matrix is normally used instead of liquid samples and a laser beam is applied to facilitate vaporization and ionization of the samples. Compared with ESI, MALDI interface are more tolerant of the modest presence of salts and/or detergents (Breux et al., 2000) owing to the fact that analytes can be vaporized away from impurities during ionization process, while impurities can compete with the analytes when using ESI as they are likely to be co-injected through spraying. However, such problem can be easily resolved by applying multi-dimensional LC separation techniques, which can significantly remove salt and other impurities in the sample before spraying into mass analyzer. ESI, theoretically, has unlimited mass range because it tends to produce multiple charged species for proteins or peptides, and proteins with large molecular weights can display at lower m/z values. Since ESI enables to generate ions directly from liquid solution, it is therefore more readily coupled to liquid-based separation tools. In comparison, MALDI, tends to generate single charged species and this feature is greatly important, particularly for identifying the molecular ion of proteins, carbohydrates and lipids (El-Aneel et al., 2009).

For identification of peptides and proteins, various types of mass analyzer are used in proteomics research including quadrupole, reflector time-of-flight (TOF), quadrupole ion trap, Fourier transform ion cyclotron (FT-ICR) and orbitrap mass analyzers, as illustrated in detail in Figure 1.6 (Aebbersold and Mann, 2003). In order to perform tandem mass analysis with quadrupole instruments, it is necessary to place two or more quadrupoles in series. In the first quadrupole (Q_1), the time-varying electric field

Figure 1.6 Mass spectrometers used in proteome research. The left and right upper panels illustrate the sample introduction and ionization process in ESI and MALDI. The different instrumental designs (a–f) are featured with their typical ion source. a, The reflector time-of-flight instrument; b, The TOF-TOF instrument; c, The quadrupole instrument or linear ion trap; d, The quadrupole TOF instrument ; e, The (three-dimensional) ion trap ; f, The FT-MS instrument. (Adapted from: Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature*. 2003; 422: 198-207.)

between four rods is applied to scan across ions of a preset m/z range and select an ion of a particular m/z . The second quadrupole, known as the collision cell (q_2), introduces the collision gas and fragmented the selected ions, and the third quadrupole (Q_3) serves to read out the fragmented ions. For ion trap analyzer, the physics behind is similar to quadrupole analyzer except that the fragmented ions are trapped in a radio frequency field rather than passing through a quadrupole. For FT-ICR analyzer, it harnesses the strong magnetic fields to trap the ions instead. In TOF analyzer, the ions are accelerated with high kinetic energy, separated along a flight tube based on their different velocities. These ions returned from a reflector with different kinetic energies finally reach on the detector that amplifies and registers arriving ions. The tandem mass spectrometry, quadrupole/TOF, regarded as a triple quadrupole with the third quadrupole replaced by an orthogonal TOF, has found applications in rapid *de novo* peptide sequencing (Morris et al., 1996; Shevchenko et al., 1997). The orbitrap mass analyzer is composed of an outer barrel-like electrode and an inner spindle-like central electrode. Tangentially injected ions can rotate around the central electrode and be trapped in the electrostatic field between the outer and central electrodes when an appropriate voltage is applied. The frequencies of axial oscillations of ions with specific m/z ratio can be detected and subjected to mass analysis.

Since the introduction of mass spectrometry-based proteomics, various approaches for separating the complex mixture of proteins before subjected to identification by mass spectrometry have been emerged. Traditionally, two-dimensional gel electrophoresis (2-DE) is used to separate the complex protein samples. In this approach, a mixture of proteins labeled with different dye is first separated by charge on an immobilized pH gradient strip and further separated by molecular weight on sodium dodecyl

sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) slab gel. Following comparison of the dye patterns of these individual proteins from multiple samples, the differentially expressed proteins of interest are excised from the gel, cleaved into peptides using proteolytic enzyme such as trypsin, and subjected to MS analysis for identification. The overall workflow for the 2-DE approach seems to be straightforward process. However, this approach suffers poor reproducibility and difficulty in automation for high throughput. Moreover, several practical limitations remain, including co-migration problem, difficulty in focusing highly basic and acidic proteins, low resolution against hydrophobic and membrane proteins and inadequate sensitivity (Lilley et al., 2002; Timperman and Aebersold, 2000). Due to these limitations, alternative approaches involves the use of liquid-based techniques such as capillary electrophoresis and HPLC have gradually substituted the gel-based separation to meet the increasing demand for resolving the high degree complexity of cellular proteomes and detect the low abundance proteins. In the LC-based approach, the mixture of the cleaved peptides can be separated sequentially on the multi-dimensional columns based on charge or hydrophobicity of the ionized peptides, eluted into the MS for identification.

Currently, various methods using stable isotope-based quantification have been developed for LC-based proteomics applications. Briefly, stable isotope labels can be introduced onto the peptide or protein by the following four approaches: (i) Spiking in an isotopically labeled peptide which was chemically synthesized as internal standards with known quantities (Kusmierz et al., 1990), (ii) incorporation through an enzyme via transfer of ^{18}O from water to peptides during protein digestion (Mirgorodskaya et al., 2000; Yao et al., 2001), (iii) introducing isotope-tagging chemistries onto peptides

or proteins and (iv) metabolic incorporation of the stable isotope to the cells in particular, stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002).

SILAC allows pooling of labeled and unlabelled cell population and subsequent fractionation/purification of the pooled sample can be simply performed, which lowers the possibility in introducing errors in quantitation. However, there are limitations for SILAC labeling approach as this method is not suitable for living cells, body fluids and secretory proteome analysis. Among all the developed stable isotope-based quantification methods, isobaric tag for relative and absolute quantitation (iTRAQ) has recently gained popularity as it allows up to multiple samples to be examined within one experiment, increasing analytical throughput and resulting in great reduction of workload. The only limitation for this method is that the quantitation has to be performed at the MS/MS stage rather than in MS and it has to be equipped with mass spectrometers capable of detecting low mass range during MS/MS analysis (Putz et al., 2005).

As shown in Figure 1.7A, the complete iTRAQ molecule consists of a reporter group (based on N-methylpiperazine), a mass balance group (carbonyl) and a peptide-reactive group (NHS ester) (Ross et al., 2004a). The overall mass of reporter group ion and mass balance group of the molecule are kept isobaric by using the differential isotopic enrichment by ^{18}O , ^{15}N and ^{13}C atoms. Specifically, the mass of the reporter group ranges from m/z 114.1 to 117.1, while the balance group ranges in mass from 28 to 31 Da, such that the combined mass for individual reagent remained constant (145.1 Da). When a tag reacted with a peptide, an amide linkage was formed

to any peptide amine (N-terminal or ϵ amino group of lysine) shown in Figure 1.7B. In such manner, the mixture of four identical peptides, each labeled with one member of the multiplex set, will appear as a single unresolved precursor ion in MS (indistinguishable m/z). When subjected to collision-induced dissociation (CID) MS/MS analysis of the precursor ion, the amide linkages will fragment in a similar manner as the backbone peptide bonds. However, the balance moiety will be lost, while charge is retained by reporter group, which leads to the detection of four reporter ions separate as distinct masses (114–117 Da) (Fig. 1.7C). In contrast, all other sequence-instructive fragment ions (b -, y -, etc.) are still isobaric. The relative abundance of the peptides thus can be deduced from the relative intensities of the corresponding reporter ions. Noteworthy, quantitation for iTRAQ technique has to be performed at the MS/MS stage rather than in MS.

Example MS/MS spectrum of peptide TPHPALTEAK from a protein digest mixture prepared with separate digests labeled with each member of the four isobaric tags and combined in a ratio of 1:1:1:1 is shown in Figure 1.8 (Ross et al., 2004a). Specifically, components illustrated in Figure 1.8 are (i) the isotopic distribution of the precursor with a m/z of 1352.84 ($[M+H]^+$), (ii) the reporter ions in the low mass region used for quantitation, (iii) isotopic distribution of the b_6 fragment ion, and (iv) isotopic distribution of the y_7 fragment ion. Since the peptide is labeled by isobaric tags at both the N terminus and C-terminal lysine side chain, the resulting precursor ion and all the internal fragment ions denoted as b^- and y^- would remain isobaric. Both the precursor ion and the internal fragment ions could be used for peptide identification. On the other hand, the reporter ions at the lower mass portion could be harnessed for the quantification the abundance of individual peptide in the mixture.

Figure 1.7 A schematic diagram showing the components and chemistry of the multiplexed isobaric tagging technique. (Adapted from: Ross et al. Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol Cell Proteomics*. 2004; 3: 1154-1169.)

Figure 1.8 Example MS/MS spectrum of peptide TPHPALTEAK derived from a mixture of protein digests after labeling with iTRAQ reagents. Four individual cleaved peptides were each labeled with isobaric reagent tag in a ratio of 1:1:1:1. (Adapted from: Ross *et al.* Multiplexed Protein Quantitation in *Saccharomyces cerevisiae* Using Amine-reactive Isobaric Tagging Reagents. *Mol Cell Proteomics*. 2004; 3: 1154-1169.)

1.5 Objectives of research project

For the bio-related application, CNTs/graphene and their functionalized derivatives are in constant interaction with living cells and tissues. The cytotoxicity and long-term impact of these nanomaterials on human health need to be characterized very carefully before applying them *in vivo*. It is important to document these interactions so that new approaches and technologies can be developed and applied to medical science and disease treatment.

Comparative proteome analysis has been widely used to study the cellular functions in response to various external stimuli. The change of protein profile directly reflects the biological processes the cell undergoes. Thus it will be of great interest to obtain the protein profile change of living systems caused by nanomaterials and have a fundamental understanding of the interactions between these nanomaterials and living systems at the proteome level.

In the present study, we aim to apply the isobaric tagged relative and absolute quantification (iTRAQ) based comparative proteomics technique to simultaneously detect and quantify the differences of protein expression levels in living systems after the treatment of various graphitic nanomaterials, namely, SWCNTs, oxidized SWCNTs, graphene and GO. Human hepatoma HepG2 cells, which have high degree of morphological and functional differentiation *in vitro*, are chosen as the model system for studying the potential cytotoxicity on primary organ of liver.

The objective of the project is to study the differentially expressed proteins within the proteome of human hepatoma HepG2 cells in response to these nanomaterials using

quantitative proteomics techniques and thus infer the possible mode of action for individual nanomaterial. By overall comparison of the differentially expressed proteins in HepG2 cells incubated with these nanomaterials, the similarities and differences can be used to evaluate the potential cytotoxic effects that might be caused by these nanomaterials.

Experimentally, to better resolve the peptides during the LC separation step and get more high confidence peptides, 2-dimensional (2D) LC workflow is carefully designed. In this approach, a strong cation exchange (SCX) column and HPLC-Chip (Large capacity chip II, Agilent Technologies) are used to set up the working platform. In the first dimension, the sample peptides are all loaded on the SCX columns, eluted by stepwise injections of salt plugs. The eluted peptides are trapped on the short enrichment column during the enrichment mode. Subsequently, the enrichment column is switched to analytic mode and the peptides trapped on enrichment column are eluted by a nanoflow gradient of acetonitrile and further separated on the reverse phase column incorporated in HPLC-Chip. Working on this sophisticated approach, we might be able to get more high confidence proteins which can shed new lights on biological responses to these nanomaterials and thereby provide a fundamental understanding of interactions between the living system and these nanomaterials. Further functional assays such as cell proliferation assay, Western blotting analysis, apoptosis assay and cell cycle analysis are also carried out with the purpose of validating the data obtained from proteomics approach.

Chapter 2 Cytotoxicity of SWCNTs on human HepG2 Cells by proteome analysis

2.1 Introduction

Due to the fascinating physical, chemical, electrical, and mechanical properties of nanomaterials, the nanotechnology is emerging as part of a new industrial revolution being applied to a diverse array of consumer products and medical applications. Among all nanomaterials, carbon nanotubes (CNTs) have recently gain popularity in the scientific community due to its cancer killing and drug delivery capabilities (Chakravarty et al., 2008; Liu et al., 2008a; Liu et al., 2009b). However, with the growing potential biomedical applications and the rising societal concerns on nanosafety, mechanistic understanding of the interactions between nanomaterials and living systems has become imperative (Chou et al., 2008; Ding et al., 2005; Manna et al., 2005; Mu et al., 2009; Zhang et al., 2006; Zhou et al., 2008). Previous studies show that both pristine SWCNTs and oxidized SWCNTs were able to generate reactive oxygen species (ROS) and cause nuclear factor kappa B (NF- κ B) activation (Manna et al., 2005; Ye et al., 2011). Nevertheless, few detailed studies related to the systematic characterization of the interactions between living systems and SWCNTs at the molecular level have been performed, especially at the proteome level.

Proteome analysis has proven to be an effective approach towards a comprehensive analysis of the cellular function in response to different stimulus and protein changes reflect biological responses. Among various existing comparative proteomics techniques, isobaric tagged relative and absolute quantification (iTRAQ) technique is

a liquid chromatography (LC) based method which has been gradually gaining popularity (Khan et al., 2010; Ross et al., 2004b; Sui et al., 2007; Xu et al., 2008). The system enables multiple samples to be analyzed within one single experiment. The procedure of this simple and robust system includes liquid chromatography separation of isotopically labeled peptides followed by MS/MS analysis for peptide identification and quantification.

In the present study, we exploited the iTRAQ-coupled 2-D LC-MS/MS analysis to study the global protein profile in human hepatoma HepG2 cells treated with SWCNTs, with purpose of elucidating the potential cytotoxicity of SWCNTs on living systems at the molecular level. To better resolve the peptides, two-dimensional (2D) liquid chromatography for separation of peptides was applied. In this approach, a strong cation exchange (SCX) column was used for the first dimension, a reversed-phase (RP) column was used for the second, and a short enrichment column was used for trapping the peptides. In the first dimension, the sample peptides were all loaded on the SCX columns, eluted by stepwise injection of salt plugs. The eluted peptides were first trapped on the short enrichment column during the enrichment mode. Subsequently, the short enrichment column was switched to analytic mode and the peptides trapped on enrichment column were eluted and further separated on the nanoRP column in the second dimension. The overall workflow can be simplified as shown in Figure 2.1.

From our preliminary data, overall 51 differentially expressed proteins that involved in metabolic pathway, redox regulation, signaling pathway, cytoskeleton formation and cell growth were identified. We found SWCNTs triggered the up-regulation of

Figure 2.1 Schematic diagram showing the workflow for the proteomics study of cellular functions in response to SWCNTs.

metabolic enzymes, heat shock proteins and proteins involved in redox regulation, which indicated SWCNTs could introduce reactive oxygen species, cause DNA damage, perturb protein synthesis and cellular metabolism. Our data also suggested that SWCNTs might trigger the activation of apoptosis signal-regulating kinase 1, result in the activation of downstream signaling cascades such as the c-Jun N-terminal kinase and p38 pathway, and finally lead to stress-induced apoptosis. The comparative protein profile provides us new insight into the cellular response at the proteome level, which should be very useful to understand the interaction between these nanomaterials and mammalian cells.

2.2 Materials and methods

2.2.1 Preparation and characterization of SWCNTs

High purity SWCNTs (purified HiPco, Unidym) were used in the present study. These SWCNTs were produced by chemical vapor deposition (CVD) by means of the HiPco process. The process produces SWCNTs with a Gaussian distribution of diameters, with a maximum peak around 0.8~1.2 nm. A wet chemical technique was used to remove the catalyst and nontubular carbon species. Briefly, the powder was heated under reflux in concentrated HCl for 6 h at 110 °C to dissolve the metal oxide particles and finally filtered under vacuum, washed with water, and dried at 110 °C for 6 h. Following purification, the quality of purified SWCNTs was checked using atomic force microscopy (AFM) and Fourier transform infrared (FTIR) spectroscopy. The AFM was taken using a MFP3D microscope (Asylum Research) with a silicon cantilever operating in tapping mode. FTIR spectra were carried on a Perkin Elmer

Spectrum One Spectrometer.

2.2.2 Cell cultures

Human hepatoma HepG2 cells, obtained from the American Type Culture Collection, were cultured in Dulbecco's modified Eagle's medium (supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin). Cells were maintained at 37 °C in an atmosphere of 5% CO₂. All culture media and media supplements were purchased from Invitrogen. As pristine SWCNTs are prone to agglomerate and agglomerants tend to settle onto the cell surface, low concentration with well-dispersed SWCNTs was used in the present study. Specifically, the media supplemented with surfactant (0.2% Tween 80) were used for the preparation of SWCNTs containing media and the final concentration of 1 µg/mL was tested stable under the subsequent culture condition. After reaching 80% confluence, the HepG2 cells were incubated 48 h with fresh medium containing 1 µg/mL of SWCNTs. For the control cells, the fresh media supplemented with surfactant (0.2% Tween 80) were used.

2.2.3 MTT assay

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) test assesses cell viability based on the ability of the mitochondrial succinate-tetrazolium reductase to convert the yellow dye (MTT) to a purple-colored formazan in living cells. The viability of the cell can be derived from the color density formed. Briefly, following HepG2 cells incubation with SWCNTs for 48 h, the media were aspirated and replaced with 90 µL of serum-free media. Then 10 µL of MTT

stock solution (5 mg/mL) was added, followed by incubation for 4 h at 37 °C. The supernatant was then removed, and cells were lysed with 100 µL of dimethylsulfoxide (DMSO) and the absorbance was recorded at 550 nm on a microplate reader (Benchmark Plus).

2.2.4 Cell lysis

After treated with SWCNTs for 48 h, the cells were first dissociated from the flasks through trypsinization. Briefly, the culture media were removed, the cells were washed with phosphate buffered saline (PBS), and 1 mL of trypsin-EDTA mixture was added to make the cells detach from the bottom of the flask. After incubation at 37 °C for 3-5 min, 5 mL of DMEM media were added to stop the reaction. The suspended cells were centrifuged at 2000 rpm for 1 min, the supernatant was discarded and the cell pellet was washed three times with 1X PBS (pH 7.2). The cells were collected by centrifugation at 2,000 rpm for 5 min at 4 °C. This was followed by an addition of 300 µL of lysis buffer composed of 8 M urea, 4% (w/v) CHAPS, and 0.05% SDS (w/v) (GE Healthcare). Briefly, samples were vigorously vortexed for 1 min and kept on ice for 30 min with regular vortexing. Next, samples were centrifuged at $16,100 \times g$ for 60 min at 4 °C, supernatant containing the total proteins was collected and stored at -80 °C for future use.

2.2.5 Protein quantification

2-D Quant Kit (GE Healthcare) was used for the accurate determination of protein concentration in samples. The procedure uses a combination of a unique precipitant and co-precipitant to quantitatively precipitate proteins while leaving interfering

substances in solution. The protein is pelleted by centrifugation and resuspended in an alkaline solution of cupric ions. The cupric ions bind to the polypeptide backbones and a colorimetric agent which reacts with unbound cupric ions is then added. Thus the color density formed is inversely related to the concentration of protein in the sample. Specifically, 0, 5, 10, 15, 20, 25 μL of 2 mg/mL BSA standard solution were aliquoted into six tubes separately for preparing the standard curve. And 1-50 μL lysed sample was assayed. Since the useful range of the assay is 0.5-50 μg , more than one sample volume were assayed for each sample to ensure that the assay falls within this range and duplicates were carried out to prevent false positive results. For each of tubes (including the standard curve tubes), a volume of 500 μL precipitant was added, vortexed briefly and incubated for 2–3 min at ambient temperature, followed by an addition of 500 μL co-precipitant. Then the tubes were centrifuged at $15,000 \times g$ for 5 min to precipitate the protein. After centrifugation, the supernatants were decanted and a small pellet of protein should be visible. The tubes were carefully repositioned in the microcentrifuge as before and briefly centrifuged to bring remaining liquid to the bottom of the tube. Any remaining supernatant was aspirated using micropipette. 100 μL of copper solution and 400 μL of distilled water were added to each tube and vortex briefly to dissolve the precipitated protein. Finally, 1 mL of working colorimetric reagent was added to each tube, followed by vortexing. The tubes were then incubated at room temperature for 15 min before the absorbance of each tube was read at a wavelength of 480 nm using water as the reference. The protein concentration of the samples was determined based on the standard curve plotted.

