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Development of Non-Covalent Functionalization of Carbon Nanotubes for siRNA Delivery

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Graduate Program in Pathology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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by

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A thesis submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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Abstract

RNA interference (RNAi) therapy is promising for treating various diseases but the delivery of small interfering RNA (siRNA) is difficult. To overcome the technical difficulties of siRNA delivery, an efficient and targeted delivery of siRNA is required for efficient RNAi therapy. Single-walled carbon nanotubes (CNT) has been used for nucleic acid delivery such as siRNA delivery. It has been found that CNT can gain entry into the cells by a diffusion-like mechanism which was called "nano-needle". However, the solubility of CNT is low in most of the solvents including water. Functionalization of CNT can be carried out to enhance the solubility of the CNT in water and non-covalent functionalization of CNT is easy to be carried out. Poly(ethylenimine) (PEI) is a cationic polymer and it has been shown to disperse CNT in water. Also, it can deliver nucleic acids including siRNA. However, it is not very efficient at delivering siRNA unless adequately modified.

Three different modifications on PEI were carried out. These polymers and the dispersed CNT were characterized and siRNA delivery capacities of the CNTs were examined *in vitro* and *in vivo*. The first was lipid-poly(ethylene glycol) (PEG) conjugated PEI. The second polymer was succinic acid conjugated PEI and the dispersed CNT was used topically in a melanoma model delivering Braf siRNA. The tumor progression was reduced dramatically. Following this work, folic acid, a ligand which target cancer cells was conjugated to the PEG and succinic acid modified PEI. The dispersed CNT was used for systematic delivery of mTOR siRNA in a melanoma model. The tumor progression was inhibited significantly.

Keywords

Co-Authorship Statement

Most of the work in this thesis was carried out by the author. Dr. Wei-ping Min is the supervisor of the author. Dr. Xiufen Zhang gave suggestions to the thesis and experiments. Dr. Elizabeth Gillies gave suggestions to the thesis and edited the manuscripts. Coworkers Dr. Di Chen and Mr. Xusheng Zheng; Ms. Winnie Liu departmental of Pathology and Ms. Aneta Borecki in Dr. Gillies' lab contributed in experiments. The detail is as follow:

Chapter 1 was written by the author.

Chapter 2 described a project proposed by the author and most of the experimental work was carried out by the author under the supervision and guidance of Dr. Min. The *in vivo* injection and qRT-PCR on mouse liver was done by Mr. Xusheng Zhang. Ms. Aneta Borecki did the SEC experiment. Dr. Min, Dr. Xiufen Zhang and Dr. Elizabeth Gillies edited the manuscript.

The work in chapter 3 was proposed by the author and most of the experimental work was carried out by the author under the supervision and guidance of Dr. Min. Crycosection of topical siRNA delivery of skin was carried out by Dr. Di Chen and H&E staining of the tumor was done by Ms. Winnie Liu. Dr. Min, Dr. Xiufen Zhang and Dr. Elizabeth Gillies edited the manuscript.

Chapter 4 described a project that was proposed by the author and most of the experimental work was carried out by the author under the supervision and guidance of Dr. Min. *In vivo* injections and the western blot were carried out by Mr. Xusheng Zhang, H&E staining was done by Ms. Winnie Liu and Ms. Aneta Borecki did the SEC experiment.

The study here in Canada is never easy because it is called PhD. It is something I like to do (probably not again tho) as to establishing as a person, who is able to think, to ask and to answer questions. It is an exciting journey in my life and I wish to enjoy it until the very end. The best thing I have learnt is not only English but also the way how the others think questions from different background and perspective.

Finally, I can proudly say "I think, therefore I am". Time stamp is 4:45 am……that's the life of PhD?

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List of Abbreviations

7AAD	7-Aminoactinomycin D
A	Absorbance
ADA	Adenosine deaminase
AFM	Atomic force microscope
AGO2	Argonaute
ALS	Amyotrophic lateral sclerosis
AMD	age-related macular degeneration
B16-F10	Mouse melanoma cell line
b-PEI	Branched polyethylenimine
Braf	proto-oncogene B-Raf
CF	Cystic fibrosis
CME	Clarthrin-mediated endocytosis
CNT	Carbon nanotube
CPP	Cell penetrating peptide
CTB	Cholera toxin beta subunit
CvME	Caveolae-mediated endocytosis
Da	Dalton
DEPTOR	DEP domain containing mTOR-interacting protein
DGI	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol)-poly(ethylenimine)
DGI/C	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol)-poly(ethylenimine) /carbon nanotubes
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOPE	1,2-Dioleoylsn- glycero-3-phosphatidylethanolamine
DSPE-PEG-COOH	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-carboxy-poly(ethylene glycol)-2000 ammoniium salt
dsRNA	Double-strain ribonucleic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EEA-1	Early endosome antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinases
EtBr	Ethidium-bromide
FBS	Fetal Bovine Serum

FGIS	Folated succinated poly(ethylenimine)-poly(ethylene glycol)
FGIS/C	Folated succinated poly(ethylenimine)-poly(ethylene glycol)/Carbon nanotubes
FKBP12	FK506-binding protein
FRB	FKBP12-rapamycin binding domain
FT-IR	Fourier transform infrared
GA	Glutaric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
SEC	Size exclusion chromatography
GIS	Succinated poly(ethylenimine)-poly(ethylene glycol)
GIS/C	Succinated poly(ethylenimine)-poly(ethylene glycol)/Carbon nanotubes
GPI	Glycosphingolipids
H&E	Hematoxylin and eosin
HIV	Human immunodeficiency virus
IS/C	Succinated poly(ethylenimine)/Carbon nanotube
KB	Human Papilloma carcinoma cell line, HeLa contaminant
LDL-C	Low density lipoprotein cholesterol
l-PEI	Linear polyethylenimine
MAPK	Mitogen-activated protein kinase
MbCD	Methyl-β-cyclodextrin
MEK	Mitogen-activated protein kinase kinase
MEM	Minimum essential medium
MES	2-(N-morpholino)ethanesulfonic acid.
miRNA	Micro ribonucleic acid
M_n	Number averaged molecular weight
mRNA	messager ribonucleic acid
mTOR	Mammalian target of Rapamicin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
M_w	Weight averaged molecular weight
MWCNTs	Multiple-walled carbon nanotubes
MWCO	Molecular weight cut off
NHS	N-hydroxysuccinimide
NLS	Nuclear localization signals
NMR	Nuclear magnetic resonance
OCT	Optimal Cutting Temperature compound
P-bodies	Processing bodies
PBS	Phosphate buffered saline

PDGFR	Platelet-derived growth factor receptor
pDNA	Plasmid deoxyribonucleic acid
PEG	Poly(ethylene glycol)
PEI	Poly(ethylenimine)
PEI-SA	Succinated poly(ethylenimine)
PIKK	Phosphatidylinositol 3-kinase-related kinase
PIWI	P-element induced wimpy testis
PLL	Poly(L-lysine)
PTD	Protein transduction domain
PTEN	Phosphatase and tensin homolog
PTGS	Post transcriptional gene silencing
qRT-PCR	Quantitative reverse transcription polymer chain reaction
Raf	Rapidly Accelerated Fibrosarcoma
RES	Reticuloendotheial system
RGD	Arginine-Glycine-Aspartic acid
RID	Reflective index detector
RISC	RNA-induced silencing complex
RLC	RISC loading complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
rRNA	Ribosomal ribonucleic acid
SA	Succinic acid
SC	Stratum corneum
SD	Standard deviation
SEM	Standard error of mean
shRNA	Small hairpin ribonucleic acid
siBraif	Braf siRNA
si-mTOR	mTOR siRNA
siRNA	Small interfering ribonucleic acid
SWCNT	Single walled carbon nanotube
T cell	Thymus cell
TEM	Transmission Electron Microscope
TFR	Transferrin receptor
Tris HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UV	Ultra violet
Vis	Visible

Chapter 1

1 Introduction to RNA interference and Carbon Nanotubes-Based Nucleic Acids Delivery

1.1 Background of RNA Interference

1.1.1 Brief Introduction to Gene Therapy

Gene therapy is the use of nucleic acids to generate therapeutic effect by correcting the existing abnormality or providing the cells with new functions. The idea of utilizing genetic materials for therapy is not new, and was proposed by Tatum in 1966 [1]. Virus-based delivery vectors for transferring disease-related gene into mammalian cells were carried out in the 1970s to 1980s [2]. The first somatic gene therapy protocol in humans was approved and carried out in 1990. Retrovirus modified tumor-infiltrating lymphocytes were used for melanoma immunotherapy [3] and retrovirus-mediated transfer of adenosine deaminase (ADA) gene to the T-lymphocyte was employed for the treatment of ADA deficiency children [4-9]. Over the past few decades our knowledge of molecular mechanisms of gene function has increased substantially. The discovery of RNAi in the late 90s has caught the attention of scientists.