2.2.6 Protein purification and precipitation

Since protein samples commonly contain substances that interfere with downstream applications, thus 2-D clean up kit (GE healthcare) was further applied to purify the protein samples before used for generating iTRAQ labeled peptides. Briefly, a total of 100 µg of each sample was precipitated by adding 300 µL of precipitant, followed by incubation on ice for 15 min. Next, 300 µL of co-precipitant was added to further assist the precipitation of protein sample. After centrifugation at 12000 rpm for 5 min at 4 °C, any remaining liquid was discarded and the pellet of protein sample was dispersed in 25 µL of distilled water through vortexing. Next, 1 mL of wash buffer (pre-chilled at least 1 h at -20 °C) and 5 µL wash additive was added to the sample, and vortex until the pellet was fully dispersed. Following vortexing, the sample tubes were incubated at -20 °C for 1 h with regular vortex every 10 min. After centrifugation at $16,100 \times g$ for 10 min, the supernatant was discarded and the protein pellet was allowed to air dry for 5 min. The protein pellet was resuspended in 20 µL of 500 mM triethylammonium bicarbonate buffer supplemented with 0.1% SDS and stored at -80 °C for later analysis.

2.2.7 Protein digestion, and labeling with iTRAQ reagents

The iTRAQ labeling process was carried out according to the manufacturer's protocol. Specifically, a total of 100 µg of each sample (dissolved in 20 µL of triethylammonium bicarbonate buffer) was subjected to reduction by tris(2-carboxyethyl)phosphine (TCEP) at 60 °C for 1 h, followed by alkylation using methylmethanethiosulfonate (MMTS) provided in the iTRAQ labeling kit (iTRAQ

Reagents Multiplex kit, Applied Biosystems, Foster City, CA). The proteins were then digested to peptides using 20 μL of 0.25 $\mu\text{g}/\mu\text{L}$ sequencing grade modified trypsin (Promega, Madison, WI) at 37 $^{\circ}\text{C}$ overnight. The digested peptides were labeled with the iTRAQ tags as follows: control HepG2 cells, iTRAQ 114; HepG2 cells incubated with 1 $\mu\text{g}/\text{mL}$ SWCNTs, iTRAQ 115. The labeled samples were pooled, vacuum dried and resuspended in 200 μL ddH₂O containing 0.1% formic acid and 5% acetonitrile to achieve roughly 1 $\mu\text{g}/\mu\text{L}$ of peptide mixture. The resuspended sample was filtered through Amicon Ultra filter with 10 kDa molecular weight cut off (Millipore) to remove bulky particles before analysis.

2.2.8 Online 2D LC-MS/MS Spectrometry analysis

The analysis was performed on LC-MS/MS system with Agilent 1200 Series liquid chromatograph, HPLC-Chip Cube MS interface, and 6530 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). The system was equipped with PolySulfoethyl A SCX column (0.32 $\times$ 50 mm, 5 μm particles, PolyLC Inc.) and HPLC-Chip (Large capacity chip II, Agilent Technologies). The HPLC chip was incorporated a 160-nL enrichment column and a 150 mm $\times$ 75 μm reverse phase column that was packed with Zorbax 300SB-C18 5 μm particles. In the first dimension, a total of 2 μL of the reconstituted peptide mixture was loaded onto the PolySulfoethyl A SCX column and was stepwise eluted by injecting 8 μL salt plugs of nine different molar concentrations of 20, 40, 60, 80, 100, 150, 300, 500, and 1000 mM ammonium formate solution. The sequentially eluted peptides from the SCX column were trapped onto the Zorbax 300SB-C18 enrichment column during the enrichment mode (Fig. 2.2A) and washed isocratically with buffer containing 5%

acetonitrile and 0.1% formic acid at 0.004 mL/min for additional 30 min to remove any excess reagent. In the second dimension, the HPLC-Chip was switched to analysis mode (Fig. 2.2B) and the enrichment column was eluted with buffer B (0.1% formic acid) and buffer C (a nanoflow gradient of 5–80% acetonitrile + 0.1% formic acid) at a flow rate of 300 nL/min. Further separation was achieved onto the analytical Zorbax 300SB C18 reversed-phase column (75 μ m $\times$ 150 mm, 5 μ m particles). The total time, including column reconditioning, was 90 min to complete one single run. The column effluent was then directly analyzed by the 6530 Q-TOF mass spectrometer interfaced through an HPLC-Chip Cube nanospray source.

For the mass spectrometer, it was operated at a capillary voltage of 1800 V with a capillary current of 1.1 μ A in 1 GHz. The MS data were acquired in the positive ionization mode using Agilent MassHunter Workstation Q-TOF B.02.01. During the course of data acquisition, the fragmentor voltage, skimmer voltage, and octopole RF were set to 175, 65, and 750 V, respectively. Auto-MS/MS was performed with a total cycle time of 1.225 s. In each cycle, MS spectra were acquired at 8 Hz (eight spectra/s) (m/z 100–2000), and the four most abundant ions (with charge states 2+, 3+, and >3+) exceeding 1000 counts were selected for MS/MS at 4 Hz (four spectra/s) (m/z 50–2000). A medium isolation (4 m/z) window was used for precursor isolation. Collision energy with slope of 3.6 V/100 Da and offset of 4 V was used for fragmentation. Reference mass correction was activated using reference mass of 121 and 922. Precursors were set in an exclusion list for 0.5 min after two MS/MS spectra. Overall, 10 runs with different fraction of peptides eluted from SCX column were performed to finish one experiment. To gain statistical evidence for differential expression of proteins, another two experiments were performed as described above.

Figure 2.2 Schematic graphs of strong cation exchange (SCX) column coupled with HPLC-Chip for online 2D separation of peptides. (A) Enrichment mode for sample loading; (B) analytical mode for analysis. Enrichment column and analytical column were denoted as 1 and 2, respectively. (Adapted from: Yuan et al. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011;15; 207(3); 213-21.)

2.2.9 Data Analysis and Interpretation

Peptide and protein identifications were run automatically with the Spectrum Mill Proteomics Workbench (Rev A.03.03.084 SR4) software from Agilent Technologies. Peak lists were created with the Spectrum Mill data extractor program with the following parameters: scans with the same precursor ± 1.4 m/z were merged within a time frame of ± 15 s; precursor ions needed to have a minimum S/N of 25; charges up to a maximum of 7 were assigned to the precursor ion and the ^{12}C peak was also

determined by the Data Extractor; $[M+H]^+$ comprised between 600 and 4000, and a scan time comprised between 0 and 300 min was used. Peptides were automatically identified by the Spectrum Mill Proteomics Workbench (Rev A.03.03.084 SR4) software using UniProtKB/Swiss-Prot protein database (Geneva, Switzerland) search for tryptic peptides with the restriction to Homo sapiens. A mass tolerance of ± 2.5 Da for the precursor ions and a tolerance of ± 0.7 Da for the fragment product ions were used. Two missed cleavages were allowed. MMTS(L) and iTRAQ (N-term, K) were set as fixed modification. Spectrum Mill scores above 25 for complete proteins with a minimum score of 10 and minimum scored peak intensity (SPI) of 70% for individual peptides were used as auto-validation criteria. These criteria provide high confidence for the searched proteins which always represent valid results. Auto-validation was necessary to ensure the same data analysis conditions for all the LC-MS/MS runs done in this work and to assure a fair comparison of the results.

Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and was the ratio of the areas under the peaks at 114.1 and 115.1 Da which were the masses of the tags that correspond to the iTRAQ reagents. The calculated peak area ratios were corrected for overlapping isotopic contributions, and were used to estimate the relative abundances of a particular peptide. For proteins with two or more qualified peptide matches, multiple average peak area ratios were calculated using the peak area ratios of the peptides originating from the same protein. The standard deviation (SD), the coefficient of variance (CV) for each average peak area ratio, and the average CV using the CVs for the average peak area ratios for each protein were also calculated. In the present study, only protein quantification data with relative expression of >1.25 or <0.8 were chosen for further analysis.

Data were displayed as mean $\pm$ SD from three independent experiments. Student's *t*-test was employed to test the deviation between different batches. Values were only considered to be statistically significant when *p*-values were less than 0.05.

2.3 Results

2.3.1 Characterization of purified SWCNTs

The height profile from AFM result revealed that the SWCNT bundles had diameters in the range of 1.0–8.0 nm, which correspond to the expected height of individual and small bundles of SWCNTs (Fig. 2.3). The lengths of the prepared SWCNTs were found to be around 2000 to 6000 nm similar to the pristine SWCNTs. It indicated that the soft oxidizing process did not truncate SWCNTs during the process. Moreover, this multistep process involving soft oxidation of the metal particles and subsequent removal of metal oxides was effective at removing both carbonaceous and catalytic impurities. The FTIR spectra further confirmed that the multistep soft oxidation process did not introduce any functional group to the surface of SWCNTs as no band corresponding to the functional group was observed in the spectrum (Data not shown).

2.3.2 MTT assay result

MTT assay is a common method to evaluate the toxicity of nanomaterials by measuring the mitochondria function of the cell. After 48 h of exposure to SWCNTs, we observed that the cells exposed to SWCNTs appeared with decreased metabolic activity when compared to the control cells. Specifically, the cells treated with SWCNTs loss 27 $\pm$ 7% of viability based on the MTT assay result. Given the tubular

Figure 2.3 Tapping-mode AFM image (top panel) and height profile (bottom panel) of purified SWCNTs. (Adapted from: Yuan et al. Cytotoxicity of single-walled carbon nanotubes on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology In Vitro*. 2011; 25(8): 1820-7.)

shape of SWCNTs, we expected that it promoted penetration of membranes and strong interactions with intracellular systems, which might cause damage to cellular metabolism, protein synthesis and DNA replication.

2.3.3 HPLC-Chip coupled 2D LC-MS/MS analysis result

In the present study, PolySulfoethyl A SCX column (0.32 × 50 mm, 5 µm particles, PolyLC Inc.) was used as the first dimension for the initial fraction of peptides. PolySulfoethyl A is a silica-based material with a bonded coating of a hydrophilic, anionic polymer: poly(2-sulfoethyl aspartamide). This material has been developed specifically for cation-exchange of peptides in the pH range 2.7-3.0. At pH 3, basic residues in peptides (His, Arg, Lys) are positively charged, as are N-termini. Acidic residues (Asp, Glu) are uncharged, and the C-termini are predominantly uncharged. Thus, most peptides with free N-termini will have net charges of at least +1 and will bind to PolySulfoethyl A. In case of a typical tryptic peptide which has been reduced and cysteine blocked, it has a charge of +2 at pH 3, due to the N-terminus at one end and the Lys or Arg at the other. Otherwise, linking two such peptides with a disulfide or other bridge results in a peptide with a charge of +4 and such peptides are eluted later than the typical tryptic peptide. These peptides can be eluted with a salt gradient in order of increasing net positive charge. Since the present approach applied the online 2D LC-MS/MS system for the identification and quantification of peptides, a volatile mobile phase is desirable and ammonium formate was used as both the buffering and gradient salt.

As can be seen in Fig. 2.4, chromatogram result indicated that the peptides were

successfully fractionated by using the SCX column as the first dimension. There were peptide signals at the initial loading step, which should be attributed to the weak binding peptides. Majority of the peptides were detected from fractions eluted by 40-500 mM ammonium formate. Only trace peptide signals were detected from the fraction eluted by 1 M ammonium formate. This chromatogram profile was very informative as the peptide signals could be directly read from the diagram, which could be harnessed for the future optimization of the fraction procedure to achieve higher resolution and better separation of peptides. Moreover, a representative MS window indicated that four precursor peptide ions, each marked with red diamond, were detected at 18.21 min (Fig. 2.5A). The ions with 121 m/z and 922 m/z appeared in the window were the reference mass ions, which were used to monitor the working condition of MS and tune the signal intensity throughout the running process. From the MS/MS spectrum shown in Fig. 2.5B, a representative selected precursor ion with 630.7 m/z was fragmented for peptide identification and quantification. Specifically, the identity of peptides can be derived from the fragmentation pattern and the relative abundance of the peptides can be calculated from the relative ratio of reporter ions. As can be seen from Figure 2.5C, the iTRAQ reporter ions appeared at mass range (100-200 m/z) in the enlarged portion from the MS/MS spectrum (Fig. 2.5C).

2.3.4 iTRAQ analysis and differentially expressed proteins to SWCNTs exposure

To establish the biological difference between the control HepG2 cells and SWCNTs treated cells, the comparative protein profile was obtained by iTRAQ-coupled 2D LC-MS/MS method. With a minimum score of 10 and a minimum SPI of 70% for

Figure 2.4 Chromatogram result of peptide signal from ten fractions eluted by ammonium formate.

Figure 2.5 (A) Representative MS window showing four peptide precursors marked by red diamond were detected at 18.21 min from the fraction eluted by 150 mM ammonium formate. (B) Representative MS/MS spectrum of one selected precursor ion and the precursor ion was marked with blue diamond. (C) Enlarged portion from MS/MS spectrum showing the iTRAQ reporter ions.

individual peptides and protein scores above 25 for complete proteins, such stringent criteria yielded more than 200 proteins from each of three independent experiments conducted (228, 229 and 233, respectively). According to the manufacturer, stringent criteria set as above always provide high confidence for the searched proteins (above 99% confidence of the results). After compared between the results obtained from independent experiments, these proteins appeared in all groups with consistent trends of variation were analyzed and the proteins with significant changes were further classified into distinct categories according to their cellular functions. These included 10 metabolic enzymes (Table 2.1), 10 proteins involved in cytoskeleton formation (Table 2.2), 13 proteins participated in signaling pathway (Table 2.3) and other 18 growth related proteins (Table 2.4).

Differentially expressed metabolic enzymes

As listed in Table 2.1, the expression levels of the metabolic enzymes involved in glycolysis were significantly up-regulated. These included fructose-bisphosphate aldolase A, which splits the hexose ring into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate; glyceraldehyde-3-phosphate dehydrogenase, which converts glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate; phosphoglycerate mutase that converts 3-phosphoglycerate to 2-phosphoglycerate and gamma-enolase that forms phosphoenolpyruvate from 2-phosphoglycerate. Since cellular catabolism yields ATP for the general cellular activities such as cell adhesion and proliferation, the increased level of these four metabolic enzymes participated in glycolysis suggested that there might be increasing energy demand after the cells exposure to SWCNTs.

Interestingly, aldose reductase, an NADPH-dependent oxidoreductase that catalyzes the reduction of a variety of aldehydes and carbonyls (Petrash, 2004), and glutathione S-transferase Mu 3 (GSTMu) involved in detoxification of endogenous compounds by conjugating of reduced glutathione to a wide number of hydrophobic electrophiles (Patskovsky et al., 1999), were both significantly down-regulated by 0.63-fold. These findings indicated the SWCNTs might not trigger aldehydes and carbonyls alike cellular responses and the detoxification process was not through the conjugation of reduced glutathione to SWCNTs by GSTMu. However, since GSTMu was also reported to modulate the stress-activated signals by suppressing apoptosis signal-regulating kinase 1 (ASK1) (Cho et al., 2001), we expected that the down-regulation of GSTMu might activate ASK1, result in the activation of downstream signaling cascades such as the c-Jun N-terminal kinase and p38 pathway, and finally lead to stress-induced apoptosis. Moreover, farnesyl pyrophosphate synthase, a key enzyme in isoprenoid biosynthesis which catalyzes the formation of farnesyl diphosphate (FPP) (Kavanagh et al., 2006), was significantly down-regulated by 0.79-fold. FPP is a precursor for several classes of essential metabolites including sterols, carotenoids, and ubiquinones and also serves as substrate for protein farnesylation and geranylgeranylation. Its down-regulation might be related to the cell membrane structure as previous investigation has reported that the inactivation of farnesyl pyrophosphate synthase could result in the disruption of lipid rafts at the plasma membrane (Wang et al., 2007c). However, the exact role for the down-regulation of farnesyl pyrophosphate synthase in response to the SWCNTs exposure remains to be further studies.

Table 2.1

List of differentially expressed metabolic enzymes in HepG2 cells incubated with SWCNTs*

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD
P04075	Fructose-bisphosphate aldolase A	133	10	30	1.58±0.21
P15121	Aldose reductase	28	2	6	0.63±0.16
P04406	Glyceraldehyde-3-phosphate dehydrogenase	139	9	35	1.42±0.13
P21266	Glutathione S-transferase Mu 3	38	3	16	0.63±0.11
P14324	Farnesyl pyrophosphate synthase	27	2	6	0.79±0.08
P09104	Gamma-enolase	38	3	8	1.47±0.09
Q14697	Neutral alpha-glucosidase AB	41	3	3	0.78±0.06
Q8N0Y7	Phosphoglycerate mutase 4	31	2	13	1.52±0.09
P18669	Phosphoglycerate mutase 1	42	3	18	2.41±0.21
P15259	Phosphoglycerate mutase 2	27	2	9	2.46±0.36

*Adapted from: Yuan et al. Cytotoxicity of single-walled carbon nanotubes on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology In Vitro*. 2011; 25(8): 1820-7.

Up-regulation of cytoskeletal proteins

As can be seen in Table 2.2, all the cytoskeletal proteins observed were significantly up-regulated. Keratin-18 together with keratin-8 are involved in interleukin-6 (IL-6)-mediated barrier protection and interleukin-6 induces keratin expression in epithelial cells (Wang et al., 2007a). Previous investigations have reported that the exposure of human cells to carboxylated SWCNTs and MWCNTs led to elevated level of interleukin-6 (IL-6) and IL-8 expression (Monteiro-Riviere et al., 2005; Ye et al., 2011). Considering the similarity in their physical properties, pristine SWCNTs might also induce an inflammatory response and trigger the expression of IL-6/8, which explained the up-regulation of keratin-18 and keratin-8 observed here.

Moreover, since intermediate filament components organize the internal tridimensional structure of the cell, anchoring organelles and serving as structural components (Herrmann et al., 2007), the variation of keratin expression might play an important role in maintaining the cell structure and counter the mechanistic stress caused by SWCNTs.

Profilin-1, a ubiquitous actin monomer-binding protein belonging to the profiling family, is thought to regulate actin polymerization in response to extra cellular signals (Mahoney et al., 1997). Since profilin prevents the polymerization of actin at high concentrations, we assumed that the significant up-regulation of profilin-1 by 1.35-fold might prevent the polymerization of actin after the cells exposed to SWCNT. The calcium binding tropomyosin is another cytoskeletal protein which implicated in stabilizing cytoskeleton actin filaments in non-muscle cells (Crabos et al., 1991). Since microfilaments act as tracks for the myosin mediated vesicle transport (Fath et al., 1993), it seems tropomyosin synergistically works with profilin to reorganize the cell cytoskeletal microfilament network, which led to altered cargo transport inside the cells. Moreover, the expression level of other actin binding proteins such as radixin, ezrin and moesin that function as cross-linkers between plasma membranes and actin-based cytoskeletons (Gary and Bretscher, 1995; Yonemura et al., 1998) were also found to be elevated from 1.27- to 1.32-fold.

Table 2.2

List of differentially expressed cytoskeletal proteins in HepG2 cells incubated with SWCNTs*

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD
P05783	Keratin, type I cytoskeletal 18	101	7	16	1.52±0.28
P05787	Keratin, type II cytoskeletal 8	294	21	42	1.35±0.11
P13797	Plastin-3	36	3	5	1.42±0.08
P07737	Profilin-1	76	5	42	1.35±0.16
P67936	Tropomyosin alpha-4 chain	89	7	27	1.35±0.16
P06753	Tropomyosin alpha-3 chain	53	4	11	1.32±0.07
P09493	Tropomyosin alpha-1 chain	52	4	14	1.28±0.09
P15311	Ezrin	64	5	1	1.31±0.11
P35241	Radixin	52	4	1	1.32±0.18
P26038	Moesin	75	6	10	1.27±0.11

*Adapted from: Yuan et al. Cytotoxicity of single-walled carbon nanotubes on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology In Vitro*. 2011; 25(8): 1820-7.

Differentially expressed proteins involved in signaling pathway

Among the proteins listed in Table 2.3, the signaling proteins related to calcium binding, 14-3-3 protein family and proteins implicated in redox regulation of the cell were identified in the present study. We found both thioredoxin and peroxiredoxin involved in redox regulation of the cell (Nordberg and Arnér, 2001; Sue et al., 2005) were all significantly up-regulated by approximately 1.5-fold. The up-regulation of redox protein indicated that SWCNTs induced oxidative stress inside the cells and these redox protein might play an vital role in eliminating the ROS introduced by SWCNTs (Manna et al., 2005) to protect the cells from oxidative damage. Since peroxiredoxin also controls cytokine-induced peroxide levels and mediates signal

transduction in mammalian cells (Sue et al., 2005), its cellular function beyond the clear up of ROS remains to be further studied.

S100 proteins are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation (Donato, 2001, 2003; Zimmer et al., 1995). Interestingly, calcyclin that promotes the cell growth and calgizzarin which deters cell growth were found to be altered by 1.21- and 0.63-fold, respectively. Since the reciprocal regulation of calcyclin and calgizzarin controls the metabolic rate and cell growth, we expected there might be increasing energy demand needed for cellular activities to counter the damage caused by SWCNTs, which also explained the elevated level of metabolic enzymes involved in glycolysis for ATP generation. Notably, calnexin that interacts with newly synthesized glycoproteins in the endoplasmic reticulum (ER) and plays a major role in the quality control apparatus of the ER (Rajagopalan et al., 1994) was found to be significantly up-regulated by 1.42-fold. The elevated level of calnexin suggested that the SWCNT might severely interfere protein folding and lead to increased level of unassembled proteins that retained in ER. Moreover, we observed that the expression level of other signaling proteins such as calmodulin and protein 14-3-3 were significantly increased. Since calmodulin regulates a number of different protein targets (Chin and Means, 2000) as well as protein 14-3-3 family that bind a multitude of functionally diverse signaling proteins (Liao and Omary, 1996), the exact role of calmodulin and protein 14-3-3 family involved in modulating the cellular functions in response to SWCNTs exposure remains to be determined.

Table 2.3

List of differentially expressed signaling proteins in HepG2 cells incubated with SWCNTs*

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD
P32119	Peroxiredoxin-2	51	4	24	1.52±0.11
P30041	Peroxiredoxin-6	48	4	16	1.53±0.13
P10599	Thioredoxin	45	3	29	1.51±0.15
O43852	Calumenin	27	2	7	1.41±0.09
P31949	Calgizzarin	28	2	14	0.63±0.08
P06703	Calcyclin	37	3	26	1.21±0.12
P27824	Calnexin	74	6	10	1.42±0.13
P08758	Annexin A5	62	5	15	1.26±0.16
P62158	Calmodulin	41	3	18	1.50±0.15
P31946	14-3-3 protein beta/alpha	79	6	19	1.64±0.09
P31947	14-3-3 protein sigma	84	6	19	1.71±0.13
P62258	14-3-3 protein epsilon	94	7	29	1.48±0.09
P61981	14-3-3 protein gamma	69	5	15	1.71±0.21

*Adapted from: Yuan et al. Cytotoxicity of single-walled carbon nanotubes on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology In Vitro*. 2011; 25(8): 1820-7.

Differentially expressed growth related proteins

Among the proteins listed in Table 2.4, the up-regulation of heat shock proteins was a good indicator that the cells were under stress after exposed to SWCNTs since heat shock proteins function as intracellular chaperones and assist protein folding (Schlesinger, 1990). Histone proteins, the core components of nucleosome, were found to be up-regulated. Since nucleosomes wrap and compact DNA into chromatin and regulate gene expression (Kornberg, 1974; Schiessel, 2003), limiting DNA accessibility to SWCNTs might play a central role in maintaining chromosomal stability and prevent the SWCNT induced damage to DNA. We also observed that the

Table 2.4

List of differentially expressed growth related proteins in HepG2 cells incubated with SWCNTs*

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD
P62826	GTP-binding nuclear protein Ran	49	3	14	1.45±0.06
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	109	8	22	1.35±0.11
P61978	Heterogeneous nuclear ribonucleoprotein K	58	5	9	1.34±0.12
P52597	Heterogeneous nuclear ribonucleoprotein F	26	2	6	0.67±0.08
P14866	Heterogeneous nuclear ribonucleoprotein L	49	4	9	1.35±0.16
Q15084	Protein disulfide isomerase A6	38	3	10	0.71±0.08
P62820	Ras-related protein Rab-1A	28	2	13	0.69±0.06
Q9GZV4	Eukaryotic translation initiation factor	28	2	13	1.53±0.08
P62988	Ubiquitin	48	3	44	1.46±0.18
P39019	40S ribosomal protein S19	51	4	28	1.44±0.15
P25398	40S ribosomal protein S12	29	2	13	1.62±0.13
P15880	40S ribosomal protein S2	59	5	17	2.71±0.19
P46777	60S ribosomal protein L5	40	3	9	0.78±0.13
P38646	Stress-70 protein, mitochondrial	76	6	8	1.58±0.16
P10809	60 kDa heat shock protein, mitochondrial	170	12	21	1.41±0.26
Q58FF6	Putative heat shock protein HSP 90	75	5	7	1.21±0.09
Q96A08	Histone H2B type 1-A	50	3	21	1.28±0.09
P57053	Histone H2B type F-S	99	6	41	1.27±0.12

*Adapted from: Yuan et al. Cytotoxicity of single-walled carbon nanotubes on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology In Vitro*. 2011; 25(8): 1820-7.

expression levels of the ribonucleoproteins implicated in pre-mRNA processing were significantly elevated, which indicated that the mRNA processing might be also interfered by SWCNTs and/or there might be increased demand for functional proteins as SWCNTs led to mounting unfolded proteins as discussed in previous sections. Protein disulfide isomerase A6 is an enzyme in the endoplasmic reticulum, which acts as molecular chaperone for protein folding (Gruber et al., 2006). Its down-regulation

was unexpected since we expected that cellular responses should instinctively protect the cells from the cytotoxicity caused by SWCNTs exposure. Nevertheless, the down-regulation of protein disulfide isomerase A6 might lead to increased level of incorrectly folded proteins in ER, which might explain the up-regulation of calnexin that functions as quality control of ER. Further evidence such as increased level of ribosomal proteins, ubiquitin and eukaryotic translation initiation factor also suggested that there might be increased level of incorrectly folded proteins and the need for protein synthesis, which might also explain the elevated expression level of the metabolic enzymes involved in glycolysis for the energy molecule generation.

2.4 Discussion

By applying iTRAQ-coupled 2D LC-MS/MS proteomics approach, we successfully identified 51 differentially expressed proteins in the cells treated with SWCNTs, which should be highly correlated with the poor cell viability obtained from the MTT assay. Previous investigations have demonstrated that CNTs could enter the cell actively and passively, frequently inserting through the plasma membrane into the cytoplasm and the nucleus (Cheng et al., 2009; Porter et al., 2009). Based on the protein profile, our data suggested that SWCNTs retained inside the cells could induce oxidative stress and interfere protein synthesis, mRNA processing, signaling pathway and cytoskeleton system (Fig. 2.6). Since oligonucleotides and proteins could be adsorbed by CNTs, these macromolecules have been exploited for the dispersion of CNTs in aqueous solution in order to deliver CNTs to the target organs (Cathcart et al., 2007; Edri and Regev, 2009; Gigliotti et al., 2006; Zhao et al., 2006). It was self-evident that these SWCNTs penetrated inside the cells would strongly interact

Figure 2.6 Schematic diagram showing the cellular functions in response to SWCNT exposure. (Adapted from: Yuan et al. Cytotoxicity of single-walled carbon nanotubes on human hepatoma HepG2 cells: an iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology In Vitro*. 2011; 25(8): 1820-7.)

with the intracellular systems through passive adsorption and mechanical interruption. Specifically, the increased level of chaperones and other protein components such as heat shock proteins indicated that the cells were under stress after exposure to SWCNTs. The elevated level of these intracellular chaperones assisted protein folding to protect the cells from the damage caused by SWCNTs. Moreover, the metabolic enzymes implicated in the glycolysis were significantly up-regulated, which could be explained by the increased energy demand for protein synthesis and other cellular activities.

From the protein profile, we also successfully identified the key proteins participated in the redox regulation of the cell to mediate the oxidative stress. We found thioredoxin and peroxiredoxin system instead of other oxidoreductases played the main role to eliminate the ROS generated by SWCNTs. Moreover, GSTMu that

modulates the stress-activated signals by suppressing ASK1 (Cho et al., 2001) was found to be significantly down-regulated by 0.63-fold after exposure to SWCNTs, its down-regulation might activate ASK1, result in the activation of downstream signaling cascades such as the c-Jun N-terminal kinase and p38 pathway, and finally lead to stress-induced apoptosis. As previous investigation have demonstrated that the stimulation of p38 activates the transcription factor NF- κ B (Craig et al., 2000), our findings strongly suggested that the previously reported activation of transcription factor NF- κ B in human keratinocytes after SWCNTs exposure (Manna et al., 2005) should be mediated by p38 signaling pathway and the collaborative action of these signals led to the interleukin-6/8 mediated inflammatory responses.

Interestingly, the expression level of various calcium binding proteins and protein 14-3-3 family involved in the signaling pathway were also found to be altered. Calcyclin that promotes the cell growth and calcizzarin which deters cell growth were found to be altered by 1.21- and 0.63-fold, respectively. Moreover, the expression level of annexin A5, which has been linked with inhibition of phospholipase activity, exocytosis and endocytosis, signal transduction, organization of the extracellular matrix and resistance to ROS (Benz and Hofmann, 1997; Laohavisit and Davies, 2011), was found to be elevated by 1.26-fold. The role of annexin on resistance to ROS was self-evident as previous report indicated that ROS could be introduced by SWCNTs (Manna et al., 2005). Considering these signaling proteins involved in wide range of cellular functions (Chin and Means, 2000; Liao and Omary, 1996), their exact role in modulating the intracellular systems in response to the SWCNTs exposure remains to be determined.

Chapter 3 Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes

3.1 Introduction

Graphitic nanomaterials, such as carbon nanotubes (CNTs) and graphene, are one of the most fascinating nanostructures with unique physical, chemical, electrical, and mechanical properties. Due to these properties, graphene and CNTs are considered to be promising materials in high-energy physics (Novoselov et al., 2005; Zhang et al., 2005), material science (Akhavan, 2010; Akhavan et al., 2010; Bai et al., 2010; Hu et al., 2010; Shang et al., 2008; Wu et al., 2009a), and a wide range of technological applications, such as biosensor, bio-imaging, tissue engineering and drug delivery (Choi et al., 2010; Liu et al., 2008a; Liu et al., 2009b; Sun et al., 2008b; Veetil and Ye, 2009). Previous investigations have demonstrated that single-walled carbon nanotubes (SWCNTs) induce a noticeable cytotoxicity to mammalian cells (Baktur et al., 2011; Manna et al., 2005; Raja et al., 2007; Shvedova et al., 2003; Wang et al., 2011; Zeni et al., 2008). Among the suggested toxicity mechanisms such as oxidative stress, rupture of cell membrane and interfering intracellular metabolic routes, the oxidative stress has been considered to be the most acceptable mechanism explaining the cytotoxicity of CNTs. Although some properties of graphene nanosheets are similar to the properties of CNTs, the biocompatibility of graphene remains to be determined. Moreover, few detailed studies on the mechanistic understanding of the interaction

between living systems and these nanomaterials at molecular level have been performed to date.

Proteome analysis has been widely used to establish the cellular functions in response to various external stimuli and the variation of protein expression level reflects biological responses. Previously, our group has set up the workbench of iTRAQ based 2D LC-MS/MS approach for comparative proteome analysis with pristine SWCNTs as the testing material. In this system, multiple samples can be analyzed in a single run, which significantly reduces the time for multiple sample analysis. Briefly, protein samples are first digested into peptides and then isotopically labeled iTRAQ reagent such that all derivative peptides will have the same retention time and mass to charge ratio in MS analysis. Following MS/MS fragmentation of the precursor ion, the reporter groups appear as distinct ions and the relative abundance of the peptides can be derived from the intensity of the corresponding reporter ions.

In the present study, we used iTRAQ technique to simultaneously detect and quantify the differences of protein expression levels in control HepG2 cells and these treated with graphene and SWCNTs, with the purpose of elucidating the modes of action regarding these distinct nanomaterials. From our comparative protein profile obtained here, 37 proteins with significant change of expression level were identified from either graphene or SWCNTs treated cells. These included metabolic enzymes, signaling proteins, cytoskeletal proteins and these proteins involved in cell growth and redox regulation of the cell. Distinct pattern of protein profile was observed for the cells exposed to graphene and SWCNTs. The possible mode of action for these nanomaterials was also discussed based on the protein profile obtained. Our finding

provided molecular evidence to the cellular functions in response to graphene/SWCNTs, which should be of great importance to evaluate biocompatibility of these nanomaterials.

3.2 Materials and methods

3.2.1 Preparation and Characterization of graphene and SWCNTs

Purified HiPco SWCNTs (Unidym) were used in the present study. These SWCNTs were produced by chemical vapor deposition (CVD) by means of the HiPco process. The process yields SWCNTs with a Gaussian distribution of diameters, with a maximum peak around 0.8~1.2 nm. To further purify SWCNTs, a wet chemical technique that has been tested effective at removing both carbonaceous and catalytic impurities was applied (Porter et al., 2009). Briefly, the powder was heated under reflux in concentrated HCl for 6 h at 110 °C. Then the sample was filtered under vacuum, washed with water, and dried at 110 °C for 6 h. Following purification, the quality of SWCNTs was assessed with atomic force microscopy (AFM).

In order to get aqueous solution of graphene, the graphite oxide (GO) was first synthesized from synthetic graphite powder (SigmaAldrich) based on a modified Hummers method (Kovtyukhova, 1999). Then the exfoliation of GO was achieved by ultrasonication for 2 h. Graphene aqueous solution was prepared using the chemical reduction method as reported earlier (Li et al., 2008). The pH of as-prepared graphite oxide solution was adjusted to ~10 with 25% ammonia solution, followed by adding 35% hydrazine aqueous solution under stirring. The reduced graphene was obtained by incubating a hydrazine-added graphene solution in an oil bath at 95 °C for 1 h. Any

remaining chemical residuals were removed by dialysis using distilled water. The quality of graphene was assessed using AFM, Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy.

The AFM was taken using a MFP3D microscope (Asylum Research) with a silicon cantilever operating in tapping mode. FTIR spectra were carried on a Perkin Elmer Spectrum One Spectrometer. Raman spectra were acquired with a micro-Raman spectrometer (Reinshaw Raman Scope RM3000) at room temperature in the backscattering configuration using an Ar-ion laser with wavelength 514.5 nm.

3.2.2 Cell cultures

Human hepatoma HepG2 cells obtained from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin). Cells were maintained at 37 °C in an atmosphere of 5% CO₂. All culture media and media supplements were purchased from Invitrogen. As SWCNTs and graphene are prone to agglomerate and tend to settle on the cell surface, surfactant containing media were used to disperse graphene and SWCNTs. Specifically, the media supplemented with 0.2% Tween 80 were used and 1 µg/mL of nanomaterial containing media were tested as stable under the subsequent culture condition. After reaching 80% confluence, the cells were treated with dispersed graphene and SWCNTs for 48 h, respectively. For the control cells, the fresh media supplemented with surfactant (0.2% Tween 80) were used.

3.2.3 MTT assay

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) test was performed to assess the metabolic activity of the cell. The assay was based on the ability of the mitochondrial dehydrogenase to convert MTT, a yellow color dye to a purple-colored formazan. The metabolic activity of the cell can be derived from the color density formed. Briefly, following HepG2 cells incubation with graphene or SWCNTs for 48 h, the media were aspirated and replaced with 90 μ L of serum-free media. Then 10 μ L of MTT stock solution (5 mg/mL) was added to each well, followed by incubation for 4 h at 37 °C. The supernatant was then removed, and cells were lysed with 100 μ L of dimethylsulfoxide (DMSO) and the absorbance was recorded at 550 nm.

3.2.4 Cell lysis, protein digestion and labeling with iTRAQ reagent

After exposure to nanomaterials for 48 h, the cells were harvested and lysed in 300 μ L of 8 M urea, 4% (w/v) CHAPS, and 0.05% SDS (w/v) on ice for 30 min with regular vortexing. Samples were centrifuged at 16,100 g for 60 min at 4 °C. The supernatants were collected and the amount of protein present in each sample was quantified using the 2-D Quant kit (GE healthcare). A total of 100 μ g of each sample was further purified using 2-D clean up kit (GE healthcare) before used to generate iTRAQ-labeled peptides. The purified protein samples were resuspended in 20 μ L of 500 mM triethylammonium bicarbonate buffer supplemented with 0.1% SDS. Then the resuspended samples were subjected to reduction by dithiothreitol at 60 °C for 1 h, followed by alkylation using methylmethanethiosulfonate (MMTS) provided in the

iTRAQ labeling kit. Proteins were then digested to peptides using 10 μL 0.5 $\mu\text{g}/\mu\text{L}$ sequencing grade modified trypsin (Promega, Madison, WI) at 37 °C overnight. The digested protein samples were labeled with the iTRAQ tags as follows: control HepG2 cells, iTRAQ 114; HepG2 cells incubated with 1 $\mu\text{g}/\text{mL}$ SWCNTs, iTRAQ 115; HepG2 cells incubated with 1 $\mu\text{g}/\text{mL}$ graphene, iTRAQ 116. The labeled samples were pooled, vacuum dried and resuspended in 300 μL ddH₂O containing 0.1% formic acid and 5% acetonitrile, to achieve roughly 1 $\mu\text{g}/\mu\text{L}$ peptide mixture.

3.2.5 Online 2D LC-MS/MS analysis

The analysis was performed on LC-MS/MS system with Agilent 1200 Series liquid chromatograph and 6530 Q-TOF mass spectrometer interfaced with HPLC-Chip Cube (Agilent Technologies, Santa Clara, CA). The system was equipped with PolySulfoethyl A SCX column (0.32 $\times$ 50 mm, 5 μm particles, PolyLC Inc.) and HPLC-Chip (Large capacity chip II, Agilent Technologies). Notably, the HPLC chip was incorporated a 160-nL enrichment column and a 150 mm $\times$ 75 μm reverse phase column that was packed with Zorbax 300SB-C18 5 μm particles. In the first dimension, a total of 2 μg of the reconstituted peptide mixture was loaded onto the PolySulfoethyl A SCX column and was stepwise eluted by injecting 8 μL salt plugs of 9 different molar concentrations of 20, 40, 60, 80, 100, 150, 300, 500, and 1000 mM ammonium formate solution.

In the second dimension, the sequentially eluted peptides from the SCX column were trapped onto the Zorbax 300SB-C18 enrichment column during the enrichment mode (Fig. 4.1A) and washed isocratically with buffer containing 5% acetonitrile and 0.1%

formic acid at 0.004 mL/min for additional 30 min in order to remove any excess reagent. Next, the HPLC-Chip was switched to analysis mode (Fig. 4.1B) and eluted with buffer A (0.1% formic acid) and buffer B (90% acetonitrile + 0.1% formic acid) at a flow rate of 300 nL/min. The nanoflow gradient was from 5% to 45% buffer B during the initial 40 min, followed by a sharp increase to 90% buffer B over 5 min. The flow was hold for additional 5 min at 90% buffer B and then allowed for reconditioning to initial state for 10 min. Further separation was achieved onto the analytical Zorbax 300SB C18 reversed-phase column (75 μm $\times$ 150 mm, 5 μm particles). The total time, including column reconditioning, was 90 min to complete one single run. The column effluent was directly analyzed by the 6530 Q-TOF mass spectrometer interfaced through an HPLC-Chip Cube nanospray source. Overall, 10 runs with different fraction of peptides eluted from SCX column were performed to finish one experiment.