1.1.2 Introduction to RNA Interference

In 1990, Napoli tried to genetically engineer a kind of flower, petunias to produce more pigment by introducing some extra copies of the gene which encode chalcone synthase, the pigment producing protein. However, the result showed that the extra copies of the gene blocked the production of chalcone synthase instead and it was named co-suppression [10]. A similar phenomenon was reported by Krol in the same year [11]. In 1993, micro RNA (miRNA), a non-coding gene for regulating gene expression was discovered [12, 13]. In 1998, double-stranded RNA (dsRNA) mediated specific RNA interference (RNAi) which was demonstrated by Fire and Mello in Caenorhabditis elegans [14]. Later, it was found that these processes shared most of the core components in gene expression regulation [15]. In 1999, small antisense RNA was described to be the component which induces the RNAi [16]. Later, this small RNA was found to trigger

RNAi and was called small interfering RNA (siRNA) [17, 18]. In 2001, RNAi was observed in cultured mammalian cells [19]. In 2004, the first RNAi-based clinical trial had been started. It was aimed at treating age-related macular degeneration (AMD) by intravitreal injection of siRNA-027 by reducing the growth of pathological blood vessels [20]. In the last decade, the mechanisms of RNAi have become clearer and scientists have explored the possibility of utilizing it as a tool for research and for therapy.

1.1.3 Principle and Application of RNAi

RNAi is also called post transcriptional gene silencing (PTGS), which is a type of anti-sense mechanism [21]. It is well-conserved and can be found in plants, viruses, invertebrates and protozoa [21, 22]. Two major classes of small RNA which induce RNAi are present, siRNA and miRNA. siRNA is 21-23 nucleotides long and its sequences are perfectly complementary to the target mRNA [17, 18] while mature miRNA have 21-23 nucleotides and its sequences are only partially complementary to the target mRNA [12, 23]. siRNA is endogenous and it usually leads to mRNA degradation while miRNA usually leads to translational inhibition [24] and sometimes mRNA degradation [25]. In plants, one of the functions of RNAi is for innate immune defense against viral infection which produces siRNA to inhibit the translation of viral RNA [26]. siRNA has also been found in mammals and one of their known functions is to suppress retrotransposition [27, 28]. siRNA is the most important component to induce RNAi [16-18, 29] and it can be synthesized and introduced into cells directly. RNAi can also be induced by the introduction of dsRNA, which is cut into siRNA by Dicer [30]. Another approach is small hairpin RNA (shRNA) which was designed to produce siRNA. It is an insert into plasmid construct such that a small fragment of RNA with a stem-loop or hairpin loop is produced. Then the hairpin loop is cut by Drosha into the designed siRNA [31, 32]. A brief mechanism of RNAi is as follows. After the dsRNA or shRNA is cut into siRNA by the Drosha (Step 1), or the siRNA gained into the cells, the siRNA duplex and DCR2–R2D2 heterodimer forms RNA-induced silencing complex (RISC) loading complex or RLC [33] (Step 2). Then the complexes load the RNA duplex into the argonaute protein (AGO2 for mammals) which turn into inactive RISC [34] (Step 3). The guide strand also named as the antisense strand, which has a complementary

sequence to the target RNA, stays in the RLC and the other strand which is called passenger strand or sense strand is degraded, this forms an active RISC [35, 36] (Step 4). The siRNA guide strand recognizes the mRNA by a sequence specific manner using Watson-Crick base pairing (Step 5) and the PIWI domain of the RISC initiates the mRNA cleavage between 10th and 11th nucleotides from 5'-end counted from the guide strand [37] (Step 6). The cleaved mRNA is then released and the RISC is recycled to cleave another copy of mRNA [25] (Step 7). (Fig. 1.1)
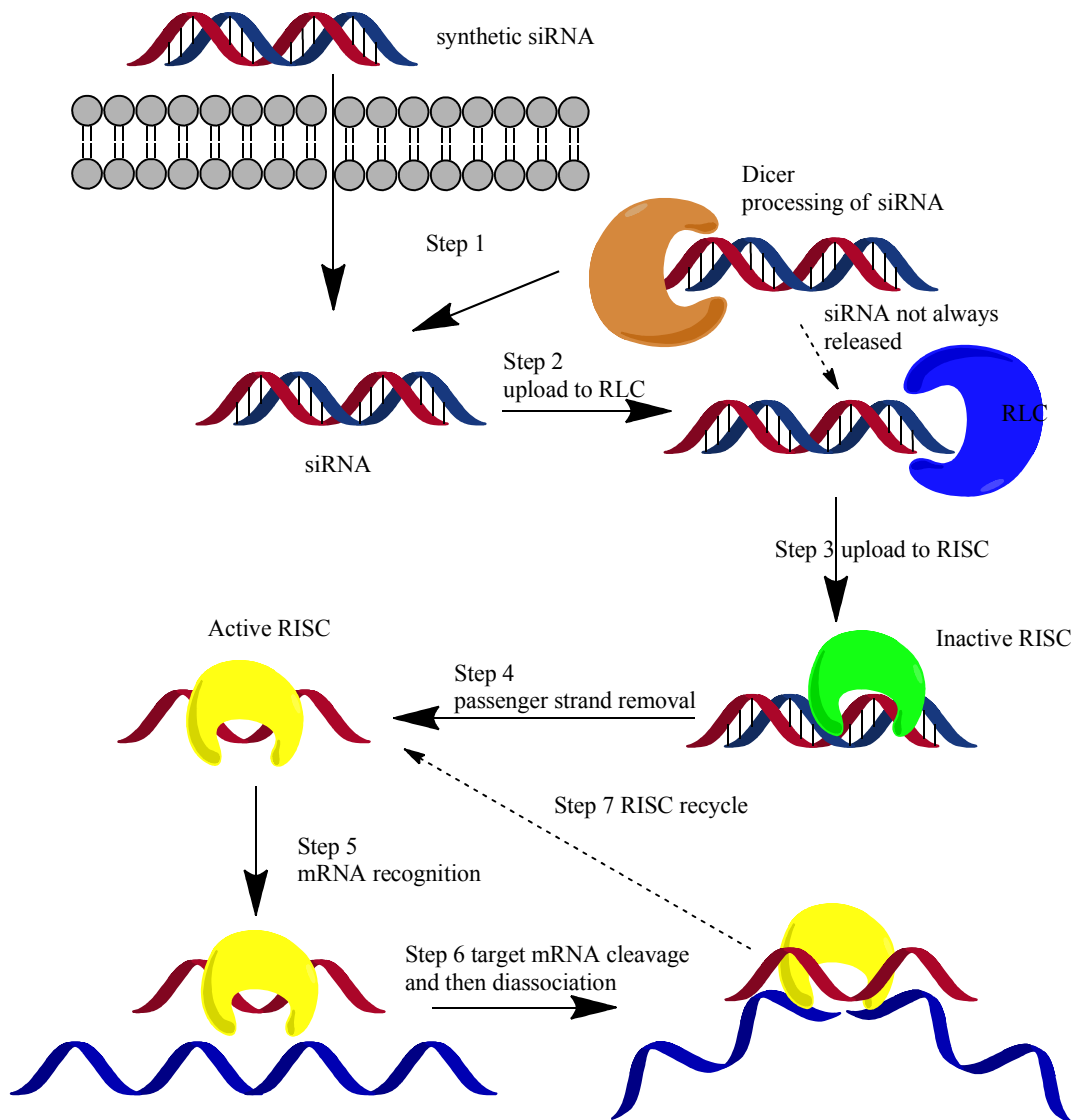
Figure 1.1 Generlized mechanism of RNAi

The structures for Dicer, RLC, inactive RISC and active RISC are simplified

Nowadays, numerous research and clinical trials on RNAi therapy have been actively carried out [38, 39]. On October 2013, there are almost 14500 results for the search of "RNAi" in PubMed. Unlike conventional gene delivery or therapy, which is gain-of-function, RNAi is loss-of-function. It provides a new approach in research as well as in treating diseases. As the innate antiviral mechanism in plant, researchers used it as a tool for understanding and treating virus induced diseased such as HIV, influenza, hepatitis, transplantation and autoimmune diseases [40-46]. Also, it can be used for treating diseases which are related to overexpression of a single protein [47] such as Alzheimer's disease [48], amyotrophic lateral sclerosis (ALS) [49] and cancer [47, 50]. Various clinical trials have been carried out aiming at treating various disease like HIV, cancer, hypercholesterolemia and asthma [39]. One of the notable clinical trials on RNAi therapy is using siRNA "ALN-PCS" which inhibits PCSK9 synthesis and in turn reduces the blood concentration of low density lipoprotein cholesterol (LDL-C). Phase I clinical trial is just finished and it reduced the LDL-C with no major toxicity observed [51].