For the mass spectrometer, it was operated at a capillary voltage of 1800 V. The MS data were acquired in the positive ionization mode using Agilent MassHunter Workstation Q-TOF B.02.01. During the course of data acquisition, the fragmentor voltage, skimmer voltage, and octopole RF were set to 175, 65, and 750 V, respectively. Auto-MS/MS was performed with a total cycle time of 1.225 s. In each cycle, MS spectra were acquired at 8 Hz (eight spectra/s) (m/z 100–2000), and the four most abundant ions (with charge states 2+ and 3+) exceeding 1000 counts were selected for MS/MS at 4 Hz (four spectra/s) (m/z 50–2000). A medium isolation (4 m/z) window was used for precursor isolation. Collision energy with slope of 3.6 V/100 Da and offset of 4 V was used for fragmentation. Reference mass correction

was activated using reference mass of 121 and 922. Precursors were set in an exclusion list for 0.5 min after two MS/MS spectra.

3.2.6 Data analysis and interpretation

Peptide and protein identifications were run automatically with the Spectrum Mill Proteomics Workbench (Rev A.03.03.084 SR4) software from Agilent Technologies. Peak lists were created with the Spectrum Mill data extractor program using the default settings. Peptides were automatically identified by the Spectrum Mill Proteomics Workbench software using UniProtKB/Swiss-Prot protein database (Geneva, Switzerland) search for tryptic peptides with the restriction to Homo sapiens. A mass tolerance of ± 2.5 Da for the precursor ions and a tolerance of ± 0.7 Da for the fragment product ions were used. Two missed cleavages were allowed. MMTS(L) and iTRAQ (N-term, K) were set as fixed modification. Auto-validation was carried out to search the proteins and these criteria provided high confidence for the searched proteins which always represent valid results (above 99% confidence). To assure a fair comparison of the results, same criteria for auto-validation were chosen to ensure the same data analysis conditions for independent batches.

Relative quantification of proteins was performed on the MS/MS scans and was the ratio of the areas under the peaks at 114.1, 115.1 and 116.1 Da which correspond to the iTRAQ reagents. The calculated peak area ratios were corrected for overlapping isotopic contributions, and were used to estimate the relative abundances of a particular peptide. For proteins with two or more qualified peptide matches, multiple average peak area ratios were calculated using the peak area ratios of the peptides

originating from the same protein. To account for small differences in protein loading, all protein ratios were normalized by the overall intensity of reporter ions from individual sample as recommended by the manufacturer. The standard deviation (SD), the coefficient of variance (CV) for each average peak area ratio, and the average CV using the CVs for the average peak area ratios for each protein were also calculated. In the present study, only protein quantification data with relative expression of >1.25 or <0.8 were chosen for further analysis.

3.2.7 Statistical analysis

To gain statistical evidence for differential expression of proteins, another two separate experiments were performed as described above. Data were displayed as mean $\pm$ SD from three independent experiments. Student's *t*-test was employed to test the deviation between different batches. Values were only considered to be statistically significant when *p*-values were less than 0.05.

3.3 Results

3.3.1 Characterization of graphene and SWCNTs

The AFM image revealed that resulting graphene with lateral dimensions of hundreds nanometers was observed (Fig. 3.1A). The majority of graphene nanosheets were single-layered with a mean height of ~ 0.8 nm. This value was smaller than that of GO, which can be explained by the removal of covalently bound oxygen functional groups and elimination of the displacements of the sp^3 hybridized carbon atoms (Wu et al., 2009b). The height profile from AFM result revealed that the SWCNT bundles had

diameters in the range of 1.0–12.0 nm, which correspond to the expected height of individual and small bundles of SWCNTs (Fig. 3.1B). The lengths of the prepared SWCNTs were found to be around 2000 to 6000 nm similar to the pristine SWCNTs. It indicated that the soft oxidizing process did not truncate SWCNTs during the process.

From the FTIR result shown in Fig. 3.2A, the GO spectrum appeared strong bands corresponding to the C=O stretching vibration of the COOH groups at 1730 cm^{-1} , the O-H deformations of the C-OH groups at 1370 cm^{-1} , the epoxide groups at 1220 cm^{-1} , and the C-O stretching vibration at 1050 cm^{-1} (Chen and Yan, 2010). These bonds were completely disappeared from the spectrum of reduced GO, thus confirming the conversion of GO to graphene after hydrazine reduction. The peak at 1620 cm^{-1} for the skeletal vibration of graphitic domains remained in the FTIR spectra after the reduction.

Figure 3.1 Tapping-mode AFM image and height profile of graphene (A) and SWCNTs (B). (Adapted from: Yuan et al. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011;15; 207(3); 213-21.)

Figure 3.2 FTIR spectra (A) and Raman spectra (B) of GO and graphene. (Adapted from: Yuan et al. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011;15; 207(3); 213-21.)

Raman spectroscopy is a direct and non-destructive technique to characterize the structural and electronic properties of graphene. The Raman spectrum of graphene is characterized by two main features, the G peak representing the E_{2g} vibration modes within aromatic carbon rings, and the D peak giving a relative measure of the amount of sp³ carbons in the surroundings. The spectrum of the exfoliated GO showed a broadened and blue-shifted G (1586 cm⁻¹), and the D band at (1354 cm⁻¹) (Ferrari, 2007). After the hydrazine reduction, the Raman spectrum of the as-prepared graphene also exhibits the presence of D and G bands at 1588 and 1345 cm⁻¹, respectively (Fig. 3.2B). The increase in the D/G intensity ratio indicates a decrease in the size of the in-plane sp² domains and a partially ordered crystal structure of the graphene (Yang et al., 2009).

3.3.2 MTT assay result

MTT assay is a common method to evaluate the toxicity of nanomaterials by measuring the mitochondria function of the cell. After 48 h of exposure to either graphene or SWCNTs, we observed that both the cells treated with nanomaterials appeared to be decreased metabolic activities when compared to the control group. Specifically, the cells treated with SWCNTs loss 26±5% of viability and the cells exposed to graphene loss about 17±3% based on the MTT assay result. The difference of metabolic activities observed might be explained by the physical properties of individual materials and their biological interaction with the cellular systems. Given the SWCNTs with tubular shape, it might promote penetration of membranes and strong interactions with various cellular systems as previously suggested (Zhang et al.,

2010c). However, for the non-tubular shape material like graphene, the “snaking” effect of graphene was expected to be much weaker in comparison with SWCNTs.

3.3.3 Protein profile obtained from iTRAQ based 2D LC-MS/MS analysis

From the chromatogram results, we confirmed the successful application of online 2D LC-MS/MS using SCX column coupled with HPLC-Chip for online fraction and separation of tryptic peptides. There were trace amount of peptide signal detected in the initial loading step, which should be the weak binding short peptides. Majority of the peptides were eluted at the salt concentration between 40-500 mM ammonium formate. As can be seen in Fig. 3.3A, a representative chromatogram result showed that the peptides trapped at the enrichment column were eluted around 8-44 min. A representative MS window indicated that four precursor peptide ions, each marked with red diamond, were detected at 984.5 s (Fig. 3.3B). From the MS/MS spectrum shown in Fig. 3.3C, a representative peptide VVFVFGPDK from peroxiredoxin-6 was fragmented for peptide identification and quantification. Specifically, the peptide identity was based on the fragmentation pattern and the relative ratio of peptides was derived from the relative ratio of reporter ions.

Since normal searching criteria for complete proteins yielded too many proteins, stringent auto validation criteria which always provide high confidence of searched results were carried out. Roughly 500 proteins were identified from each of the three independent experiments conducted. After compared between the results obtained from independent experiments, these proteins appeared in all groups with consistent

Figure 3.3 (A) Representative chromatogram result of peptides eluted by 300 mM ammonium formate. (B) Representative MS window showing four peptide precursors marked by red diamond were detected at 984.5 sec. (C) Representative MS/MS spectrum of peptide VVFVFGPDK from peroxiredoxin-6. (Adapted from: Yuan et al. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011;15; 207(3); 213-21.)

trends of variation were analyzed and the proteins with significant changes were further classified into distinct categories according to their cellular functions. These included 7 proteins involved in redox regulation of the cell (Table 3.1), 9 proteins participated in cytoskeleton formation and calcium related signaling pathway (Table 3.2), 6 metabolic enzymes (Fig. 3.4) and 15 other growth related proteins (Table 3.3).

As listed in Table 3.1, we successfully identified seven proteins implicated in redox regulation of the cell. Peroxiredoxin family proteins are thioredoxin peroxidases participating directly in eliminating hydrogen peroxide (H_2O_2) and neutralizing other reactive oxygen species (ROS) (Nordberg and Arnér, 2001; Sue et al., 2005). The up-regulation of peroxiredoxin proteins coupled with thioredoxin for the cells treated with SWCNTs indicated that it might play an important role in eliminating the ROS introduced by SWCNTs to protect the cells from oxidative damage. However, there was only moderate change of thioredoxin and peroxiredoxin levels for the batch treated with graphene as shown in Table 3.1, which suggested that the low dose of graphene might not introduce intense ROS as SWCNTs. The peroxiredoxin 1 (Prx-1) was found to be involved in apoptosis signal-regulating kinase 1 (ASK1)-mediated signaling pathway (Kim et al., 2008). In Prx-1 knockdown cells, ASK1, p38, and JNK were quickly activated, leading to apoptosis in response to H_2O_2 . Thus the up-regulation of Prx-1 inhibited the activation of ASK1, and resulted in the inhibition of downstream signaling cascades such as the MKK3/6 and p38 pathway. Previous investigation has demonstrated that peroxiredoxin 5 (Prx-5) is a thioredoxin peroxidase that inhibits p53-induced apoptosis (Kropotov et al., 2006; Zhou et al., 2000). Thus the down-regulation of Prx-5 suggested that the SWCNTs exposure might activate p53-mediated DNA damage checkpoint signals and lead to apoptosis if the

Table 3.1

List of differentially expressed proteins involved in redox regulation of the HepG2 cells after treated with graphene and SWCNTs^a

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD	(116/114)±SD
Q06830	Peroxiredoxin-1	135	8	40	1.41±0.11	1.01±0.12
P30044	Peroxiredoxin-5	96	6	39	0.73±0.13	1.03±0.07
P30041	Peroxiredoxin-6	119	9	39	1.63±0.18	1.15±0.13
Q16881	Thioredoxin reductase 1	66	5	11	1.63±0.09	0.74±0.19
P10599	Thioredoxin	53	3	31	1.55±0.15	0.98±0.03
P21266	Glutathione S-transferase Mu 3	86	6	32	0.70±0.09	0.63±0.11
P04179	Superoxide dismutase (Stankovich et al.)	24	2	9	0.47±0.09	1.12±0.11

^a 115/114 is the ratio of different protein expression level in the cells treated with SWCNTs relative to the control cells;

116/114 is the ratio of different protein expression level in the cells treated with graphene relative to the control cells.

Adapted from: Yuan et al. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes:

An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011;15; 207(3); 213-21.)

DNA damage proved to be irreparable. Moreover, p53 can induce growth arrest by holding the cell cycle at the G₁/S regulation point on DNA damage recognition, which might also explain the G₁ arrest of HEK293 cells as reported earlier (Cui et al., 2005).

Glutathione S-transferase Mu, involved in detoxification of endogenous compounds by conjugating of reduced glutathione to a wide number of hydrophobic electrophiles (Patskovsky et al., 1999), was significantly down-regulated by 0.7-fold for both graphene and SWCNTs treated cells. It indicated that the detoxification of these nanomaterials might not be through the glutathione S-transferase mediated pathway. Moreover, manganese superoxide dismutase (MnSOD) which catalyzes the dismutation of superoxide into oxygen and H₂O₂ was found to be up-regulated by 1.12-fold for graphene treated cells, but not for SWCNTs treated cells. MnSOD, the product of superoxide dismutase 2 (SOD2) gene, is one of the major cellular defenses against oxidative stress (Li et al., 1995). Previous investigation has demonstrated that p53 is able to repress SOD2 gene expression and the reciprocal regulation of p53 and SOD2 gene expressions leads to the modulation of various cellular processes including apoptosis (Drane et al., 2001). The significant down-regulation of MnSOD by 0.47-fold for the cells treated with SWCNTs further supported the hypothesis that the apoptosis observed by previous study (Cui et al., 2005) might be mediated through p53 signaling pathway.

Among the proteins listed in Table 3.2, three cytoskeleton related proteins, namely, keratin, profilin and moesin were found to be up-regulated for the batch of cells treated with SWCNTs. Keratin-18 together with keratin-8 are involved in interleukin-6 (IL-6)-mediated barrier protection and IL-6 induces keratin expression in

epithelial cells (Wang et al., 2007a). Previous investigations have shown that exposure of human primary monocytes to carboxylated SWCNTs and exposure of A549 cells to MWCNTs led to IL-6 and IL-8 expression and the inflammatory response in these cells were through oxidative stress and NF- κ B activation (Ye et al., 2011; Ye et al., 2009). Our results suggested that pristine SWCNTs might interact with the cellular system in a similar way as carboxylated SWCNTs and MWCNTs and also induce inflammatory response through NF- κ B activation (Baktur et al., 2011). Profilin, a ubiquitous actin monomer-binding protein, is thought to regulate actin polymerization in response to extra cellular signals (Mahoney et al., 1997) and its up-regulation might prevent the polymerization of actin when cells were exposed to SWCNTs. Another actin binding protein moesin that functions as cross-linkers between plasma membranes and actin-based cytoskeletons was also found to up-regulated by 1.35-fold. These findings suggested that SWCNTs had profound effects on the cellular cytoskeleton organization, the cell-cell recognition and cell movement. However, no obvious effect on cytoskeletal proteins for graphene treated cells was observed, which indicated weak interactions of graphene with the cellular cytoskeleton system.

A close analysis of the metabolic enzymes listed in Fig. 3.4 revealed distinct pattern of changes for metabolic enzymes between graphene and SWCNTs treated cells, which suggested different mode of action for these nanomaterials. Overall six differentially expressed metabolic enzymes were identified in the present study. These included glucose-6-phosphate isomerase that catalyzes the conversion of glucose-6-phosphate into fructose 6-phosphate; fructose-bisphosphate aldolase, which splits the fructose 1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate;

Table 3.2

List of differentially expressed calcium-binding proteins and cytoskeletal proteins in HepG2 cells incubated with graphene and SWCNTs^a

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD	(116/114)±SD
P05783	Keratin, type I cytoskeletal 18	180	11	25	1.39±0.08	1.15±0.09
P07737	Profilin-1	70	4	42	1.39±0.12	1.02±0.06
P26038	Moesin	73	7	10	1.25±0.11	1.01±0.03
P31949	Protein S100-A11	25	2	17	0.69±0.18	1.17±0.03
P06703	Protein S100-A6	23	2	14	1.23±0.12	0.73±0.09
P25815	Protein S100-P	30	2	24	1.43±0.09	1.01±0.11
P04083	Annexin A1	102	7	28	1.29±0.08	1.37±0.23
P08758	Annexin A5	149	12	40	1.31±0.12	1.10±0.08
P62158	Calmodulin	26	2	11	1.29±0.11	0.86±0.06

^a 115/114 is the ratio of different protein expression level in the cells treated with SWCNTs relative to the control cells;

116/114 is the ratio of different protein expression level in the cells treated with graphene relative to the control cells.

Adapted from: Yuan et al. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes:

An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011;15; 207(3); 213-21.

Figure 3.4 Differentially expressed metabolic enzymes in HepG2 cells incubated with graphene and SWCNTs. CNT:C is the ratio of protein expression level in the cells treated with SWCNTs relative to the control cells; G:C is the ratio of protein expression level in the cells treated with graphene relative to the control cells. (Adapted from: Yuan et al. Comparative protein profile of human HepG2 cells treated with graphene and single-walled carbon nanotubes: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicology Letters*. 2011;15; 207(3); 213-21.)

glyceraldehyde 3-phosphate dehydrogenase, that converts glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate; triosephosphate isomerase that catalyzes the reversible interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate; and phosphoglycerate kinase that transfers a phosphate group from 1,3-bisphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. Majority of these proteins involved in the glycolysis for the batch of cells treated with SWCNTs were found to be up-regulated around 1.25- to 1.58-fold (Fig. 3.4). The elevated level

of these metabolic enzymes indicated the increasing demand for energy molecules like NADH and ATP for cellular activities. In contrast, these metabolic enzymes for the batch of cells treated with graphene appeared to be the opposite trend, which indicated low metabolic rate after exposure to graphene.

As listed in Table 3.2, various calcium-binding proteins were identified in the current study. S100 proteins are involved in regulation of protein phosphorylation, transcription factors, calcium homeostasis, the dynamics of cytoskeleton constituents, cell growth and the inflammatory response (Donato, 1999, 2001, 2003; Zimmer et al., 1995). Interestingly, S100-A6 that promotes cell growth and S100-A11 which deters cell growth were found to be altered by 1.23- and 0.69-fold for the batch treated with SWCNTs. In contrast, these S100 proteins showed the opposite trend of variation for the graphene treated cells as protein S100-A6 and S100-A11 were altered by 0.73- and 1.17-fold, respectively. As the reciprocal regulation of protein S100-A6 and S100-A11 determines the metabolic rate and energy demand of the cell, thus the trend regarding S100 family proteins was consistent with that of the metabolic enzymes as previously discussed. We suspected that SWCNTs might severely interfere with intracellular systems and cause false-folding non-functional protein accumulations, which might lead to the increasing demand for energy molecules like NADH and ATP for protein synthesis. Furthermore, calmodulin, an ubiquitous intracellular Ca^{2+} receptor that regulates various cellular functions (Chin and Means, 2000), was also found to be altered in the opposite trend, which further supported the hypothesis of different mode of action regarding these nanomaterials. Interestingly, another calcium binding protein S100-P was found to be elevated by 1.43-fold for the SWCNTs treated cells only. Protein S100-P was previously report to stimulate the activation of

transcription factor NF- κ B (Arumugam et al., 2004), thus the elevated level of protein S100-P further supported that SWCNTs might activate the NF- κ B signaling pathway for the HepG2 cells as discussed above. Moreover, we found the expression levels of annexins, another family of calcium-binding proteins, were both elevated for graphene and SWCNTs treated cells. Annexins have been linked with inhibition of phospholipase activity, exocytosis and endocytosis, signal transduction, organization of the extracellular matrix and resistance to ROS (Benz and Hofmann, 1997; Laohavisit and Davies, 2011). The role of annexins on resistance to ROS was self-evident as previous reports indicated that ROS can be generated by SWCNTs and graphene (Zhang et al., 2010c). However, since the functions regarding to these calcium-binding proteins involved in signaling pathways were extremely complex, their exact role involved in the cellular responses to SWCNTs and graphene remains to be further studied.

To further strengthen the hypothesis that graphene might be more biocompatible in comparison to SWCNTs, we examined other growth related proteins as listed in Table 3.3. We found heat shock proteins were significantly up-regulated with iTRAQ ratios ranging from 1.37- to 1.54-fold for the cells treated with SWCNTs. In contrast, stress-70 protein and 60 kDa heat shock protein were only raised by 1.12- and 1.16-fold for the graphene treated cells. Since heat shock proteins function as intra-cellular chaperones and assist protein folding (Schlesinger, 1990), the significantly higher level of heat shock proteins for SWCNTs treated cells indicated that SWCNTs induced more severe stress in comparison to graphene, which might also explain the poor viability for SWCNTs treated cells as observed from the MTT assay. A closer analysis of the proteins listed in Table 3.3 further revealed that the

expression levels of ribosomal proteins, ribonucleoproteins, histone, ubiquitin and translation initiation factor also appeared to be much higher for the cells exposed to SWCNTs over graphene. Previous studies have shown that CNTs could enter the cell and frequently insert through the plasma membrane into the cytoplasm and the nucleus (Cheng et al., 2009) and oligonucleotides/proteins could be absorbed by CNTs for debundling nanotubes (Cathcart et al., 2007; Edri and Regev, 2009; Gigliotti et al., 2006). Our data suggested these SWCNTs retained inside the cells might strongly interact with the cellular systems and interfere mRNA processing, protein folding and synthesis. However, graphene might still cause stress and perturb the translation process and protein folding. Since the 78 kDa glucose-regulated protein (GRP78), a key marker of ER stress, was found to be the significant up-regulated for both graphene and SWCNTs treated cells, which indicated that both nanomaterials might cause large amount of unfolding or false-folding protein accumulation in the ER. Evidence like the elevated levels of translation initiation factor and protein disulfide-isomerase for the graphene treated cells further supported the idea that graphene might still cause moderate cytotoxicity to the mammalian cells. Moreover, proliferation associated protein 2G4, an RNA-binding protein involved in growth regulation (Squatrino et al., 2004), was found to be significantly up-regulated for both graphene and SWCNTs treated cells. The elevated level of proliferation associated protein 2G4 observed in the present study strongly suggested that the growth rate of HepG2 cells might also be perturbed after exposure to graphene and SWCNTs.