1.1.4 Advantages and Disadvantages of RNAi

There are a lot of advantages of using RNAi for research as well as for therapy. Firstly, RNAi can be highly target specific. Cleavage may not result for some mismatches by a single nucleotide between siRNA and the RNA target [52, 53]. It is a promising tool for studying the interaction between proteins as well as treating disease induced by gain of function mutations such as cancer [54, 55]. Secondly, small molecules or biologics are limited by "undruggable" targets which are possible targets for RNAi [47, 56]. Some of the interaction of proteins cannot be inhibited by small molecules or biologics but the protein can be removed by RNAi and thus, the interaction of proteins can be studied. The screening of small molecules to inhibit a known pathological protein is expensive and inefficient [57] while RNAi can provide as a powerful alternative to treat diseases if the target is known. Thirdly, RNAi can be introduced transiently by siRNA or permanently by shRNA respectively for acute or chronic pathological condition [58]. In theory, a single treatment could cure chronic disease and the cost of treatment can be reduced drastically while the technology is still transferrable for curing acute disease like

conventional drug. A short list of the difference between RNAi and traditional small molecular drug was listed as below (Table. 1.1).

	RNAi	Small Molecule
Molecular Weight	~15k Da for siRNA, over a million Da for shRNA	Range from several hundred Da to a thousand Da
Solubility in Water	Highly soluble	Varies
Stability in circulation	Susceptible to be degraded by nuclease	Usually stable
Target specificity	Higher	Lower
Easy to manufacture	Easy	Can be complicated
Delivery	Difficult	Easy

Table 1.1 Comparision of RNAi and conventional small molecular drug

Every coin has two sides; there are also limitations of RNAi. Firstly, there is an intrinsic difference between the response of human or mammals and plants or other organisms to dsRNA. There is interferon response, which is a cytokine for fighting against viral infection in mammals [59]. It would respond to dsRNA which is longer than 30 nucleotides [60] but even for siRNA which has less than 30 nucleotides, it might also induce interferon response [61]. Also, activation of toll-like receptors has been reported [62]. It is becoming clearer that sequences like 5'-r(GUCCUUCAA) or 5'-r(UGUGU) could induce interferon [63] which can be avoided by dsRNA design. Secondly, there is a possibility for RNAi to be off target, that is, it might inhibit non-targeted genes. As mentioned earlier for miRNA in mammals, perfect complementary is usually not observed. Translational suppression is possible when there is partial complementation of

the seed region of the guide strand, which are the 2nd to 8th nucleotides counted from 5'-end [64] to non-targeted mRNA. It is obvious that the foreign siRNA we introduced might trigger non-targeted miRNA translational inhibition instead of going through the designed RNAi pathway. Therefore, careful design and testing on the sequences are necessary. Thirdly, the way how RNAi, siRNA or shRNA were introduced may saturate the cellular RNAi machine. siRNA completes with miRNA for RISC, which the RISC maybe be saturated and the miRNA cannot function for normal gene regulation. Lethality has not been observed for low dosage of shRNA, however, death of mice was observed by prolonged exposure to high doses of shRNA [65]. The effect of long term saturation of the RNAi machinery is largely unknown and transient siRNA or triggered shRNA might be a better option for therapy before the long term effect was uncovered and fully understood. Lastly, the delivery of siRNA or shRNA is either potentially harmful or inefficient, which will be covered in next section.

1.2   Challenges in Nucleic Acid Delivery

1.2.1 Difficulties of Nucleic Acids Delivery

The stumbling block of gene therapy as well as RNAi application is the delivery of intact nucleic acids into the right place effectively and safely. The delivery of nucleic acids into mammalian cells, a process called transfection is still difficult. It is obvious that the human body and its defense mechanism have evolved for millions of years, the entry of potentially harmful foreign substances as well as foreign genetic materials which might harm our cells or genome is actively or passively impeded by cellular membrane. Traditional therapeutic small molecules are usually made up of different structure and they bind to the protein or pathological components which lead to therapeutic effects. Also, small molecules can gain entry into the cells by diffusion because of their low molecular weight (usually lower than 1000 Da) and lipophilicity. Most of them cannot be degraded easily but by the enzyme in a specific site. In contrast, nucleic acids such as plasmid DNA, messenger RNA, oligo RNA and siRNA are made up of nucleotides. They have similar structure and the genetic codes which have to be processed by the cellular machinery in order to generate the therapeutic effect. They cannot gain into the cells by diffusion easily because of their high molecular weight. The higher molecular weight the

nucleic acids are, the more difficult for them to diffuse into the cells. So, lower molecular weight siRNA which has 21 to 23 nucleotides is easier to be delivered compared to shRNA which is inserted into a plasmid construct which has thousands of nucleotides. In addition, they can be degraded easily when exposed to intracellular and extracellular nuclease [66-72]. One of the biggest barriers for utilizing nucleic acids as drug is to bring them across the cell membrane.

The calculated length of dsDNA with 20 base pair is 7.5 nm [73] and siRNA should be similar to dsDNA. However, nucleic acids are polyanion and the cellular surface is also negatively charged. Spontaneous entry of siRNA into cells is unlikely due to electrostatic repulsion. The same is true for pDNA or shRNA but they are larger which further hinders their delivery. The desired function cannot be carried out if the nucleic acids are excluded from the cytosol [68, 72, 74]. Its function can only be brought out by reaching the right location: cytosol for RNA and nuclear envelope for DNA.

Although the nucleic acids do not gain entry easily into the cell and carry out our desired therapeutic function, scientists have endeavored various way to deliver nucleic acids into the cell [1, 68, 74-76].

1.2.2 Cellular Uptake Mechanism and Cellular Trafficking for Non-Viral Nucleic Acid Delivery

The cellular uptake mechanism is the most important barrier scientists have to understand and overcome for efficient non-viral nucleic acid delivery. The cell is a well-guided biochemical plant which highly regulates the entry of foreign substances. There are several different routes for the cells to take up a substance and some of the important routes are phagocytosis, Clathrin-mediated endocytosis (CME), Caveolae-mediated endocytosis (CvME) and marcopinocytosis (Fig. 1.2). Some of the well-studied endocytosis routes related to non-viral DNA or RNA delivery are summarized as follow.
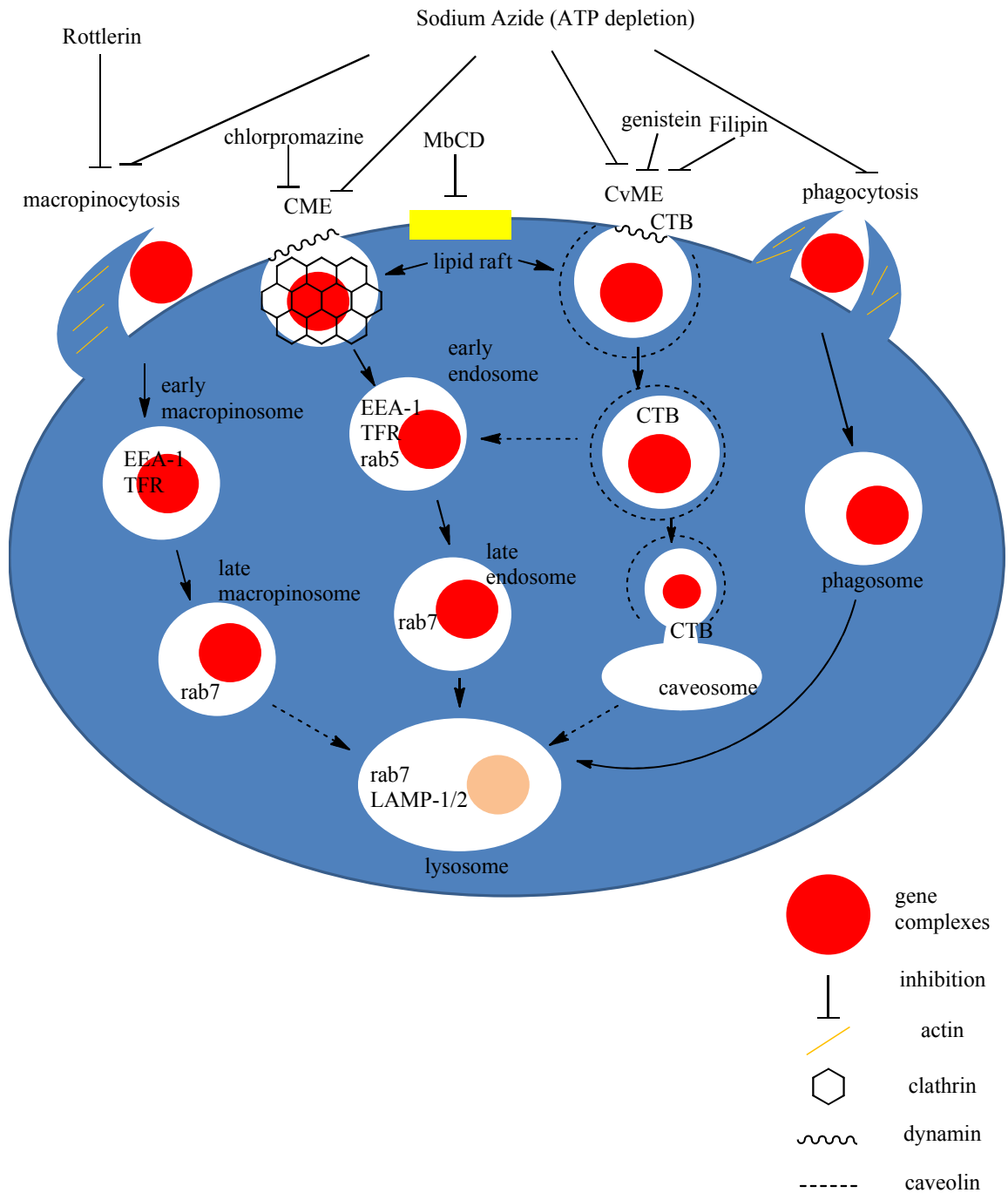
Figure 1.2 Cellular uptake mechanism

The CME is Clarthrin-mediated endocytosis; CvME is Caveolae-mediated endocytosis. Cholera toxin beta subunit (CTB) and transferrin receptor (TFR) are classical markers for CvME and CME respectively. There are also different markers like early endosome antigen 1 (EEA-1) and Rab5 for early endosome, Rab7 is the maker for late endosome.

There are different inhibitors for studying the cellular uptake pathway like Rottlerlin for marcopinocytosis; chlorpromazine for CME; methyl-β-cyclodextrin (MbCD), genistein, Filipin for CvME.

(Created with reference to the review of Xiang et al. [77])

Phagocytosis is exhibited by some specialized cells such as macrophages, dendritic cells, mast cells, monocytes and neutrophils [78]. It is usually for bacteria after opsonization [79]. Sizes up to 10 μm have been reported which can be taken up by phagocytosis [80] while 3 μm was reported as the most efficient [81]. It can be exploited as a targeted delivery method to cells exhibiting phagocytosis like antigen presenting cells [82] and macrophages [80].

Clathrin-mediated endocytosis is the most well-characterized pathway and a lot of nanoparticles were designed for cellular uptake by this pathway [83]. It is one of the most important routes for the internalization of nutrients [84]. This route is exploited by a lot of viruses for cellular entry [85]. The maximum size of Clathrin-mediated endocytosis reported was around 200 nm [86].

Caveolae-mediated endocytosis is the most studied clathrin-independent endocytosis mechanism. It is one of the lipid rafts, which is a combination of sphingolipids, cholesterol and sometimes glycosphingolipids (GPI) linked protein with the exclusion of unsaturated lipoprotein to form a denser outer membrane leaflet of cell membranes [87]. This route is also exploited by viruses for cellular entry [88]. The size of caveolae-mediated endocytosis observed with TEM was around 70-100 nm [89, 90]. Caveolae-mediated endocytosis has been observed in most cells especially endothelial cells and adipocytes [87].

Marcopinocytosis is a signal dependent process and usually macrophages or cancer cells exhibit it [77] when there is activation of receptor tyrosine kinases like epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor (PDGFR). It has a diameter greater than 0.2 μm and most of the studies on it are related to antigen presentation [91].

After endocytosis, the components are engulfed in phagosome or early endosome which is topologically outside the cell. The nucleic acids still cannot function because of the partition of endosome or phagosome. For early endosome, it will be transferred to later endosome and later lysosome for endosome and phagosome. The content inside is then degraded [92]. The escape from the endosome or late endosome is therefore necessary [93]. The pH reduces to facilitate the action of degrading enzymes from endosome to lysosome [92]. Additionally, caveolae-mediated endocytosis uptake will lead to caveosome which has a neutral pH [94].

There are three major mechanisms for delivery vectors to efficiently escape from the endosome or lysosome: flip-flop, proton sponge and expanded umbrella. The flip-flop mechanism is mostly exhibited by liposome. It was predicted by a computational model [95] and later confirmed by Xu et al [96]. The lipid of liposomes interact with the lipid in cell membrane and because of the pH sensitive lipids, the liposome flip-flop from the lamellar phrase to inverted hexagonal phrase [97] which was also observed under cryo-TEM by changing the temperature of the lipid [98, 99].

Proton sponge effect, the exceptional transfection capacity by polyethylenimine (PEI) was hypothesized by Behr. Amine groups in PEI neutralized the proton in endosomes by its wide buffering range (pH 3-10). Chloride anions gain entry into the endosome because of the charge balance and water gains into the endosome by water potential. The PEI/DNA complexes are then released due to the rupture of the endosome by the accumulated osmotic pressure [100]. This hypothesis was supported by computational model [101, 102] but it is still controversial due to various methods of observing or examining the proton sponge effect [103-106]. This hypothesis was also used for explaining the exceptional transfection capacity of non PEI vectors [107, 108].

Expanded umbrella is the extended version of proton sponge hypothesis. The influx of proton neutralized by the basic polymer again but the originally flexible complex (polymer/DNA complexes) expanded and extended due to the repulsion between the positively charged polymer chains. The endosome burst because of the rigid polymer by increased volume and space [109].

Cell penetrating peptide (CPP) or protein transduction domain (PTD) is another important class of materials which is known to facilitate endosomal escapes or directs cell penetration [108, 110]. Some reported that CPP can mediate both at the same time [111]. The first CPP discovered was Trans-Activator of Transcription (Tat) peptide, which is derived from HIV-1 [112]. Some well-studied CPP are pennetratin, derived from the third helix of Antennapdeia homeodomain [113]; oligoarginine, the many arginine domain developed from Tat [114-116]; GALA, a peptide derived from virus protein which was synthesized for interacting with cellular membranes [117], transportan which is a chimeric peptide of galanin (a peptide from G protein-coupled receptors mainly found in central nervous system) and Mastoparan (peptide of wasp venom) [118, 119], pVEC peptide [120] and tryptophan rich peptide derived from pVEC [121] and more [108, 110, 122]. Some of the proposed mechanisms for the "cell penetrating" properties are inverted micelle formation [123, 124], pore-formation [125-127] and adaptive translocation [128, 129]. Indeed, various mechanisms are proposed and some of them enter the cell via multiple pathways including energy dependent endocytotic pathways [129-131].

After the escape from the endosome, the sites of delivery for different nucleic acids (RNA or DNA) are different. For RNA delivery, introduction of RNA to cytosol or siRNA to processing bodies (P-bodies) are good enough for the RNA to carry out the desired function while for DNA delivery, the DNA has to enter the nucleus for transcription into mRNA or shRNA followed by translation or further processing in cytosol. Two possible pathways could be involved for the nuclear entry of DNA. The first one is the release of DNA from complexes to cytosol and the DNA gains entry into the nucleus. This is similar to injection of naked DNA into cytosol. Alternatively, the complexes can transport near the nucleus by microtubules and then release the DNA or are directly transported into the nucleus. Some complexes can be transported actively to the nucleus [132] which is believed to be more efficient for DNA delivery since DNA molecules longer than 2000 base pairs are immobile in the cytosol [133]. Also, the DNA needs to be protected from nuclease degradation in the cytosol by the complexes before it reaches the nucleus [134]. Furthermore, DNA usually has a size larger than 10 nm which is the size limit for passive diffusion through the nuclear pore complex (NPC) in

interphase [135]. If the cell is undergoing mitosis, DNA can get into the nuclear complex as the nuclear membrane disassembles [136]. However, most of the cells are not undergoing mitosis and efficient nuclear entry is necessary. Nuclear localization signals (NLS) maybe used to facilitate nucleus entry [137, 138]. This can extend the NPC up to a diameter of 26 nm or 8 million Da in different stages in cultured cell [139].