Table 3.3

List of differentially expressed growth related proteins in HepG2 cells incubated with graphene and SWCNTs^a

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD	(116/114)±SD
P07237	Protein disulfide-isomerase	120	8	19	1.68±0.07	1.35±0.11
P62826	GTP-binding nuclear protein Ran	69	4	20	1.50±0.06	1.09±0.16
P31948	Stress-induced-phosphoprotein 1	73	5	10	1.84±0.16	0.89±0.28
P22626	Nuclear ribonucleoproteins A2/B1	135	10	28	1.33±0.14	1.17±0.06
P62820	Ras-related protein Rab-1A	44	3	17	0.69±0.05	0.85±0.09
P05198	Translation initiation factor 2	24	2	6	1.47±0.08	1.29±0.08
P62988	Ubiquitin	95	6	76	1.25±0.13	1.05±0.10
P11021	78 kDa glucose-regulated protein	264	15	31	1.39±0.15	1.25±0.12
Q9UQ80	Proliferation-associated protein 2G4	62	2	15	1.37±0.26	1.43±0.14
P39019	40S ribosomal protein S19	39	3	17	1.46±0.12	1.26±0.15
P62917	60S ribosomal protein L8	52	4	25	1.45±0.13	1.01±0.08
P38646	Stress-70 protein	132	10	12	1.37±0.15	1.12±0.11
P10809	60 kDa heat shock protein	288	18	38	1.54±0.26	1.16±0.08
Q96A08	Histone H2B type 1	58	4	24	1.21±0.08	1.05±0.09
Q96QV6	Histone H2A type 1	44	3	29	1.49±0.25	1.02±0.12

^a 115/114 is the ratio of different protein expression level in the cells treated with SWCNTs relative to the control cells;

116/114 is the ratio of different protein expression level in the cells treated with graphene relative to the control cells.

Adapted from: Yuan et al. *Toxicology Letters*. 2011:15; 207(3); 213-21.

3.4 Discussion

Taken together, our group successfully demonstrated the application of iTRAQ based comparative proteomics to study the cellular functions in response to graphene and SWCNTs. We were able to identify 37 differentially expressed proteins in cells either treated with graphene or SWCNTs. Given the tubular shape of SWCNTs, it might promote penetration of membranes, uptake by the cells, and strong interactions with various cellular systems. These SWCNTs retained inside the cells might interfere metabolic routes, protein synthesis, mRNA processing, cytoskeleton system and signaling pathway. However, from the proteomics data, graphene showed moderate effect on the cellular functions compared to SWCNTs, which indicated low cytotoxicity as previously suggested (Ryoo et al., 2010). For the non-tubular shape of graphene, the “snaking” effect of graphene appeared to be much weaker in comparison with SWCNTs, thus caused less severe toxic effect to the cells. Specifically, we successfully identified the key enzymes involved in the redox regulation of the cell, and our findings strongly suggested that SWCNTs might trigger apoptosis of the cell through p53 signaling pathway. In contrast, graphene did not trigger the up-regulation of the thioredoxin-peroxiredoxin system to counter the ROS stress or induce the apoptosis based on the protein profile. Moreover, the expression levels of calcium-binding proteins were significantly altered for both the cells treated with SWCNTs and graphene. The pattern of variation regarding calcium-binding proteins was distinct between cells treated with graphene and SWCNTs, which also indicated the different mode of action with the cellular systems for these nanomaterials. However, the exact role of calcium-binding proteins involved the cellular functions in response to SWCNTs and graphene remains to be further studied.

Chapter 4 Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human HepG2 cells

4.1 Introduction

Recently, the functionalized derivatives of carbon nanotubes (CNTs) and graphene nanomaterials have been extensively studied for bio-related applications such as drug delivery, tissue engineering, biomaterials, biosensor and bio-imaging (Choi et al., 2010; Hu et al., 2010; Liu et al., 2010; Liu et al., 2008a; Robinson et al., 2008; Sun et al., 2008b; Veetil and Ye, 2009). With the growing potential bio-related applications of these nanomaterials and the mounting societal concern about the nanosafety, it has become imperative to evaluate the potential risks that might be caused by these nanomaterials and have a detailed mechanistic understanding of the interactions between these nanomaterials and living systems (Chou et al., 2008; Ding et al., 2005; Manna et al., 2005; Mu et al., 2009; Zhang et al., 2006; Zhou et al., 2008). Previous investigations have demonstrated that single-walled carbon nanotubes (SWCNTs) induce a noticeable cytotoxicity to mammalian cells (Ding et al., 2005; Magrez et al., 2006; Manna et al., 2005; Pulskamp et al., 2007; Raja et al., 2007; Shvedova et al., 2003). More recently, graphene has also been reported to induce certain degree of cellular toxicity (Hu et al., 2010; Ryoo et al., 2010). Among the suggested toxicity mechanisms, oxidative stress and mechanical rupture of cell membrane have been considered to be the most acceptable mechanisms to explain the cytotoxicity introduced by these nanomaterials. However, for the functionalized graphitic nanomaterials that have great potential for bio-related applications, such information

is generally lacking to date.

Previously, our group has successfully demonstrated using the isobaric tagged relative and absolute quantification (iTRAQ) based proteomics approach to study the cellular functions in response to various external stimuli such as pristine SWCNTs and graphene. Since the change of protein profile directly reflects the biological processes the cell undergoes, it provided useful information on the interactions between the living systems and these nanomaterials at the molecular level. In the present study, we further applied the iTRAQ based comparative proteomics technique to simultaneously detect and quantify the differences of protein expression levels in living systems after the treatment of the functionalized graphitic nanomaterials. Briefly, the iTRAQ technique is a liquid chromatography (LC) based approach, which includes LC separation of isotopically labeled peptides followed by peptide identification and quantification using MS/MS analysis (Ross et al., 2004b). In this study, two commonly used derivatives of graphitic nanomaterials, namely, oxidized SWCNTs and graphene oxide (GO), were selected as the testing materials and human hepatoma HepG2 cells were chosen as the *in vitro* model for studying the potential cytotoxicity on primary organ of liver.

From our comparative protein profile obtained here, overall 30 proteins with significant change of expression level were identified from either the oxidized SWCNTs or GO treated cells. These included metabolic enzymes, signaling proteins, cytoskeletal proteins and these proteins involved in cell growth and redox regulation of the cell. Based on the protein profile, oxidized SWCNTs induced oxidative stress and perturbed the intracellular metabolic routes, protein synthesis and cytoskeletal systems. Further experiments such as reactive oxygen species (ROS) measurement

and apoptosis detection confirmed that oxidized SWCNTs did generate elevated level of ROS and lead to a significant increase in the proportion of apoptotic cells. Cell cycle analysis showed that cells were arrested at of G₂/M phase after exposure to oxidized SWCNTs. Further Western blotting analysis confirmed that oxidized SWCNTs triggered the cell apoptosis through the phosphorylation of the stress-activated MAP kinase p38 and the p53 signaling pathway. In contrast, only moderate variation of protein levels for the cells treated with GO was observed. Functional assays further confirmed there was no significant increase percentage of apoptotic cells and cell cycle was not severely perturbed. Our data indicated GO was more biocompatible and could be promising candidate for bio-related applications. The possible mode of action for these functionalized graphitic nanomaterials that led to distinct pattern of protein profile was also discussed. Our finding provided molecular evidence to the cellular functions in response to oxidized SWCNTs and GO, which should be of great importance to evaluate biocompatibility of these nanomaterials.

4.2 Materials and methods

4.2.1 Preparation and Characterization of oxidized SWCNTs and GO

Purified HiPco SWCNTs (Unidym) produced by chemical vapor deposition (CVD) were used in the present study. These SWCNTs appeared to have a Gaussian distribution of diameters, with a maximum peak around 0.8~1.2 nm. A wet chemical technique that has been tested effective at removing both carbonaceous and catalytic impurities was applied to further purify SWCNTs (Porter *et al.*, 2009) before used to generate functionalized SWCNTs. Oxidized SWCNTs were produced by refluxing in

a mixture of concentrated sulfuric and nitric acids at 70 °C for 4 h, as described in detail elsewhere (Zhang *et al.*, 2007). Following the oxidizing process, the prepared oxidized SWCNTs were extensively washed with distilled water to remove any remaining impurities meanwhile adjust pH to 7. The GO was synthesized from synthetic graphite powder (SigmaAldrich) based on a modified Hummers method (Kovtyukhova, 1999). The exfoliation of GO was achieved by ultrasonication of the dispersion for 2 h. Any remaining chemicals or residuals were removed via dialysis with distilled water meanwhile adjusted pH to 7.

Both the quality of oxidized SWCNTs and GO were checked using atomic force microscopy (AFM), Fourier transform infrared (FTIR) spectroscopy and energy dispersive X-ray spectroscopy (EDS). The AFM was carried on a MFP3D microscope (Asylum Research) with a silicon cantilever operating in tapping mode. FTIR spectra were obtained on a Perkin Elmer Spectrum One Spectrometer. EDS was obtained using JEOL scanning electron microscope (JSM-6390), equipped to perform elemental chemical analysis.

4.2.2 Cell cultures

Human hepatoma HepG2 cells were obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagle's medium (supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin) and maintained at 37 °C in an atmosphere of 5% CO₂. All culture media and media supplements were obtained from Life Technologies. Both oxidized SWCNTs and GO were ultrasonicated 1 h for the dispersion before added to the culture media. The readily water soluble oxidized SWCNTs and GO were then diluted to 1 µg/mL in DMEM medium supplemented with 10% FBS and mixed

thoroughly. And these nanomaterial supplemented culture media appeared to be stable under the subsequent experimental condition. After reaching 80% confluence, the cells were treated with nanomaterials by replacing with oxidized SWCNTs or GO supplemented media, respectively. For the control cells, the fresh media without any nanomaterial supplementation were used.

4.2.3 Cell lysis, protein digestion and labeling with iTRAQ reagent

After exposure to oxidized SWCNTs or GO for 48 h, the cells were collected and lysed in 300 μ L of 8 M urea, 4% (w/v) CHAPS, and 0.05% SDS (w/v) on ice for 30 min with regular vortexing. Samples were centrifuged at 16,100 g for 60 min at 4 °C, supernatant was collected, and the protein concentrations were measured by the Quick Start Bradford Protein Assay (Bio-Rad). A standard curve was made using BSA as a control. A total of 100 μ g of each sample was further purified by 2-D clean up kit (GE healthcare) before used to generate iTRAQ-labeled peptides. The iTRAQ labeling process was carried out according to the manufacturer's protocol (iTRAQ Reagents Multiplex kit, Applied Biosystems, Foster City, CA). Specifically, the purified proteins were first resuspended in 20 μ L of triethylammonium bicarbonate buffer supplemented with 0.1% SDS. To fully dissolve proteins, the sample tubes were put into a sonicator bath for an additional 1 min. Then the samples were subjected to reduction by tris(2-carboxyethyl)phosphine (TCEP) at 60 °C for 1 h, followed by alkylation using methylmethanethiosulfonate (MMTS) at room temperature for 15 min. Next, the cysteine-blocked protein samples were digested to peptides using 10 μ L of 0.5 μ g/ μ L sequencing grade modified trypsin (Promega, Madison, WI) at 37 °C overnight. The digested samples were labeled with the iTRAQ tags as follows: control HepG2 cells, iTRAQ 114; HepG2 cells incubated with 1 μ g/mL oxidized SWCNTs,

iTRAQ 115; HepG2 cells incubated with 1 $\mu\text{g/mL}$ GO, iTRAQ 116. After incubated at room temperature for 1 h, the labeled samples were pooled, vacuum dried and resuspended in 300 μL ddH₂O containing 0.1% formic acid and 5% acetonitrile, to achieve approximately 1 $\mu\text{g}/\mu\text{L}$ peptide mixture. The resuspended sample was filtered to remove bulky particles before loading on LC-MS/MS for analysis.

4.2.5 HPLC-Chip coupled 2D LC-MS/MS analysis

After exposure to oxidized SWCNTs or GO for 48 h, the cells were collected and lysed in 300 μL of 8 M urea, 4% (w/v) CHAPS, and 0.05% SDS (w/v) on ice for 30 min with regular vortexing. Samples were centrifuged at 16,100 g for 60 min at 4 °C, supernatant was collected, and the protein concentrations were measured by the Quick Start Bradford Protein Assay (Bio-Rad). A standard curve was made using BSA as a control. A total of 100 μg of each sample was further purified by 2-D clean up kit (GE healthcare) before used to generate iTRAQ-labeled peptides. The iTRAQ labeling process was carried out according to the manufacturer's protocol (iTRAQ Reagents Multiplex kit, Applied Biosystems, Foster City, CA). Specifically, the purified proteins were first resuspended in 20 μL of triethylammonium bicarbonate buffer supplemented with 0.1% SDS. To fully dissolve proteins, the sample tubes were put into a sonicator bath for an additional 1 min. Then the samples were subjected to reduction by tris(2-carboxyethyl)phosphine (TCEP) at 60 °C for 1 h, followed by alkylation using methylmethanethiosulfonate (MMTS) at room temperature for 15 min. Next, the cysteine-blocked protein samples were digested to peptides using 10 μL of 0.5 $\mu\text{g}/\mu\text{L}$ sequencing grade modified trypsin (Promega, Madison, WI) at 37 °C overnight. The digested samples were labeled with the iTRAQ tags as follows: control HepG2 cells, iTRAQ 114; HepG2 cells incubated with 1 $\mu\text{g/mL}$ oxidized SWCNTs,

iTRAQ 115; HepG2 cells incubated with 1 $\mu\text{g/mL}$ GO, iTRAQ 116. After incubated at room temperature for 1 h, the labeled samples were pooled, vacuum dried and resuspended in 300 μL ddH₂O containing 0.1% formic acid and 5% acetonitrile, to achieve approximately 1 $\mu\text{g}/\mu\text{L}$ peptide mixture. The resuspended sample was filtered to remove bulky particles before loading on LC-MS/MS for analysis.

The LC-MS/MS system was equipped with Agilent 1200 Series liquid chromatograph and 6530 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA). The LC system for separation of peptides included a PolySulfoethyl A SCX column (0.32×50 mm, 5 μm particles, PolyLC Inc.) and HPLC-Chip (Large capacity chip II, Agilent Technologies) as discussed before. The sequentially eluted peptides was first trapped onto the Zorbax 300SB-C18 enrichment column during the enrichment mode of HPLC-Chip and washed isocratically with buffer containing 5% acetonitrile and 0.1% formic acid at 0.004 mL/min for additional 30 min in order to remove any excess reagent. Next, the HPLC-Chip was changed to analysis mode and the peptides binding to enrichment column was subjected for further separation in the second dimension. Briefly, the enrichment column was switched from the capillary flow path connected with SCX column to the nanoflow pathway followed by an analytical column. The peptides trapped onto the enrichment column were eluted with buffer A (0.1% formic acid) and buffer B (90% acetonitrile + 0.1% formic acid) at a flow rate of 300 nL/min. A linear gradient with two segments was used. One segment was from 5% to 45% buffer B during the initial 40 min, followed by a second segment with sharp increase to 90% buffer B over 5 min. The flow was held for additional 5 min at 90% buffer B to elute any remaining substances that might bind to the columns and then allowed for reconditioning to initial state for 10 min. Further separation was achieved onto the analytical Zorbax 300SB C18 reversed-phase column ($75 \mu\text{m} \times 150$ mm, 5 μm

particles). The column effluent was directly analyzed by the 6530 Q-TOF mass spectrometer interfaced through an HPLC-Chip Cube nanospray source. Overall, 10 runs with different fraction of peptides eluted from SCX column were performed to complete one experiment.

For the mass spectrometer, the capillary voltage was set to 1800 V to achieve good and stable spray of effluent. The MS data were acquired in the positive ionization mode using Agilent MassHunter Workstation Q-TOF B.02.01. The fragmentor voltage, skimmer voltage, and octopole RF were set to 175, 65, and 750 V during the data acquisition process. Auto-MS/MS with a total cycle time of 1.225 s was chosen for MS/MS acquisition. Specifically, MS spectra were acquired at 8 Hz (eight spectra/s) (m/z 100–2000) in each cycle, and the four most abundant ions (with charge states 2+ and 3+) exceeding 1000 counts were selected for MS/MS analysis at 4 Hz (four spectra/s) (m/z 50–2000). A medium isolation (4 m/z) window was used for precursor isolation. Collision energy with slope of 3.6 V/100 Da and offset of 4 V was used for the fragmentation of selected precursor peptide ions, which was generally enough to achieve iTRAQ reporter ions were amongst the most intense ions in the spectra. Reference mass correction was activated using reference mass of 121 and 922. Precursors were set in an exclusion list for 0.5 min after two MS/MS spectra.

4.2.6 Data analysis and interpretation

Peptide and protein identifications were carried out by the Spectrum Mill Proteomics Workbench (Rev A.03.03.084 SR4) software from Agilent Technologies. Default settings on Spectrum Mill data extractor program were used to generate the peak list. Peptides were automatically identified by the Spectrum Mill Proteomics Workbench software using UniProtKB/Swiss-Prot protein database (Geneva, Switzerland) search

for species of *Homo sapiens*. The search criteria were set using the following parameters: a mass tolerance of ± 2.5 Da for the precursor ions and a tolerance of ± 0.7 Da for the fragment product ions were used; two missed cleavages were allowed; methylmethanethiosulfate-labeled cysteine and iTRAQ modification at the amino group were set as fixed modification. Auto-validation was carried out to search the proteins and these criteria provided high confidence for the searched proteins which always represent valid results (above 99% confidence). To assure a fair comparison of the results, the same criteria were chosen to the analysis of other independent batches.

Relative quantification of peptide was obtained from the MS/MS spectra and was the ratio of the peak intensity at 114.1, 115.1 and 116.1 Da that correspond to the iTRAQ reporter ions. The relative abundance of specific protein was calculated using the relative ratios of all the peptides originating from the same protein. To account for small variation during the sample preparation process, the relative ratios of proteins were normalized based on the overall ratios of the iTRAQ reporter ions from the MS/MS spectra. In the present study, only proteins with two or more qualified peptide matches and relative ratio of >1.2 or <0.8 were subjected to further analysis.

4.2.7 Cell metabolic activity assay and ROS measurement

The cell proliferation was evaluated by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) test. Briefly, following HepG2 cells incubation with oxidized SWCNTs or GO for 48 h, the media were aspirated and replaced with 90 μ L of serum-free media. Then 10 μ L of MTT stock solution (5 mg/mL) was added to each well, followed by incubation for 4 h at 37°C. The supernatant was then removed, and cells were lysed with 100 μ L of dimethylsulfoxide (DMSO) and the absorbance was recorded at 550 nm on a

microplate reader (Benchmark Plus).