1.2.3 Overview of Nucleic Acids Delivery Methods

There are two main approaches which can be used for nucleic acid delivery, physical methods and non-physical methods. Physical methods are the use of the physical means to deliver nucleic acids into cells such as intra-muscular injection of naked plasmid DNA or siRNA, gene gun, massage, sonication, hydrodynamic injection and electroporation [1, 74, 140-142]. Non-physical methods included the use of viruses [143-149], bacteria [150], chemically modified nucleic acids for siRNA [151], chemicals [152-154], exosome generated by cells [155], fragment antigen-binding [156] to facilitate the delivery of nucleic acids. The reagents for delivering nucleic acids are also called "transfection vector" or "vector". Based on convention, there are two types of vectors, viral and non-viral.

1.2.4 Limitation of Viral Vectors

Viruses are a biological agent that carry nucleic acids and have evolved to equip effective methods to evade host immune response and replicate themselves by utilizing the cellular materials, and often hijacking the cellular machinery. They are the expert in delivering nucleic acids. Various viruses have been used in both research and clinical trials such as retrovirus, lentivirus, adenovirus, adeno-associated virus and herpes simplex virus [157-164]. In general, viral vectors are highly effective and they have been used in clinical trials in treating various diseases such as cystic fibrosis (CF) [2, 8, 9, 165]. However, as many researchers have suggested, it suffers from the following disadvantages [1, 2, 8, 9, 68, 166]. Firstly, it induces immune response and resistance such that second administration will become less effective. Secondly, it might induce insertional mutagenesis such as turning on oncogenes or turning off anti-tumor genes. Leukemia-like illness has been developed due to insertional mutagenesis by retrovirus in clinical trials

[167, 168]. Thirdly, the choice of therapeutic gene is limited because it has limited size for loading genetic materials. Fourthly, it is difficult to produce and purify large amounts of viral vectors with consistent quality compared to chemicals. Moreover, contaminations with a few copies of wide type virus or virus which has the capacity to reproduce itself might lead to serious consequence to patients. Finally, inflammatory response by innate immune system to viral vector could lead to lethality. The first death in clinical trials was due to acute inflammatory response [169]. Although safety concerns might not abandon the idea of using highly effective viral vector, researchers are exploring safer and efficacious alternatives delivery vectors.

1.2.5 Overview of Non-Viral Vectors

An ideal vector or so-called "artificial virus" should be safe, easy and cheap to be mass-produced, have low or no toxicity to normal cells, be non-immunogenic, be stable during storage, have a reasonably long half-life in physiological conditions, be eliminated from body after administration, be able to access targeted cells or tissues, deliver the genetic materials to induce the desired therapeutic effect and does not induce genetic mutation. Inorganic nanostructures, proteins, lipid-based, polymer-based vectors and various nano-size materials such as gold nanoparticles or silicone have been used as gene delivery vectors [74, 166, 170-176]. Notably liposome using a neutral helper lipid 1,2-Dioleoylsn-glycero-3-phosphatidylethanolamine (DOPE) [177] could largely enhance the transfection. Also, a cationic polymer poly(ethylenimine) (PEI) is highly effectively in delivering pDNA by the "proton sponge" effect [178]. Carbon nanotubes (CNT) have attracted the interest of researchers for nucleic acids delivery because they can gain entry into the cells by a diffusion like mechanism as well as by endocytosis [179, 180].

1.2.6 Poly(ethylenimine) (PEI) for Nucleic Acid Delivery

There are two kinds of poly(ethylenimine) (PEI), branched PEI (b-PEI) [181] and linear PEI (l-PEI) [182]. They are water soluble, basic and positively charged in physiological pH. b-PEI has a primary, secondary and tertiary amine ratio of 1:2:1[183]. However, the commercially available b-PEI may not have the primary, secondary and tertiary amine ratio equals to 1:2:1 but 1:1:1 as suggested by the studies of von Harpe et al.[183]

PEI (M_w 800 kDa) has been introduced for pDNA delivery by Boussif et al. since 1995 [178]. According to Abdallah et al., PEI can form polyplex with pDNA that produces transgene expression levels comparable to lentiviral or adenoviral vectors via direct brain injection [184]. Twenty five kDa (M_w 25000, M_n 10000) and higher molecular weight PEI has high sufficient transfection efficiency [185], however, their cytotoxicities are also much higher than low molecular weight PEI [186, 187].

PEI is the gold standard in pDNA delivery because of its high transfection efficiency, however, it is not very efficient in delivering siRNA both *in vitro* and *in vivo* [188-190]. It is believed that the interaction between PEI and siRNA is weaker than PEI and pDNA and as a result, the protection of siRNA by PEI is too weak and the siRNA was released after reaching the cell membrane especially for l-PEI [191]. PEI modified by PEG [192, 193], neutral or anion head groups [189] and hydrophobic moiety [194] could mediate better siRNA delivering capacity compared to unmodified PEI.

1.3   Background of Carbon Nanotubes

1.3.1 Introduction to Carbon Nanotubes

CNT, together with graphite, diamond, graphene and buckminsterfullerene are a class of carbon allotrope (Fig 1.3). They were a class of nano-size materials first discovered in 1952 by Russian scientists Radushkevich and Lukyanovich, described again by Bollmann and Spreadborough in 1960 and then described in 1976 by Oberlin, Endo and Koyama [195, 196]. Finally, they were rediscovered by Iijima in 1991 and they have been caught by the attention of scientists [197]. CNT are seamless cylinders of graphene sheets. There are two kinds of CNT, single-walled carbon nanotubes (SWCNT) and multiple-walled carbon nanotubes (MWCNT). The diameter of the CNT could be up to 100 nm and the length could be up to centimeter [198-200]. The carbons in the CNT are sp^2 hybridized and most of them are in a six member ring. There are usually defects on the CNT such as pentagon-heptagon defect (or Stone-Wales rearrangement) or "elbow connection" between armchair and zigzag confirmation. They could be produced by arc discharge, chemical vapor deposition, flame and laser ablation [200]. They have three conformations based on symmetry: armchair, chiral and zigzag (Fig 1.4). They have

distinct mechanical, electrical, piezoelectric and optical properties. Various applications of CNT were proposed such as electronic components, sensors, fuel cells, fibers, bio-materials, drug delivery, catalysis and gaseous storage [200-208].

Carbon Nanotube   Fullerene   Graphene
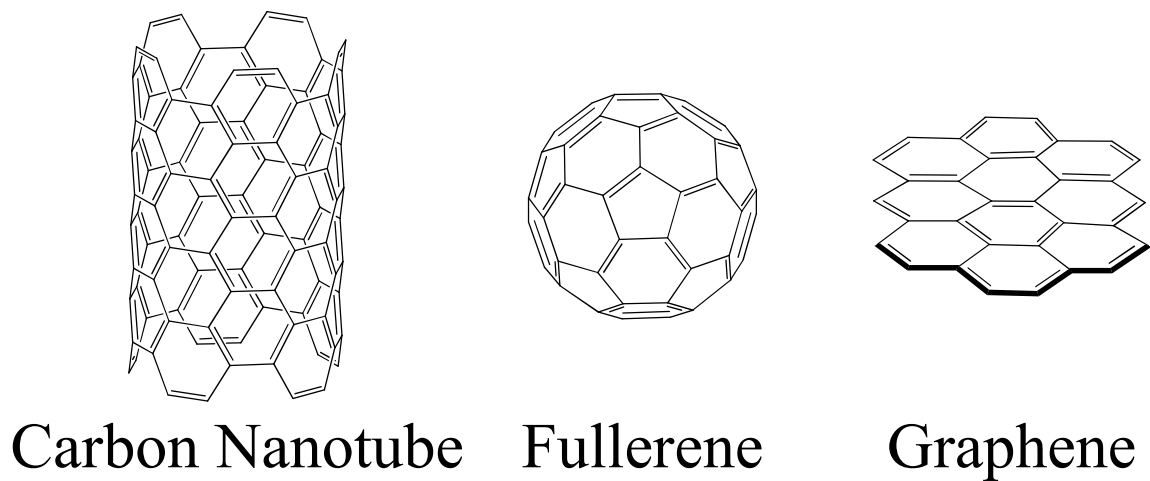
Figure 1.3 Carbon Allotropes

Armchair   Zigzag
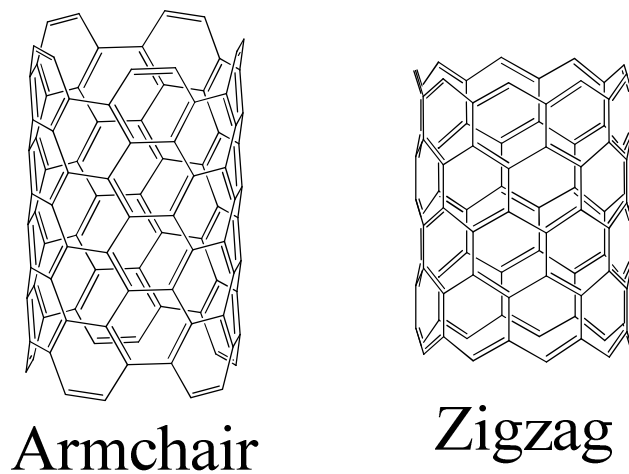
Figure 1.4 Structure of Carbon Nanotubes

1.3.2 Physical and Chemical Properties of CNT

CNT are black solids and they tend to form bundles in solid state due to strong van der Waals interaction. This is one of the biggest hurdles in CNT modification. Also, they do not dissolve well in any organic or aqueous solvent. They can be dispersed in some solvent with sonication, but they precipitate immediately after the sonication is ceased. However, they interact with various classes of compounds, which could facilitate the processing of CNT. The three main approaches of CNT modification can be categorized as follows: 1) covalent modification on the π-conjugated surface of CNT; 2) non-covalent adsorption or wrapping of molecules on the CNT surface; 3) endohedral filling of the internal cavity of CNT [209].

Covalent modification include halogenation [210], hydrogenation [211], cycloadditions [211-213], radical additions [214], nucleophilic additions [215], electrophilic additions [216], ozonolysis [217, 218], plasma activation [219], addition of inorganic compounds [220], mechanical functionalizations [221], polymer grafting [222, 223], defect site reactions [224] and biomolecule or biocompatible molecule attachment [225-233]. Non-covalent modification included the use of polynuclear aromatic compounds [234, 235], epoxy composites [236], acrylates [237], hydrocarbon polymers [238], conjugated polymers [239, 240], other synthetic polymers [241-247] and biomolecules [248-253]. Endohedral filling included fullerenes [254, 255], metals [256-258], liquids [259] and biomolecules [260-262].

1.3.3 CNT and Biological System Interaction

In molecular dynamics simulation, DNA oligonucleotides can be inserted into CNT in aqueous medium [263]. Also, CNT could be filled with small proteins [260]. These discoveries have led us to a new realm of nanomaterial and biological system interactions, such as application of CNT for biosensors [233, 247, 264] or drug delivery [265-271]. Xiao et al. used acid treated CNT for coupling with hexamethylenediamine and then reacted to FITC for the study of CNT interaction with cells [272]. Kam et al. used a lipopolymer to disperse the CNT for cancer therapy [273]. Interestingly, it has been shown that the cellular uptake of functionalized CNT is energy independent process

and is independent of cell type [180]. It was coined as "nano-needle" because the CNT gain into the cells by an orthogonal position [274]. More interesting, both of the functionalizations were done with 1,3-dipolar cycloaddition of azomethine ylides [180, 275]. On the contrary, some researchers reported that the cellular uptake of f-CNT is an energy dependent process [223]. The discrepancies between cellular entry mechanisms are probably due to the different methods of CNT functionalization. Also, the type, length, aggregation of CNT as well as the cell type tested played an important role on the cellular uptake mechanism [276, 277]. Bundled CNT and CNT longer than 1 μm would be taken up by the cell by phagocytosis. For singly dispersed CNT, it can gain entry into the cells if it is shorter than 1 μm, however, if it formed supermolecules, it will gain entry into cells by endocytosis (Fig. 1.5). Interestingly, it has been shown that CNT can be eliminated from cells and body by three different methods of functionalization as mentioned above [277-279].
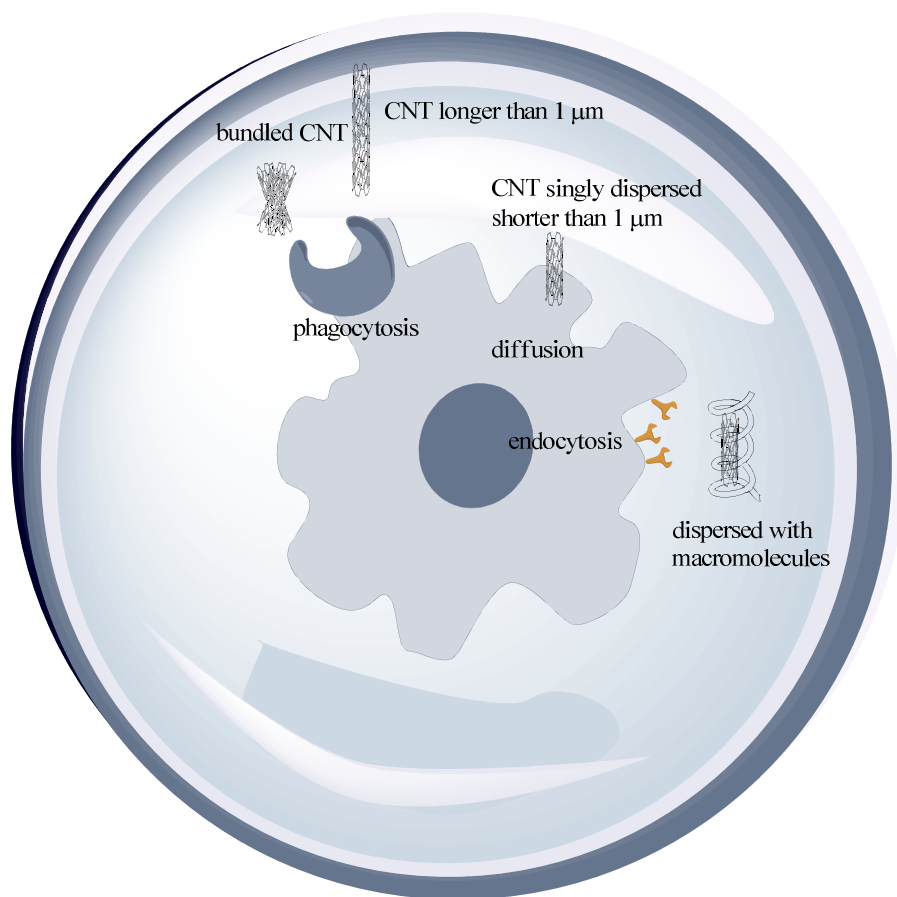
Figure 1.5 Parameters influencing the carbon nanotube internalization mechanisms.

1.3.3.1   CNT Toxicity

The toxicity of CNT is highly controversial. Some researchers have reported the toxicity of CNT [280-283]. Some researchers have reported the biocompatibility of CNT [284-288]. These contradictory reports are probably due to purity of CNT [289], method of CNT dispersion [290], dosage and assessment method [288, 291, 292]. The presence of impurities such as metal or metal oxide catalyst residues in CNT would misestimate the CNT toxicity [288, 292]. Also, pristine CNT (p-CNT) are not soluble in water and thus the toxicity of CNT in solution can only be determined with functionalization or the application of surfactants [293, 294]. Some of the surfactants are toxic themselves and thus it is necessary to do the experiment carefully whether the toxicity is due to the surfactants residue [293], undissolved CNT, the functionized CNT or CNT themselves.

1.3.3.2   CNT as Nucleic Acid Delivery Vector

The first report of using CNT for pDNA delivery into cells was by Pantarotto et al [274]. Amine functionalized SWCNT was used for the delivery and it exhibited 10 times greater transfection capacity than naked pDNA. Another approach by Liu et al. [223], used polyethylenimine grafted MWCNT for pDNA delivery. The transfection capacity was found to be better than PEI alone. Since then, various functionalized CNT were used to deliver nucleic acids *in vitro* and *in vivo* [268, 273, 295-300]. For covalent functionalization, two major methods have been used: amination or polymer conjugation. Direct amination can be done by 1,3-dipolar cycloaddition of azomethine ylides [274] or acid functionalization followed by conjugation with diamine [298]. Polymer conjugation was carried out by polymer-graft-type method like grafting to [267, 301-303] and grafting from [223] approach.