The intracellular ROS level was measured by the oxidation of the nonfluorescent probe dihydrofluorescein diacetate (DFDA) to the fluorescent metabolite fluorescein as reported earlier (Hempel *et al.*, 1999). Briefly, 1×10^5 cells were seeded in each well of 96-well plates and allowed to attach overnight. After incubation of $1 \mu\text{g/mL}$ of oxidized SWCNTs and GO for 12 h, the media were aspirated and replaced with $100 \mu\text{L}$ of $5 \mu\text{M}$ DFDA in phosphate-buffered saline (PBS). Cells were incubated with protection from light for 1 h at room temperature. For the quantification of fluorescein generated, the fluorescence intensities were acquired at 488 nm excitation and 535 nm emission with a fluorescence spectrophotometer. Specifically, the fluorescence intensities at the initial loading of probe and after 1 h incubation were both recorded. The absolute intensity of fluorescein formed by the cell was obtained by deducting the background fluorescence intensity at the initial loading of probe.

4.2.8 Apoptosis assay and cell cycle analysis

To detect apoptosis at the early stage, Annexin V-FITC Apoptosis Detection Kit from BD Pharmingen was used. Briefly, cells were seeded in each well of 12-well plates and allowed to attach overnight. After treated with oxidized SWCNTs or GO for 48 h, cells were collected after trypsin treatment. After washed twice with PBS, 1×10^5 cell were then incubated with annexin V-FITC and propidium iodide (PI) in the $100 \mu\text{L}$ binding buffer for 15 min at room temperature with protection from light. The cells were diluted by 5-fold using binding buffer before subjected to flow cytometry analysis (Bechman Coulter Altra).

For cell cycle analysis, 1×10^6 cells were collected after trypsin treatment and fixed

with 10 mL of 70% ethanol at 4 °C overnight. After repeated washes with PBS, cells were then stained with staining solution (0.1% Triton X-100, 10 µg/mL PI and 100 µg/mL RNase A in PBS) for 10 min at 37 °C. The percentage of cells in different phases of the cell cycle was measured by flow cytometric analysis.

4.2.9 Western blot analysis

For Western blot analysis, cell lysates with equal protein amount from individual samples were separated on a 12% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking the membrane for 1 h in TBST (20 mM Tris, pH 7.6, 136 mM NaCl, and 0.1% Tween-20) containing 5% nonfat milk, membranes were incubated at 4 °C overnight with primary antibody diluted in 5% milk-TBST solution. Following incubation, the membranes were washed with TBST and incubated with horseradish peroxidase conjugated secondary antibody at room temperature for 1 h. Blots were developed using Pierce SuperSignal West Pico Luminal/Enhancer solution. The following primary and secondary antibodies were used: anti-p53 (1:1000, Cell Signaling #2524), anti-phospho-p38 MAPK (1:1000, Cell Signaling #9215), anti-β-actin (1:2000, Sigma Aldrich A1978), anti-rabbit IgG-HRP and anti-mouse IgG-HRP (1:1000, Santa Cruz sc-2004 and sc-2005).

4.2.10 Statistical analysis

To gain statistical evidence for the differentially expressed proteins, three independent experiments were performed and data were displayed as mean±SD. Student's *t*-test was employed to test the deviation between different batches. Values were only considered to be statistically significant when *p*-values were less than 0.05.

4.3 Results

4.3.1 Characterization of oxidized SWCNTs and GO

As can be seen in Fig. 4.1A, the AFM result revealed that majority of these white streaks of oxidized SWCNTs appeared to be around 1000 to 2000 nm. From the height profile, we found the oxidized SWCNTs had diameters in the range of 1.0–6.0 nm, which might correspond to the expected height of individual and small bundles of oxidized SWCNTs. The resulting oxidized SWCNTs were readily soluble and stable in ddH₂O, which indicated the successful functionalization of SWCNTs as the pristine SWCNTs were more likely to form large bundles and precipitate in ddH₂O without the supplementation of surfactants. The AFM image revealed the resulting GO with lateral dimensions of hundreds nanometers was observed (Fig. 4.1B). From the height profile, the GO nanosheets were single-layered with a mean height of ~1.0 nm, which was thicker than the graphene sheet due to the presence of covalently bound oxygen functional groups and the displacements of the sp³ hybridized carbon atoms (Wu et al., 2009b).

Next, FTIR spectroscopy was used to study the oxidation of SWCNTs after strong acid treatment. As can be seen in Fig. 4.2A, FTIR results supported the theory of nanotube functionalization. From the spectrum, we observed multiple strong adsorption bands for the treated SWCNTs but not for the purified SWCNTs. Since SWCNTs themselves are not Raman active, any IR active mode for the treated SWCNTs should be associated with the attached functional groups. From the FTIR result shown in Fig. 4.2B, the GO spectrum showed strong bands corresponding to the C=O stretching vibration of the COOH groups at 1730 cm⁻¹, the O-H deformations of

the C-OH groups at 1370 cm^{-1} , the epoxide groups at 1220 cm^{-1} , and the C-O stretching vibration at 1050 cm^{-1} (Chen and Yan, 2010). In contrast, there were no such bands for graphene as shown from the spectrum. Moreover, EDS result confirmed metal catalysts that might produce reactive radicals and generate ROS were completely removed for both oxidized SWCNTs and GO (Fig. 4.3).

4.3.2 iTRAQ coupled 2D LC-MS/MS analysis results

To investigate how the protein profile changed in response to nanomaterials treatment, the global proteins were extracted from the cells and analyzed by iTRAQ-coupled 2D LC-MS/MS approach. In this approach, a strong cation exchange (SCX) column and HPLC-Chip (Large capacity chip II, Agilent Technologies) were used to set up the working platform. In the first dimension, the sample peptides were all loaded on the SCX columns, eluted by stepwise injections of salt plugs. The eluted peptides were trapped on the short enrichment column during the enrichment mode. Subsequently, the enrichment column was switched to analytic mode and the peptides trapped on enrichment column were eluted by a nanoflow gradient of acetonitrile and further separated on the reverse phase column incorporated in HPLC-Chip. As shown in Fig. 4.4A, a representative chromatogram result showed peptides signals for the fraction eluted by 100 mM ammonium formate were detected around 8-35 min. A representative MS window showed four precursor ions as marked by a red diamond were detected at 15.8 min (Fig 4.4B). These ions met the searching criteria during data acquisition process were selected for further electron bombardment. As can be seen from the MS/MS spectrum shown in Fig. 4.4C, a precursor ion with 448.3 m/z was fragmented for peptide identification and quantification. The peptide identity was derived from the fragmentation pattern of precursor ion and the relative ratio of

peptides was calculated from the relative ratio of iTRAQ reporter ions.

In the present study, stringent auto validation criteria which always provide high confidence of searched results were carried out for protein identification. Overall, more than 300 proteins were identified from each of the three independent experiments conducted. Among these proteins obtained by auto-validation searching criteria, proteins with significant changes (>1.2 or <0.8 fold) were classified into distinct categories according to their cellular functions. These included 5 proteins involved in redox regulation of the cell (Figure 4.5), 7 proteins participated in cytoskeleton formation and calcium related signaling pathway (Table 4.1), 10 metabolic enzymes (Table 4.2) and 8 other growth related proteins (Table 4.3).

Figure 4.1 Tapping-mode AFM image of oxidized SWCNTs (A) and GO (B). (Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.)

Figure 4.2 FTIR spectra of oxidized SWCNTs in comparison to purified SWCNTs (A) and GO in comparison to graphene (B). (Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.)

Figure 4.3 EDS spectra of oxidized SCWNTs (Top) and GO (Bottom). Oxidized SWCNT solution was dropped on gold coated silicon wafer. (Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.)

Differentially expressed proteins involved in redox regulation of the cell

As shown in Fig. 4.5, we successfully identified five proteins involved in redox regulation of the cell. These included peroxiredoxin (Prx) family proteins and thioredoxin. Prx family proteins are thioredoxin peroxidases involved directly in eliminating hydrogen peroxide and neutralizing other ROS (Nordberg and Arnér, 2001; Sue et al., 2005). Specifically, Prx-1, Prx-2 and Prx-6 were significantly up-regulated by 1.21- to 1.38-fold for the cells treated with oxidized SWCNTs when compared to the control cells (Fig. 4.5). Another protein involved in redox regulation of the cell, thioredoxin, was also found to be elevated by 1.20-fold. The up-regulation of these Prx proteins and thioredoxin for the cells treated with oxidized SWCNTs suggested that the cells might be under oxidative stress after exposure to oxidized SWCNTs. In comparison, the relative abundance of these Prx proteins and thioredoxin for the batch treated with GO did not show any significant change over the control cells (Fig. 4.5).

Interestingly, Prx-5 was found to be elevated for GO treated cells, but it was down-regulated for the oxidized SWCNTs treated cells. Since previous investigations have demonstrated that Prx-5 is a thioredoxin peroxidase that inhibits p53-induced apoptosis (Kropotov et al., 2006; Zhou et al., 2000), the down-regulation of Prx-5 might trigger the activation of p53-mediated DNA damage checkpoint signals and lead to apoptosis when the DNA damage appeared to be irreparable.

Figure 4.4 (A) Representative chromatogram result of peptides eluted from the fraction of 100 mM ammonium formate. (B) Representative MS window showing four peptide precursors as marked by a red diamond were detected at 15.8 min. (C) Representative MS/MS spectrum of precursor ion with 448.3 m/z . (Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.)

Figure 4.5 Differentially expressed proteins involved in redox regulation of the HepG2 cells after treated with oxidized SWCNTs and GO. CNT:C is the ratio of protein expression level in the cells treated with oxidized SWCNTs relative to the control cells; GO:C is the ratio of protein expression level in the cells treated with GO relative to the control cells. Data are shown as mean $\pm$ SD from three independent experiments. (Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.)

Differentially expressed cytoskeletal proteins and signaling proteins

Among the proteins listed in Table 4.1, two cytoskeleton related proteins, namely, profilin and filamin were found to be up-regulated by approximately 35% for the batch of cells treated with oxidized SWCNTs. Specifically, profilin is a ubiquitous actin monomer-binding protein that involved in regulating actin polymerization in response to extra cellular signals (Mahoney *et al.*, 1997), and actin binding protein filamin functions as cross-linkers between plasma membranes and actin-based cytoskeletons. The up-regulation of these cytoskeleton proteins suggested that the exposure of oxidized SWCNTs might alter the intracellular microfilament network. In contrast, no obvious effect on these cytoskeletal proteins for GO treated cells was observed, which indicated weak interactions of GO with the intracellular cytoskeleton system.

As listed in Table 4.1, several calcium-binding proteins involved in signaling pathways were found to be differentially expressed. S100 proteins are involved in regulation of protein phosphorylation, transcription factors, calcium homeostasis, the dynamics of cytoskeleton constituents, cell growth and the inflammatory response (Donato, 2003; Zimmer *et al.*, 1995). Interestingly, S100-A11 that deters the cell growth and S100-A6 that promotes cell growth were found to be 1.20- and 0.61-fold for the batch treated with oxidized SWCNTs. As the reciprocal regulation of protein S100-A6 and S100-A11 determines the metabolic rate and cell proliferation rate, our finding suggested that the cell growth might be severely deterred after exposure to oxidized SWCNTs. Furthermore, calmodulin, an ubiquitous intracellular Ca^{2+} receptor that regulates various cellular functions (Chin and Means, 2000), was also found to be down-regulated by 20% for oxidized SWCNTs treated cells. Since various

calmodulin-dependent protein kinases need to be activated in proliferating cell, the down-regulation of calmodulin further supported the exposure of oxidized SWCNTs might deter the cell proliferation rate. In contrast, these S100 proteins showed only moderate variations for the GO treated cells as protein S100-A11 and S100-A6 appeared to be 1.03- and 0.87-fold of the control cells. Moreover, we found the expression levels of annexins, another family of calcium-binding proteins, were elevated for GO and SWCNTs treated cells. As annexins have been involved in various cellular activities such as signal transduction, endocytosis, signal organization of the extracellular matrix and resistance to ROS (Benz and Hofmann, 1997; Laohavisit and Davies, 2011), their exact role in modulating the cellular functions in response to these nanomaterials remains to be further studied.

Differentially expressed metabolic enzymes

A close analysis of the metabolic enzymes listed in Table 4.2 revealed a distinct pattern of changes between oxidized SWCNTs and GO treated cells, which suggested different modes of action for these nanomaterials. Specifically, two key metabolic enzymes involved in glycolysis were found to be significantly altered for the batch of cells treated with oxidized SWCNTs. These included glyceraldehydes 3-phosphate dehydrogenase, that converts glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate; and phosphoglycerate kinase that transfers a phosphate group from 1,3-bisphosphoglycerate to ADP, forming ATP and 3-phosphoglycerate. In contrast, these metabolic enzymes for the batch of cells treated with GO appeared to be the similar level as the control cells, which indicated the cellular metabolism was not perturbed by the GO exposure.

Moreover, we found glutathione S-transferase Mu (GSTMu), involved in

detoxification of endogenous compounds through conjugating of reduced glutathione (Patskovsky *et al.*, 1999), was significantly down-regulated by 50% for the cells exposed to oxidized SWCNTs. Peptidyl-prolyl cis-trans isomerase that functions as protein folding chaperone was found to be 1.46-fold of the control cells for the oxidized SWCNTs treated cells, but not for the GO treated cells. And another enzyme that acts to catalyze protein-folding, protein disulfide isomerase A6, was also found to be up-regulated by 1.66-fold for the cells exposed to oxidized SWCNTs only. Furthermore, the expression levels of various other metabolic enzymes as listed in Table 4.2 were found to be significantly altered for the batch of cells treated with oxidized SWCNTs.

Differentially expressed other growth related proteins

To further strengthen the hypothesis that GO might be more biocompatible in comparison to oxidized SWCNTs, we examined other growth related proteins as listed in Table 4.3. We found 60 kDa heat shock protein and 78 kDa glucose-regulated protein were significantly up-regulated with iTRAQ ratios by 20% to 34% for the cells treated with oxidized SWCNTs. In contrast, these proteins remained the same level as the control cells for the GO treated cells. A closer analysis of the proteins listed in Table 4.3 further revealed that the expression levels of ribonucleoproteins, histone, and elongation factor also appeared to be much higher for the cells exposure to oxidized SWCNTs over GO. Moreover, proliferation associated protein 2G4, an RNA-binding protein involved in growth regulation, was found to be significantly up-regulated by 1.27-fold for the oxidized SWCNTs treated cells. Another cell-cycle control protein, S-phase kinase-associated protein 1 that controls the progression from G₁ phase to S phase of the mitotic cell cycle, was significantly down-regulated by

54% for the batch of cells treated with oxidized SWCNTs. The elevated level of proliferation associated protein 2G4 and the down-regulation of S-phase kinase-associated protein 1 suggested that the growth rate and/or the cell cycle might be disturbed after exposure to oxidized SWCNTs. In contrast, all these proteins mentioned in this section for the GO treated cells showed only moderate changes or remained similar levels as the control cells, which suggested that GO might cause less cytotoxicity to the cells.

4.3.3 Oxidized SWCNTs reduced cell proliferation rate and induced ROS generation

To determine whether proliferation rate of the human HepG2 cells was altered by the exposure of these nanomaterials, MTT assay that evaluates of cell proliferation rate based on the ability of the mitochondrial dehydrogenase to convert the yellow dye (MTT) to a purple-colored formazan was carried out. The potential toxic effect on cell proliferation can be calculated by comparing the amount of purple formazan produced by the cells treated with nanomaterials over the amount of formazan formed by the untreated cells. After 48 h of exposure to either oxidized SWCNTs or GO, we found

Table 4.1

List of differentially expressed calcium-binding proteins and cytoskeletal proteins in HepG2 cells incubated with oxidized SWCNTs and GO^a

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD	(116/114)±SD
P07737	Profilin-1	69	5	42	1.37±0.09	1.09±0.08
P21333	Filamin-A	197	16	8	1.35±0.06	1.04±0.11
P31949	Protein S100-A11	24	2	17	1.20±0.18	1.03±0.03
P06703	Protein S100-A6	35	3	22	0.61±0.14	0.87±0.08
P04083	Annexin A1	51	4	17	1.43±0.08	1.30±0.12
P08758	Annexin A5	87	7	23	1.21±0.15	1.13±0.08
P62158	Calmodulin	27	2	11	0.80±0.12	0.87±0.07

^a 115/114 is the ratio of different protein expression level in the cells treated with oxidized SWCNTs relative to the control cells;

116/114 is the ratio of different protein expression level in the cells treated with GO relative to the control cells.

Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.

Table 4.2

List of differentially expressed metabolic enzymes in HepG2 cells incubated with oxidized SWCNTs and GO^a

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD	(116/114)±SD
P00558	Phosphoglycerate kinase 1	193	16	37	0.80±0.12	1.13±0.16
P07205	Phosphoglycerate kinase 2	67	6	13	0.76±0.06	0.93±0.04
P15531	Nucleoside diphosphate kinase A	67	5	31	1.26±0.08	0.98±0.10
P00338	L-lactate dehydrogenase A	164	13	39	1.35±0.14	0.94±0.12
P04406	Glyceraldehyde-3-phosphate dehydrogenase	111	9	31	1.52±0.08	0.97±0.06
P21266	Glutathione S-transferase Mu 3	86	6	32	0.54±0.19	0.93±0.12
P23284	Peptidyl-prolyl cis-trans isomerase B	57	5	18	1.46±0.06	0.97±0.15
P23921	Ribonucleoside-diphosphate reductase	127	9	14	1.29±0.07	0.92±0.04
Q15084	Protein disulfide-isomerase A6	64	5	14	1.66±0.12	1.15±0.04
P05187	Alkaline phosphatase	27	2	3	1.83±0.14	1.34±0.02

^a 115/114 is the ratio of different protein expression level in the cells treated with oxidized SWCNTs relative to the control cells;

116/114 is the ratio of different protein expression level in the cells treated with GO relative to the control cells.

Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.

Table 4.3

List of differentially expressed growth related proteins in HepG2 cells incubated with oxidized SWCNTs and GO^a

Accession no.	Name	Score	No. of peptides	%AA coverage	(115/114)±SD	(116/114)±SD
P22626	Nuclear ribonucleoproteins A2/B1	132	12	37	1.25±0.14	1.04±0.06
P62820	Ras-related protein Rab-1A	26	2	13	0.81±0.05	0.97±0.09
P49411	Elongation factor Tu	65	5	13	1.42±0.14	1.05±0.06
P11021	78 kDa glucose-regulated protein	173	13	24	1.20±0.04	1.09±0.02
Q9UQ80	Proliferation-associated protein 2G4	27	2	5	1.27±0.10	1.17±0.02
P63208	S-phase kinase-associated protein 1	24	2	13	0.47±0.22	0.76±0.17
P10809	60 kDa heat shock protein	240	18	33	1.34±0.16	1.01±0.02
P0C0S8	Histone H2A type 1	46	3	23	1.50±0.16	1.04±0.12

^a 115/114 is the ratio of different protein expression level in the cells treated with oxidized SWCNTs relative to the control cells;

116/114 is the ratio of different protein expression level in the cells treated with GO relative to the control cells.

Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.

that both the cells treated with nanomaterials showed a decrease in metabolic activities when compared to the control batch (Fig. 4.6). Specifically, we found the cells treated with oxidized SWCNTs lost approximately 18% of metabolic activity and the cells exposed to GO only lost 6% based on the MTT assay result. To determine the effect of oxidized SWCNTs and GO on ROS generation in human HepG2 cells, the fluorescent probe DFDA was used to detect cell-derived oxidants. After exposure to oxidized SWCNTs and GO for 12 h, DFDA was added to the cell culture and the fluorescein formed by the cells was measured after 1 h incubation with DFDA in dark. As can be seen in Fig. 4.6, the fluorescence intensity of fluorescein produced for the cells exposure to oxidized SWCNTs was higher in comparison to the control batch, which was approximated 1.14-fold over the control cells. However, the fluorescence intensity for the cells treated with GO showed only 8% increase over the control cells. These data indicated oxidized SWCNTs induced higher level of ROS in human HepG2 cells in comparison to GO, which might explain more severe oxidative stress in these cells over GO as indicated from the protein profile.

4.3.4 Oxidized SWCNTs induced apoptosis and led to growth arrest in human HepG2 cells

To investigate whether human HepG2 cells undergo apoptosis after the treatment of oxidized SWCNTs and GO, cells treated with either of the nanomaterials were examined using annexin V-FITC/PI apoptosis detection assay. Since the appearance of phosphatidylserine (PS) residues on the surface of the cell is a signal of apoptosis before nuclear breakdown, DNA fragmentation and the appearance of apoptosis-

Figure 4.6 Relative cell proliferation rate and intracellular ROS level for the HepG2 cells treated with oxidized SWCNTs and GO. Cell proliferation was measure after 48 h treatment of oxidized SWCNTs and GO using MTT assay. Intracellular ROS level was recorded after 12 h treatment of oxidized SWCNTs and GO. Data are shown as mean $\pm$ SD from three independent experiments and p -values are less than 0.05 over the control. (Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.)

Figure 4.7 (A) Assessment of apoptosis in human HepG2 cells after exposure to oxidized SWCNTs and GO. (B) Cell cycle distribution analysis of the HepG2 cells after exposure to oxidized SWCNTs and GO. Apoptosis data are representative of three independent experiments. The quantitative data for cell cycle distribution are shown as mean $\pm$ SD from three independent experiments. (Adapted from: Yuan et al. Cytotoxicity evaluation of oxidized single-walled carbon nanotubes and graphene oxide on human hepatoma HepG2 cells: An iTRAQ-coupled 2D LC-MS/MS proteome analysis. *Toxicological Sciences*. 2012; 126(1); 149-61.)

associated molecules, annexin V-FITC that have a high affinity for binding to PS has been widely used to detect apoptotic cells in the early stage. As can be seen in Fig. 4.7A, we observed a significant increase in the proportion of cells undergoing apoptosis for the batch treated with oxidized SWCNTs. Specifically, the subpopulation of cells staining with annexin V-FITC for cells exposure to oxidized SWCNTs was 26.3% of total cells in comparison to 18.6% of total cells for the control batch. In comparison, there was no significant increase in the proportion of apoptotic cells for the GO treated batch (Fig. 4.7A).

As shown in Fig. 4.7B, cell cycle analysis showed that treatment of human HepG2 cells with oxidized SWCNTs led to a significant increase in the proportion of cells in the G₂/M phase. Specifically, the proportion of cells at G₂/M phase was 40.3% over 31.5% of the total cells for the control batch. In contrast, the treatment of human HepG2 cells with GO resulted a minor decrease in the percentage of G₂/M phase cells over the control cells.

4.3.4 Oxidized SWCNTs triggered apoptosis through p53-p38 pathways

To understand the molecular mechanism of apoptosis induced by oxidized SWCNTs, differential expression of tumor suppressor protein p53 and p38 in response to oxidized SWCNTs was analyzed by Western blot analysis. As can be seen from Fig. 4.8, there was no obvious band for p53 and phospho-p38 in the control cells (Fig. 4.8). The absence of bands for p53 and phospho-38 is due to the detection limit of Western blotting analysis, which might be overcome by increasing the initial loading of

sample or optimizing the primary and secondary concentration. However, for the lane loaded with proteins from the batch of cells treated with 1 $\mu\text{g/mL}$ of oxidized SWCNTs, there were weak bands for both p53 and phospho-38, which was visible from the film under the same exposure condition (Fig. 4.8). Notably, for the cells treated with even higher concentration of oxidized SWCNTs at 50 $\mu\text{g/mL}$, we did observe intense bands for both p53 and phospho-p38 (Fig. 4.8). The significant up-regulation of p53 for the cells treated with higher concentration of oxidized SWCNTs indicated that the previous hypothesis made from proteomics data was correct and p53 was truly involved in growth arrest and cell cycle control as previously suggested. Moreover, since p38 is a stress-activated MAP kinase that becomes activated upon phosphorylation and is involved in apoptosis initiation, the significant upregulation of phospho-p38 confirmed that p38 was involved in the SWCNTs triggered apoptosis as suggested by proteomics data as well.

Figure 4.8 Effect of oxidized SWCNTs on the expression level of p53 and phospho-p38 protein in human HepG2 cells. Lane 1, control cells without treatment; Lane 2, cells treated with 1 $\mu\text{g/mL}$ oxidized SWCNTs for 48 h. Lane 3, cells treated with 50 $\mu\text{g/mL}$ of oxidized SWCNTs.

4.4 Discussion

In the present study, our group successfully identified 30 differentially expressed proteins in cells either treated with GO or oxidized SWCNTs through iTRAQ-coupled 2D LC-MS/MS proteomics approach, which provided useful information to evaluate the potential cytotoxicity of these nanomaterials and elucidate the underlying cytotoxicity mechanisms. Since previous investigations indicated that CNTs could be taken by mammalian cells (Cheng et al., 2009; Porter et al., 2009; Ye et al., 2011), the protein profile obtained by iTRAQ based proteomics approach suggested these oxidized SWCNTs retained inside the cells might interfere metabolic routes, protein synthesis, mRNA processing, cytoskeleton system and signaling pathway. In contrast, only moderate variation of protein profile was observed for the cells exposed to GO, which suggested less cytotoxicity of GO based nanomaterials.

By applying iTRAQ coupled 2D LC-MS/MS analysis, we successfully identified changes in the levels of key proteins involved in the redox regulation of the cell. Specifically, Prx family protein and thioredoxin were found to be significantly up-regulated in the cells treated with oxidized SWCNTs, which suggested there might be elevated level of intracellular ROS after treated with oxidized SWCNTs. Further measurement of the intracellular ROS level confirmed that SWCNTs induced higher level of ROS in comparison to the control cells (Fig. 4.6), which indicated cells after exposure to oxidized SWCNTs were undergoing oxidative stress. However, Prx-5, a thioredoxin peroxidase that inhibits p53-induced apoptosis (Kropotov et al., 2006; Zhou et al., 2000), was found to be significantly down-regulated by 21% for the cells exposure to oxidized SWCNTs. Its down-regulation suggested that oxidized SWCNTs

might activate p53-mediated DNA damage checkpoint signals and lead to apoptosis. Since p53 can also induce growth arrest by holding the cell cycle at the G₁/S and G₂/M regulation point on DNA damage recognition (Agarwal *et al.*, 1995), it might explain the significant increase in the proportion G₂/M phase cells for oxidized SWCNTs treated cells as observed from the cell cycle analysis (Fig. 4.7B). Further experiment using Western blot analysis showed that cells exposed to oxidized SWCNTs did induce upregulation of tumor suppressor protein p53 (Fig. 4.8). These findings were consistent with previously study, showing that growth arrest at G₂/M phase was observed for the oxidized SWCNTs treated human primary monocytes (Ye *et al.*, 2011).

Moreover, we found GSTMu that modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1 (ASK1) (Cho *et al.*, 2001) was significantly down-regulated by 50% after exposure to oxidized SWCNTs. Its down-regulation might induce the activation of ASK1, therefore resulted in the activation of downstream signaling cascades such as the stress associated protein kinases (SAPKs) and p38 pathway, and finally led to stress-induced apoptosis. From apoptosis analysis result (Fig. 4.7A), we did find a significant increase in the proportion of cells undergoing apoptosis after the treatment of oxidized SWCNTs. Further experiment using Western blot analysis indicated that oxidized SWCNTs did induce upregulation of the stress-activated MAP kinase p38 through phosphorylation (Fig. 4.8), which confirmed the hypothesis made from the proteomics data. Moreover, as previous investigation has demonstrated that the stimulation of p38 activates the nuclear factor kappa B (NF- κ B) (Craig *et al.*, 2000), our findings suggested that the activation of transcription factor NF- κ B by oxidized SWCNTs exposure reported

earlier might be through p38 signaling pathway and the collaborative action of these signals might also explain the interleukin-6/8 mediated inflammatory response in human primary monocytes (Ye et al., 2011).

Furthermore, S100-A6 and calmodulin that promote cell growth were found to be 0.61- and 0.80-fold of the control cells for the batch treated with oxidized SWCNTs. S100-A11 protein that deters the cell growth was found to be elevated by 20% in comparison to the control cells. Since the reciprocal regulation of these growth rate control proteins decides the cell proliferation rate, our findings suggested growth rate of the cells might be reduced after exposure to oxidized SWCNTs. Further evidences on reduced proliferation rate could also be obtained from the down-regulation of the metabolic enzyme such as phosphoglycerate kinase involved in the ATP generating step, MTT assay data and cell cycle analysis result.

Furthermore, 60 kDa heat shock protein that functions as intra-cellular chaperones and assists protein folding (Schlesinger, 1990) was found to be significantly up-regulated by 34% for the cells treated with oxidized SWCNTs. 78 kDa glucose-regulated protein, one of the endoplasmic reticulum (ER) chaperones and a key marker of ER stress, was found to be elevated by 20% after the treatment of oxidized SWCNTs. Peptidyl-prolyl cis-trans isomerase that functions as protein folding chaperone was found to be 1.46-fold of the control and another enzyme that acts to catalyze protein-folding, protein disulfide isomerase A6, was also found to be up-regulated by 66% for the cells exposed to oxidized SWCNTs. The significant up-regulation of these chaperone proteins involved in assisting protein folding might be explained by elevated protein demand as various proteins were found to be significantly up-regulated after the

treatment of oxidized SWCNTs and/or oxidized SWCNTs might perturb the protein folding process.

Moreover, the cell-cycle regulation proteins such as proliferation associated protein 2G4 and S-phase kinase-associated protein 1 that controls the progression from G₁ phase to S phase of the cell cycle were found to be 1.27- and 0.47-fold of the control cells, respectively. These differentially expressed growth regulation proteins provided further evidences that oxidized SWCNTs could perturb the growth rate and cell cycle as discussed above. A closer analysis of the proteins listed in Table 4.3 revealed that the expression levels of other growth related proteins such as ribonucleoproteins, histone, and elongation factor also appeared to be much higher for the cells exposure to oxidized SWCNTs, which indicated that oxidized SWCNTs had profound effects on the intracellular systems.

Based on the information obtained from protein profile, GO showed moderate effects on the cellular functions in comparison with oxidized SWCNTs, which confirmed low cytotoxicity of GO as previously suggested (Hu et al., 2010; Ryoo et al., 2010). From the protein profile, we found S100-A11 and S100-A6 and calmodulin that promote cell growth were down-regulated by approximated 13% over the control cells. And other cell growth rate regulation proteins such as proliferation associated protein 2G4 and S-phase kinase-associated protein 1 were also found to be altered by the exposure of GO. These findings suggested growth rate and cell cycle might still be perturbed after the treatment of GO. Such hypothesis was further confirmed by functional assays as we did found a minor reduction in proliferation rate after the cells exposure to GO based on the MTT assay result (Fig. 4.6) and cell cycle was also slightly perturbed

(Fig. 4.7B). Moreover, elevated intracellular ROS level was also observed for GO treated cells. However, cellular responses between oxidized SWCNTs and GO treated cells were significantly different as we only found Prx-5 in the Prx family proteins was up-regulated in the GO treated cells. The distinct pattern of these Prx proteins might lead to distinct fate of the cells as these proteins were involved in mediating signal transduction and modulating various cellular activities in mammalian cells. Since it is nearly impossible to introduce the same degree of oxidization for these nanomaterials, it might cause some differences in the interaction with the intracellular systems. However, these subtle differences during the functionalization could hardly explain the distinct pattern of protein profiles obtained here. Previously, Cheng et al. have reported that MWCNTs entered the cell both actively and passively frequently inserting through the plasma membrane into the cytoplasm and the nucleus (Cheng et al., 2009). Considering the similarity between MWCNTs and oxidized SWCNTs regarding to their physical properties, we expected that oxidized SWCNTs might also cause incomplete phagocytosis or mechanically pierce through the plasma membrane and result in oxidative stress and cell death through apoptosis in a similar manner like MWCNTs. As GO nanosheets have also been reported to be internalized within mammalian cells by endocytosis and retained inside the endosome of the cytoplasm (Hu et al., 2010), the low cytotoxicity of GO might be explained by the poor puncturing effect and the compartmentation of GO inside the endosome significantly prevented the severe interference with other intracellular systems.

Chapter 5 Conclusion

Because of their distinctive physical and chemical properties, graphitic nanomaterials have attracted tremendous interest in the fields of biotechnology. SWCNTs, graphene and their functionalized derivatives are promising candidate for biomedical applications, such as biosensors and drug carriers (Bianco et al., 2005, 2008; Chen et al., 2008; Lacerda et al., 2006; Liu et al., 2010; Liu et al., 2008a; Sun et al., 2008b; Veetil and Ye, 2009). In such scenario, these nanomaterials are in constant interaction with living cells and tissues, which raises several concerns about their biocompatibility. With the growing potential applications in biomedical areas, it is urgently needed to evaluate the potential risks that might be introduced by these nanomaterials and have a fundamental understanding of the interaction between these nanomaterials and living systems.

In the present study, our group successfully demonstrated the application of iTRAQ-coupled 2D LC-MS/MS proteomics approach to study the cellular functions of human hepatoma HepG2 cells in response to various nanomaterials such as SWCNTs, graphene and their oxidized derivatives. We were able to identify various proteins involved in metabolic routes, protein synthesis, mRNA processing, cytoskeleton system and signaling pathway. By the close look at cellular function at the proteome level, we clearly identified the distinct pattern of cellular responses to SWCNT- and graphene-based nanomaterials.

Specifically, we successfully identified changes in the levels of key proteins involved in the redox regulation of the cell. Prx family protein and thioredoxin were found to be significantly up-regulated in the cells treated with pristine and oxidized SWCNTs,

which suggested there might be elevated level of intracellular ROS after the cells treated with pristine and oxidized SWCNTs. Further measurement of the intracellular ROS level confirmed that oxidized SWCNTs induced higher level of ROS in comparison to the control cells, which suggested cells were undergoing oxidative stress after exposure to oxidized SWCNTs. However, Prx-5, a thioredoxin peroxidase that inhibits p53-induced apoptosis (Kropotov et al., 2006; Zhou et al., 2000), was found to be significantly down-regulated for both the cells exposure to pristine and oxidized SWCNTs. Its down-regulation suggested that oxidized SWCNTs might activate p53-mediated DNA damage checkpoint signals and lead to apoptosis. Previous investigation showed superoxide dismutase 2 (SOD2) gene is able to repress p53 gene expression and the reciprocal regulation of these two genes can lead to the modulation of various cellular processes (Drane et al., 2001). Thus the significant down-regulation of SOD2 gene observed from cells treated with SWCNTs further supported that p53 signaling pathway might be activated. It is well known that p53 can also mediate growth arrest by holding the cell cycle at the G₁/S and G₂/M regulation point on DNA damage recognition (Agarwal *et al.*, 1995). The detailed signaling pathway involved in cell cycle control can be found in Figure 5. Interestingly, the 14-3-3 sigma was found to be significantly up-regulated for the batch of cells treated with SWCNTs. Considering the similar pattern of protein profile obtained from SWCNTs and oxidized SWCNTs treated cells, it is highly possible that the significant increase in the proportion G₂/M phase cells for oxidized SWCNTs treated cells as observed from the cell cycle analysis should be mediated through p53 signaling pathway. Further Western blotting analysis confirmed the significant upregulation of p53 when cells were exposed to higher concentration of oxidized SWCNTs, which suggested the good correlation of the proteomics data with

Figure 5 (A) Check points and the cell cycle. (B) The G₁/S and G₂/M DNA damage check points. (Adapted from: Michelle D. Cell cycle control and cancer. *Current Science*. 2001; 81: 515-22.)

Western blotting analysis result (Fig. 4.8).

Moreover, we found glutathione S-transferase Mu that modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1 (ASK1) (Cho *et al.*, 2001) was significantly down-regulated after exposure to pristine/oxidized SWCNTs as well graphene. Its down-regulation might induce the activation of ASK1, therefore resulted in the activation of downstream signaling cascades such as the stress associated protein kinases (SAPKs) and p38 pathway, and finally led to stress-induced apoptosis. From apoptosis analysis result (Fig. 4.7A), we did find a significant increase in the proportion of cells undergoing apoptosis after the treatment of oxidized SWCNTs. Further experiment using Western blot analysis indicated that oxidized SWCNTs did induce upregulation of the stress-activated MAP kinase p38 through phosphorylation (Fig. 4.8), which further supported the hypothesis suggested by the proteomics data. Moreover, as previous investigation has demonstrated that the stimulation of p38 activates the nuclear factor kappa B (NF- κ B) (Craig *et al.*, 2000), our findings suggested that the activation of NF- κ B by oxidized SWCNTs exposure reported earlier should be also through p38 signaling pathway and the collaborative action of these signals led to the interleukin-6/8 mediated inflammatory response in human primary monocytes (Ye *et al.*, 2011).

Furthermore, S100-A6 and calmodulin that promote cell growth were found to be down-regulated of the control cells for the batch treated with oxidized SWCNTs. S100-A11 protein that deters the cell growth was found to be elevated in comparison to the control cells. Since the reciprocal regulation of these growth rate control proteins decides the metabolic rate, our findings suggested metabolic rate of the cells might be reduced after exposure to oxidized SWCNTs. Further evidences on reduced

metabolic rate could also be obtained from the down-regulation of the metabolic enzyme such as phosphoglycerate kinase involved in the ATP generating step, MTT assay data and cell cycle analysis result. However, these proteins involved in metabolic rate control were found in the opposite trend for the batch treated with pristine SWCNTs, as well as the trend for the metabolic enzymes. So far, the underneath mechanism that leads to such distinct pattern of these proteins between SWCNTs and oxidized SWCNTs treated cells is still not clear.

Furthermore, 60 kDa heat shock protein that functions as intra-cellular chaperones and assists protein folding (Schlesinger, 1990); 78 kDa glucose-regulated protein, one of the endoplasmic reticulum (ER) chaperones and a key marker of ER stress; peptidyl-prolyl cis-trans isomerase that functions as protein folding chaperone and another enzyme that acts to catalyze protein-folding, protein disulfide isomerase A6, were found to be up-regulated for the cells exposed to SWCNTs and its oxidized derivative. The significant up-regulation of these chaperone proteins involved in assisting protein folding might be explained by elevated protein demand as various proteins were found to be significantly up-regulated after the treatment of oxidized SWCNTs and/or oxidized SWCNTs might perturb the protein folding process.

Moreover, the cell-cycle regulation proteins such as proliferation associated protein 2G4 and S-phase kinase-associated protein 1 that controls the progression from G₁ phase to S phase of the cell cycle were also found to altered after the treatment SWCNTs and oxidized SWCNTs. These differentially expressed growth regulation proteins provided further evidences that SWCNTs based nanomaterials could perturb the growth rate and cell cycle as discussed above. A closer analysis of the other growth related proteins revealed that the expression levels of these proteins such as

ribonucleoproteins, histone, and elongation factor also appeared to be much higher for the cells exposure to SWCNTs based nanomaterials.

Based on the information obtained from protein profile, both graphene and GO showed moderate effects on the cellular functions in comparison with SWCNTs based nanomaterials, which confirmed low cytotoxicity of graphene based materials as previously suggested (Hu et al., 2010; Ryoo et al., 2010). From the protein profile, we found S100-A6 and calmodulin that promote cell growth were down-regulated for batches treated with graphene based nanomaterial. And other cell growth rate regulation proteins such as proliferation associated protein 2G4 and S-phase kinase-associated protein 1 were also found to be altered by the exposure of graphene based materials. These findings suggested growth rate and cell cycle might still be perturbed after the treatment of graphene based materials. Such hypothesis was further confirmed by functional assays as we did find a minor reduction in proliferation rate after the cells exposure to graphene and GO. However, cell cycle analysis indicated that the cell cycle was only slightly perturbed for the GO treated cells (Fig. 4.7B). Interestingly, elevated intracellular ROS level was also observed for GO treated cells. However, the distinct cellular responses to SWCNTs and graphene based nanomaterials were observed, which might explain the distinct fate of the cells as discussed above. Previously, Cheng et al. have reported that MWCNTs entered the cell both actively and passively frequently inserting through the plasma membrane into the cytoplasm and the nucleus (Cheng et al., 2009). Considering the similarity between MWCNTs and oxidized SWCNTs regarding to their physical properties, we expected that both pristine and oxidized SWCNTs might also cause incomplete phagocytosis or mechanically pierce through the plasma membrane and result in oxidative stress and cell death through apoptosis in a similar manner like MWCNTs.