Non-covalently functionalized CNT which could effectively deliver siRNA has also been developed. Some of the methods [297, 299, 304] were based on Kam's method [295] by using a lipopolymer DSPE-PEG for CNT dispersion while some methods were based on the aromatic π- π stacking interaction [301, 305]. The lipopolymer method exploits the hydrophobic tail to interact with the CNT hydrophobically while the PEG helps to disperse the whole complex in water [295] and acts as a site for siRNA covalent

conjugation by disulphide bonds. This approach is easy to carry out and is highly efficient. Liu used this method and he delivered siRNA to T cells [304]. This method was further developed by Cai as an efficient magnetic CNT spearing [297] for *in vitro* transfection in T and B cells [297, 299]. Non-covalently dispersed CNT conjugated to the pDNA directly or condensed by DSPE-PEG-poly(L-lysine). The CNT was filled with nickel at the end to exhibit magnetism.

1.4   Non-Viral siRNA Delivery *in vivo*

The delivery of nucleic acids *in vivo* is important but difficult. The biggest hurdle is the bioavailability of nucleic acids is usually too low to induce therapeutic effect for systematic administration [76, 306]. Local administration to the pathological site can be carried out to increase the bioavailability while there are various methods to improve the bioavailability of nucleic acids to pathological tissues.

1.4.1 Local Administration

Local administration is a direct and effective way to delivery therapeutics to pathological tissues or cells. There are various barriers for local administration for nucleic acid delivery [307]. Depending on the location of pathological tissues or cells, some of them can be overcome by injection [308], topical application [309], iontophoresis, electroporation [310] or sonoporation [311]. Topical application for transdermal delivery is one of the most promising methods due to ease of administration and transdermal delivery of siRNA has been demonstrated [312, 313].

1.4.1.1   Transdermal Delivery

Topical application of therapeutics is an attractive strategy for treating cutaneous pathological conditions. This is because its non-invasiveness, ease of self-administration, selective targeting to pathological location and lower dosage and systematic toxicity for achieving the therapeutic effect. However, the skin acts as the hurdle for topical delivery. It is obvious that the skin serves as the first line of defense and it prevents most physical and chemical insult as well as biological invasions. Traditionally, skin was divided into three layers: epidermis, dermis and subcutaneous layer.

The outermost layer of skin is epidermis which has five layers, from outermost to innermost: stratum corneum (SC), stratum lucidum, stratum granulosum, stratum spionsum and stratum germinativum. Cells in stratum germinativum contain keratinocyte which is actively undergoing mitosis. They replicate themselves actively moving upwards towards the outer layers. The cells in stratum spinosum begin to differentiate into keratinocytes and their shape change from columnar to polygonal. The keratinocytes in stratum granulosum are undergoing apoptosis and losing their nuclei. In stratum corneum, the keratinocytes are highly keratinized and finally differentiate into anucleated corneocytes [314, 315]. There are 10-30 layers of continually shedding stacks of dead cells which is 10-20µm thick and is mechanically stiff [316]. The SC is made up of 75-85% protein and 5-15% lipids in dry weight [317] (Fig. 1.6a).

The dermis layer is 3-5 mm thick and it has blood vessels, lymph vessels, nerve endings, sense receptors, hair follicles, sebaceous glands and sweat glands (Fig 1.6b).

Topical therapeutics are mostly excluded by the epidermis, while the SC is the stumbling block for transdermal delivery [315, 318]. The transdermal delivery effectiveness is determined by several factors such as the level of hydration of skin, pH, size of molecule, charge, hydrophilicity, lipophilicity, melting point, the rate of skin renewal and the thickness of the skin [315].

Transappendageal pathway is the first known pathway for transdermal delivery. It is because of the presence of natural openings of skin like sweat glands, sebaceous glands and hair follicles. It is a relatively limited route because these openings constitute only 0.1% of the total skin surface area [314]. Transepidermal pathway can be subcategorized into two pathways: intercellular route and transcellular route. Intercellular route is the continuous diffusion of drug through the lipid domains of the cells. Transcellular route is the diffusion of drug across corneocytes and intercellular lipid lamellae [315].

Transdermal siRNA delivery was carried out by Ritprajak [313] with an emulsion while Lin carried out transdermal siRNA delivery by the aid of peptide [312].
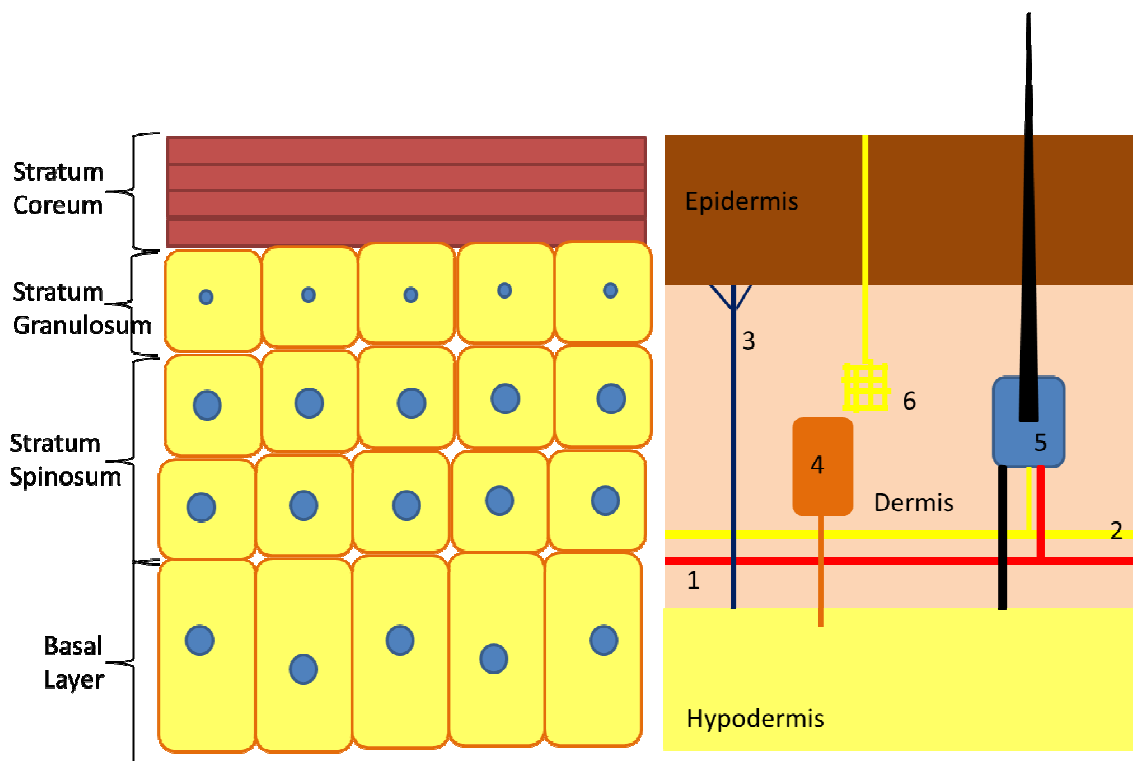
Figure 1.6 Structure of skin.