In contrast, GO nanosheets have also been reported to be internalized within mammalian cells by endocytosis and retained inside the endosome of the cytoplasm (Hu et al., 2010). The low cytotoxicity of graphene and GO observed from the protein profile might be explained by the poor puncturing effect of these nontubular shape materials and the compartmentation inside the endosome significantly prevented the severe interference with other intracellular systems.

In summary, our group has demonstrated the effectiveness of applying iTRAQ-coupled 2D LC-MS/MS proteome analysis to study the cellular functions of HepG2 cells in response to nanomaterials. By the close look at cellular functions at proteome level, we clearly identified the distinct pattern of cellular responses to SWCNTs and graphene based nanomaterials, which suggested different modes of action to the living systems. Further functional assays confirmed that oxidized SWCNTs triggered elevated level of ROS, reduced cellular metabolic activity, perturbed cell cycle and led to a significantly higher proportion of apoptotic cells. Western blot analysis confirmed oxidized SWCNTs did induce upregulation of two important molecular markers, p53 and phospho-p38 (Fig. 4.8), which validated the hypothesis proposed by the proteomics data. We envision that the systematic characterization through comparative proteomics technique coupled with functional assays would provide us a more detailed understanding of the interactions between living systems and these nanomaterials.

Chapter 6 Future Directions

6.1 Cytotoxicity evaluation of other nanomaterials on living systems

Besides pristine SWCNTs, graphene and their oxidized derivatives, there are various other approaches for the functionalization of these nanomaterials. The variation in size, shape, charge, methods of production, chemical compositions and surface chemistry introduced through functionalization may result in the interaction with living systems in different ways and the toxicity mechanism underneath may not be the same. In addition, other types of nanomaterials such as fullerene (C₆₀), metal and metal oxide particles, polymer nanoparticles, and quantum dots are also commonly used for bio-related applications. Their unique physiochemical properties (light weight, high tensile strength, thermal/chemical stability and conductivity) have generated applications in specific area. It will be very important to have a systematic characterization of the cellular functions in response to these various nanomaterials including their functionalized and modified derivatives before applying them *in vivo*.

Considering that there are various ways the nanomaterials can enter the human body, skin direct contact, inhalation and even deliberate injection for medical purpose, different cell models might be used for elucidating the interaction between these nanomaterials and living systems. Assuming inhalation as the most probable exposure scenario, A549 alveolar epithelial cells might be used as the *in vitro* model for studying the potential effects on the mammalian primary target organ of lung. For the scenario that nanoparticles are deposited into the pulmonary system followed by translocation to the circulatory system, liver (WIF-B9, Can-10, HepG2) and kidneys (NRK-52E, LLC-PK1) may be suitable as the model for studying the effects of

nanoparticles on potentially secondary target organs. Moreover, as the immune system will constantly interact with the injected nanomaterial-mediated drug/gene delivery carrier, it will also be important to study cellular functions of cells involved in the immune system in response to the nanomaterials as well. Since iTRAQ based proteomics approach is versatile, tissues, body fluids and secretory proteome analysis can also be performed using *in vivo* animal models, which will definitely boost our understanding of these nanomaterials on human health.

In the near future, we will mainly focus on studying the effects of metal oxide based nanoparticles and other SWCNTs derivatives on human HepG2 cells and other cell lines such as THP-1 derived macrophage cells. We envision data established in our study will provide us molecular evidence to the cellular functions in response to these nanomaterials, which should be of great importance to evaluate biocompatibility of these nanomaterials.

6.2 Develop new platform for safety evaluation and risk assessment

Human health risk assessment is the qualitative and quantitative evaluation of the risks posed to human health and/or the environment by the actual or potential presence and/or use of specific pollutants (Heinrich-Hirsch et al., 2001). The risk assessment process includes describing potential adverse effects, evaluating the magnitude of each risk, estimating potential exposure to the chemical or other hazard, estimating the range of likely effects given the likely exposures, and describing uncertainties (Marquis and Siek, 1988). To understand the toxic mechanisms and/or modes of action (MOAs), systematic characterization of the toxic process at the gene expression (transcriptomics), protein (proteomics), and metabolite (metabolomics) levels will definitely shed some light on key mechanisms and networks of toxicity.

Previously, Juan *et al.* has reported a profiling of human HL-60 cells using both gene and protein expression approaches, however, the data collected from these two approaches is not consistent. A total of 624 genes were altered from cDNA array analysis while 136 proteins were altered using a 2-D gel-based proteomics approach. Interestingly, only four proteins were found in common between proteomic and DNA microarray analysis, suggesting a poor correlation between mRNA and protein expression, and importance of proteomics as a more reliable approach to the study of toxic mechanisms and/or MOAs. Proteins play a central role in a system view of biological processes since they are ultimately the functional molecules for most cellular processes (Alaiya et al., 2000). Because of this, molecular characterization of the proteome is not only necessary for a complete understanding of chemical-induced toxic responses, but also the best approach for our understanding of the cellular response to the toxicants. In fact, the notion of toxicoproteomics has been established several years ago (Ge et al., 2007; Wetmore and Merrick, 2004), it is the use of proteomic technologies to better understand the toxic mechanisms, and modes of action in response to exposure to toxicants and in the long-term development of diseases caused or influenced by these exposures. Use of toxicoproteomic technologies to identify key biochemical pathways, mechanisms, and biomarkers of exposure and toxicity will definitely provide us a better approach for human health risk assessment (Ge et al., 2007).

Currently, nanomaterials are mainly involved in two research fields: biological /therapeutic applications and the impact on human health and the environment. Therefore, biocompatibility and toxicity are the critical factors that will not only determine the utility of nanoparticles in their clinical applications but in other areas. Herein, we suggest a new approach to understand the cellular function in response to

Figure 6 Workflow of iTRAQ-coupled LC-MS/MS proteomics for risk assessment.

the nanomaterials and study the toxic mechanism using iTRAQ-coupled LC-MS/MS proteomics approach (Fig. 6) as the results showed in our previous study are very promising. The data obtained from iTRAQ-coupled LC-MS/MS proteomics approach are informative for safety evaluation of these nanomaterials. In the long run, database may be constructed with toxicoproteomics data gained from various nanoparticles, which will definitely provide us valuable platform for the future design of biocompatible nanomaterials.

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APPENDIX

Appendix I: Supplementary data

Table S1 Peptide summary the differentially expressed proteins obtained from the batch of cells treated with graphene and SWCNTs

Accession	Score	Intensity SPI (%)	Sequence
Peroxiredoxin-1			
Q06830	9.6	73	IGHPAPNFK
Q06830	17.6	94	ATAVMPDGQFK
Q06830	15.6	94	DISLSYK
Q06830	17.1	93	TIAQDYGVLK
Q06830	12.9	84	ADEGISFR
Q06830	18.3	100	QITVNDLPVGR
Q06830	13.9	93	QAFQFTDK
Q06830	9.9	83	EYFSK
Peroxiredoxin-5			
P30044	17.8	100	VGDAIPAVEVFEGEPGNK
P30044	16.9	96	VNLAELFK
P30044	12.9	89	GVLFVPGAFTPGCSK
P30044	13.7	96	THLPGFVEQAEALK
P30044	11.3	76	LLADPTGAFGK
P30044	9.6	70	ETDLLLDDSLVSIFGNR
Peroxiredoxin-6			
P30041	12.3	80	DFTPVCTTELGR
P30041	11.6	81	LAPEFAK
P30041	11.5	80	DINAYNCEEPTEK
P30041	9.1	72	ELAILLGMLDPAEK
P30041	15.2	93	VVFVFGPDK
P30041	12.1	88	LSILYPATTGR
P30041	11.3	81	VATPVDWK
P30041	9.7	78	GVFTK
P30041	9.4	72	ELPSGK
Thioredoxin reductase 1			
Q16881	7.3	65	QFVPIK
Q16881	9.5	66	VEQIEAGTPGR
Q16881	9.8	76	IGLETVGVK
Q16881	8.3	64	LYAGSTVK

Q16881	18.5	88	CDYENVPTTVFTPLEYGACGLSEEK
Thioredoxin			
P10599	20.3	97.3	TAFQEALDAAGDK
P10599	15.5	93	VGEFSGANK
P10599	7.9	85	LEATINELV
Glutathione S-transferase Mu			
P21266	12.9	74	YTCGEAPDYDR
P21266	12.9	84	SQWLDVK
P21266	14.7	89	FKLDLDFPNLPYLLDGK
P21266	13.5	94	LKPQYLEELPGQLK
P21266	14.1	84	CLDEFPNLK
P21266	18.1	84	IAAYLQSDQFCK
Superoxide dismutase (Stankovich et al.)			
P04179	13.6	92	GDVTAQIALQPALK
P04179	10.1	90	GELLEAIK
Keratin, type I cytoskeletal 18			
P05783	17.6	97	AQIFANTVDNAR
P05783	15.5	93	IVLQIDNAR
P05783	10.8	74	LAADDFR
P05783	12.7	84	QSVENDIHGLR
P05783	12.1	80	VIDDTNITR
P05783	20.1	96	LQLETEIEALKEELLFMK
P05783	14.8	91	AQYDELAR
P05783	16.4	81	TVQSLEIDLDSMR
P05783	20.8	100	QAQEYEALLNIK
P05783	18.2	97	VVSETNDTK
Profilin-1			
P07737	14.7	88	DSPSVWAAVPGK
P07737	16.3	96	TFVNITPAEVGVLVGK
P07737	12.7	92	STGGAPTFNVTVTK
P07737	8.3	80	EGVHGGLINK
Moesin			
P26038	8.7	81	KENPLQFK
P26038	7.5	65	NISFNDK
P26038	10.3	72	FVIKPIDK
P26038	13.9	85	APDFVIFYAPR
P26038	7.7	61	LQDYEEK
P26038	12	83	IALLEEAR

P26038	13.2	90	QLLTLSSSELSQAR
Protein S100-A11			
P31949	13.5	92	ISSPTETER
P31949	11.2	68	DGYNYTLSK
Protein S100-A6			
P06703	9.8	81	ELIQK
P06703	12.8	90	LQDAEIR
Protein S100-P			
P25815	13.7	79	YSGSEGSTQTLTK
P25815	16.3	87	ELPGFLQSGK
Annexin A1			
P04083	19.9	92	GGPGSAVSPYPTFNPSSDVAALHK
P04083	13.4	73	GVDEATHIDILTK
P04083	15.9	86	AAYLQETGKPLDETLK
P04083	14.7	87	TPAQFDADELRL
P04083	13.2	70	DITSDTSGDFR
P04083	13.4	81	SEDFGVNEDLADSDAR
P04083	11.3	79	ALYEAGER
Annexin A5			
P08758	15.4	88	GTVTDFPGFDER
P08758	8	69	ADAETLR
P08758	16.2	74	GLGTDEESILTLLTSR
P08758	12.8	87	QEISAAPK
P08758	10.5	75	DLLDDLK
P08758	17.2	96	LYDAYELK
P08758	14.6	91	VLTEIIASR
P08758	9.2	78	TPEELR
P08758	10.4	70	MLVVLLQANR
P08758	12.4	77	DPDAGIDEAQVEQDAQALFQAGELK
P08758	9.7	61	FITIFGTR
P08758	10.2	72	SIPAYLAETLYYAMK
Calmodulin			
P62158	10.6	77	EAFSLFDK
P62158	15.8	85	DGDGTITTK
Protein disulfide-isomerase			
P07237	13.4	87	SNFAEALAAHK
P07237	13.3	87	ALAPEYAK

P07237	22.1	100	VDATEESDLAQQYGVR
P07237	19.3	90	QFLQAAEAIDDIPFGITSNSDVFSK
P07237	14.4	94	ENLLDFIK
P07237	11.5	72	ILFIFIDSDHTDNQR
P07237	12.6	84	NFEDVAFDEK
P07237	10.4	68	VHSFPTLK

GTP-binding nuclear protein Ran

P62826	18.8	97	LVLVGDGGTGK
P62826	19.5	99	FNVWDTAGQEK
P62826	13	93	VCENIPIVLCGNK
P62826	17.8	93	NLQYYDISAK

Stress-induced-phosphoprotein 1

P31948	15.2	79	ALSVGNIDDALQCYSEAIK
P31948	12.4	78	AAALEFLNR
P31948	10.4	73	IGNSYFK
P31948	21.1	100	LAYINPDLALEEK
P31948	13.8	84	DPQALSEHLK

Heterogeneous nuclear ribonucleoproteins A2/B

P22626	12.9	81	TLETVPPLER
P22626	14.9	89	NYYEQWGK
P22626	13.9	78	EESGKPGAHVTVK
P22626	12.6	89	LFVGGIK
P22626	15.4	86	DYFEEYGK
P22626	15.9	95	IDTIEITDR
P22626	13.7	92	GFGFVTFDDHDPVDK
P22626	8.9	74	IVLQK
P22626	10	63	QEMQEVQSSR
P22626	17	93	GGGGNFGPGPSNFR

Ras-related protein Rab-1A

P62820	16.3	92	LLLIGDSGVGK
P62820	10.7	82	YASENVNK
P62820	16.6	96	EFADSLGIPFLETSK

Eukaryotic translation initiation factor 2

P05198	11.4	76	INLIAPPR
P05198	13	80	VVTDTDETELAR

Ubiquitin

P62988	10.5	81	MQIFVK
P62988	20.2	97	TITLEVEPSDTIENVK

P62988	11.9	87	EGIPPDQQR
P62988	8.6	67	LIFAGK
P62988	17.1	92	TLSDYNIQK
P62988	14	90	ESTLHLVLR
78 kDa glucose-regulated protein			
P11021	15.2	91	VEIANDQGGR
P11021	13.9	88	ITPSYVAFTPEGER
P11021	16.4	96	TKPYIQVDIGGGQTK
P11021	15.2	86	ETAEAYLGK
P11021	19.3	98	VTHAVVTVPAYFNDAQR
P11021	14.9	80	IINEPTAAAIAYGLDK
P11021	12.4	84	VLEDSDLK
P11021	12.9	94	SDIDEIVLVGGSTR
P11021	11.2	85	IQQLVK
P11021	12.5	90	SQIFSTASDNQPTVTIK
P11021	14.7	77	VYEGERPLTK
P11021	12.2	75	DNHLLGTFDLTGIPPAPR
P11021	17	94	NELESYAYSLK
P11021	15.8	100	ELEEIVQPIISK
P11021	12.8	80	LYGSAGPPPTGEEDTAEK
Proliferation-associated protein 2G4			
Q9UQ80	10	76	SDQDYILK
Q9UQ80	10.2	78	HELLQPFNVLYEK
Q9UQ80	14	88	EGEFVAQFK
Q9UQ80	19	98	ITSGPFEPDLYK
Q9UQ80	10.7	76	ALLQSSASR
40S ribosomal protein S19			
P39019	15	93	ALAAFLK
P39019	13.2	78	VPEWVDTVK
P39019	10.4	72	IAGQVAAANK
60S ribosomal protein L8			
P62917	9	69	AVDFAER
P62917	14	92	ASGNYATVISHNPETK
P62917	18.3	96	AVVGVVAGGGR
P62917	10.2	73	IDKPILK
Stress-70 protein			
P38646	13	90	VLENAEGAR
P38646	12.7	80	ETAENYLGHTAK
P38646	15.8	92	NAVITVPAYFNDSQR

P38646	15.5	87	DAGQISGLNVLR
P38646	15.1	90	AQFEGIVTDLIR
P38646	13.3	84	VQQTVDLFR
P38646	11.3	83	NTTIPTK
P38646	9.2	74	LLGQFTLIGIPPAPR
P38646	15.6	89	EQQIVIQSSGGLSK
P38646	10.6	76	LFEMAYK
60 kDa heat shock protein			
P10809	15.2	86	ALMLQGVDLLADAVAVTMGPK
P10809	18.8	95	TVIEQSWGSPK
P10809	8.4	70	DGVTVAK
P10809	21.8	97	LVQDVANNTNEEAGDGTTTATVLAR
P10809	13.4	93	GANPVEIR
P10809	10.3	78	GVITVK
P10809	10.1	87	TLNDELEIEGMK
P10809	14.7	95	GYISPYFINTSK
P10809	11.9	77	ISSIQSIVPALEIANAHR
P10809	17.4	98	VGLQVVAVK
P10809	15.7	95	VGEVIVTK
P10809	16.8	85	IQEIEQLDVTSEYEK
P10809	14.3	94	LSDGVAVLK
P10809	12	69	VGGTSDVEVNEK
P10809	13.4	92	VTDALNATR
P10809	17.4	93	CIPALDSLTPANEDQK
P10809	15.4	98	IGIEIK
P10809	14.7	80	NAGVEGSLIVEK
Histone H2B type 1-A			
Q96A08	19.7	96	QVHPDTGISSK
Q96A08	14.7	98	EIQTAVR
Q96A08	17.7	97	LLPGELAK
Q96A08	6.3	65	AVTK
Histone H2A type 1-A			
Q96QV6	13.4	92	AGLQFPVGR
Q96QV6	14.5	86	NDEELNK
Q96QV6	16.3	82	LLGGVTIAQGGVLPNIQAVLLPK
Fructose-bisphosphate aldolase A			
P04075	8.8	72	ELSDIAHR
P04075	11.8	83	IVAPGK
P04075	21.1	100	GILAADESTGSIK
P04075	12.8	82	LQSIGTENTENR

P04075	12	72	ADDGRPPQVIK
P04075	13.6	82	IGEHTPSALAIMENANVLAR
P04075	13.3	87	CQYVTEK
P04075	10.5	6.9	AAQEEYVK
P04075	17.8	92	ALANSLACQGK
Triosephosphate isomerase			
P60174	19	97	QSLGELIGTLNAAK
P60174	15.9	92	VPADTEVVCAPPTAYIDFAR
P60174	18.7	96	IAVAAQNCYK
P60174	11.1	85	HVFGESDELIGQK
P60174	12.8	86	VVFEQTK
P60174	10.5	68	VIADNVK
P60174	18.2	95	VVLAYEPVWAIGTGK
P60174	16	90	TATPQQAQEVHEK
P60174	18	98	SNVSDAVAQSTR
P60174	22.1	100	IYGGSVTGATCK
P60174	19.1	95	ELASQPDVDGFLVGGASLKPEFVDIINAK
Glyceraldehyde-3-phosphate dehydrogenase			
P04406	12.8	78	AAFNSGK
P04406	14.7	81	VIISAPSADAPMFVMGVNHEK
P04406	11.9	79	YDNSLK
P04406	19.8	91	VIHDNFGIVEGLMTTVHAITATQK
P04406	12.3	73	TVDGPSGK
P04406	21.1	100	GALQNIIPASTGAAK
P04406	10	80	LTGMAFR
P04406	19.8	99	VPTANVSVVDLTCR
Phosphoglycerate kinase 1			
P00558	20.5	98	YSLEPVAVELK
P00558	16.4	94	DCVGPEVEK
P00558	12.6	80	ACANPAAGSVILLENLR
P00558	16.9	89	FHVEEEGK
P00558	17.8	96	LGDVYVNDAFGTAHR
P00558	14.8	94	ELNYFAK
P00558	12.1	80	VLNNMEIGTSLFDEEGAK
P00558	16.4	92	ITLPVDFVTADK
Glucose-6-phosphate isomerase			
P06744	14.5	88	AVLHVALR
P06744	16	95	TLAQLNPESLFIASK
P06744	18.2	80	TFTTQETITNAETAK
P06744	12.6	78	EWFLQAAK

P06744	8.8	72	ILLANFLAQTEALMR
P06744	15.8	87	VFEGNRPTNSIVFTK

Neutral alpha-glucosidase AB

Q14697	8.3	62	IDELEPR
Q14697	13.1	84	SLLSVNAR
Q14697	13.2	83	AEKDEPGAWEEETFK
Q14697	18.3	87	YSLLPFWYTLLYQAHR