(A) Layers of the epidermis: basal layer, stratum spinosum, stratum granulosum and the stratum corneum (SC). Cells found in this layer of skin are keratinocytes with different differentiation levels, Langerhans cells and melanocytes. (B) Skin layers: (1) hypodermis, dermis containing blood vessels; (2) lymph vessels; (3) nerve endings; (4) sense receptors; (5) hair follicles; (6) sebaceous glands and sweat glands and epidermis. (Made with reference to Pegoraro et al. [315])

1.4.2 Systematic Administration

Systematic administration is one of the best ways to delivery therapeutics to internal organs. For DNA or siRNA delivered with vectors, one of the most important things is the colloidal stability of the vector/nucleic acid complexes. The vector/nucleic acid must exhibit a reasonable long half-life which is stable in circulation such that the nucleic acids can be delivered. The vector/nucleic acid complexes must not aggregate in physiological

salt concentration or in the present of serum protein. Besides, there are several other concerns for systematic delivery because of the more complicated *in vivo* environment. First, there is active RNase and DNase such that protection of the siRNA or DNA is necessary. Second, there is reticuloendothelial system (RES), in which macrophages, and the liver and spleen will take up most of the particles introduced. Third, naked siRNA will be excreted because its molecular weight falls below the size of renal clearance. Therefore, the delivery vector should be able to increase the serum stability, reduce the interaction between RES and increase the circulation half-life and thus, the bioavailability of DNA as well as siRNA can be increased and they can reach the cells, gain entry into the cells and carry out the desired function (Fig 1.7).
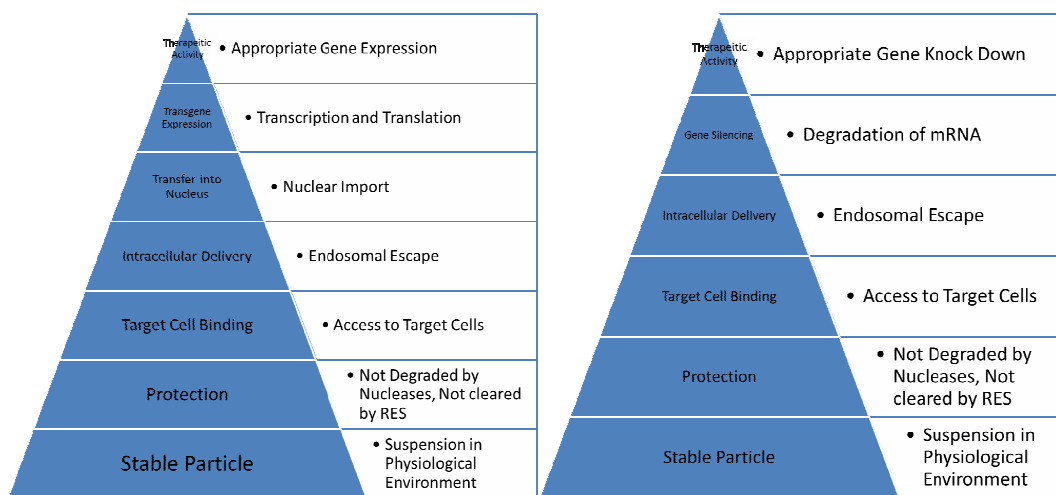
Figure 1.7 Barries for pDNA/shRNA delivery and siRNA delivery

There are different barriers for different nucleic acids. (a) The barriers for pDNA/shRNA are more than (b) siRNA because of the necessity of nuclear import for DNA. Endosomal or lysosomal escape are usually required if the vector/DNA or vector/siRNA complexes were taken up by endocytosis.

1.4.2.1   Poly(ethylene glycol) (PEG) for *in vivo* Delivery

The conjugation of poly(ethylene glycol) PEG or PEGylation is one of the most commonly used modifications for *in vivo* application. Large particles or particles which

are highly charged tend to be eliminated by the RES [319]. Also, positively charged particles will tend to aggregate with serum protein. To reduce the interaction between the delivery vehicles and RES or serum protein, a shielding domain is needed for *in vivo* application. Researchers have attached PEG to various vehicles [320]. The zeta potential of the resulting complexes can also be reduced but the colloidal stability was maintained by steric effect [321] and the interaction between immune system [322, 323] or serum protein can also be reduced due to reduction of positive zeta potential. It outperforms other polymers not only because of its commercial variability, but also its versatility. Liposome vectors [324] and even adenovirus [325] have been modified by PEG. Also, various structure of PEG can be made, block copolymer [326], alternate block copolymer [327] and graft copolymer [328] have been synthesized. The difference of molecular weight of PEG, grafting density to PEI and the relationship between biological activities for pDNA transfection have been compared by Petersen et al [329]. Generally, PEGylated copolymers reduce toxicity and prolong blood circulation half-life. It is desirable for *in vivo* application. However, it is important to note that PEG is biocompatible but not biodegradable. If the molecular weight of PEG is higher than 30 kDa, it may be retained in our body for a long time as it excesses the limit of renal elimination of water soluble polymer [169, 330]. Lower molecular weight PEG or degradable counterpart should be used for clinical application.

1.4.2.2　Ligands

Peptides, antibodies, steroids or other molecules with known biological activities that can facilitate transfection can be incorporated. It is a desirable method as the bioactive ligands can be chosen for different desired targets as well as applications [331-338].

Incorporating endogenous ligands is one of the most attractive strategies to increase transfection efficiency as well as tissue or cell type specificity for systematic delivery. Because they have high affinity with the receptors expressed on the cell surface, the vector may gain entry into the cell by receptor-mediated endocytosis. This strategy has been proved useful by Wanger et al. with transferrin conjugated PLL [339]. Various receptor specific endogenous ligands have been attached to delivery vehicle [331, 332, 340]. Notably, transferrin [331], folic acid [332], RGD peptide [333, 334], epidermal

growth factor [338], galactose [335] and mannose [341] as well as many other endogenous ligands have been used [68]. Some of the cells or tissues express certain receptors on their cell surface. For example, overexpression of folate receptors [342, 343] and epidermal growth factor receptors [344] have been detected in various cancer and cancer cell lines. Therapeutic nucleic acids can be delivered selectively or specifically with the aid of the endogenous ligand of the vector. This is preferable especially for delivering suicide genes for cancer therapy so that the therapeutic effect can be maximized and the damage to the healthy tissues can be minimized.

Some peptide ligands such as nuclear localization signaling peptide (NLS) [345] and CPP [346] have also been attached to delivery vehicle. These ligands also increase the transfection efficiencies by the virtue of the known biological activities of the peptides. However, some of them are derived from viral protein such as HIV1 TAT [347], they may induce undesirable immune responses. Also, conjugation of these viral proteins is expensive and the shelf life of storage could be reduced.

1.4.2.3   Folic Acid for Cancer Targeting

Folic acid is a water soluble vitamin. It is an aromatic compound that was first isolated from spinach in 1941 and was synthesized in 1943 [348]. It is composed of a pteridine ring, para-amino benzoic acid and glutamic acid (Fig 1.8). It is important for the synthesis of DNA. There are receptors that bind to folate (anion of folic acid) with high specificity. Folate receptor is glycosylphosphatidylinositol (GPI) membrane glycoprotein. It has a very high affinity toward folate, the association constant is 0.1-1 nM [349]. It was found to be overexpressed in various cancers and cancer cell lines [343, 350]. The high specificity of folate receptor as well as high expression level in cancer renders it as a maker of certain cancers [349]. The incorporation of folic acid is not limited to non-viral gene therapy but also to diagnosis and chemotherapy for cancer [349, 350].
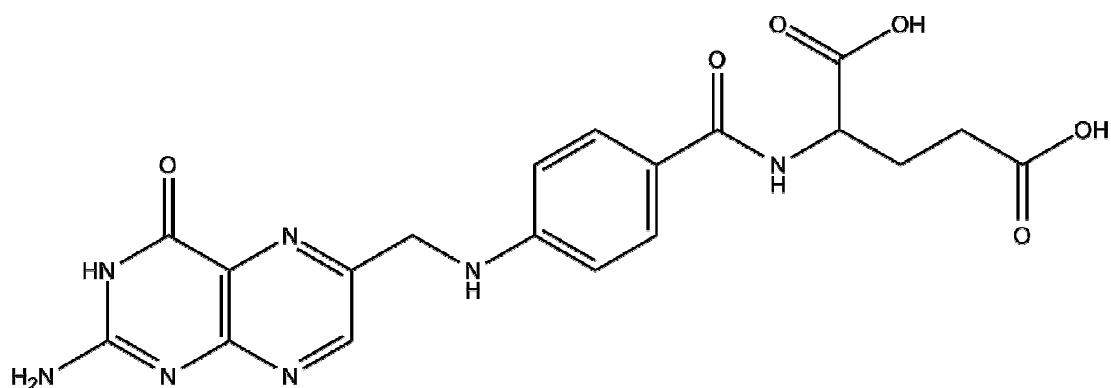
Figure 1.8 Structure of folic acid

1.5 Braf and mTOR in Cancer

1.5.1 Brief Introduction to Braf in Cancer

Proto-oncogene B-Raf (Braf) was discovered in 1988 [351]. It belongs to the Rapidly Accelerated Fibrosarcoma (Raf) family and v-Raf was the first identified member [352]. v-Raf is the mutated homolog of Raf-1 or C-Raf [353], there is also A-Raf but its function has not been well characterized. A-Raf was suggested to stabilize Craf/Braf heterodimer. Raf is serine/threonine-protein kinase and they play an important role in Ras-Raf-MEK-ERK cascade of mitogen activated protein kinase (MAPK) pathway. Braf was identified as the mutational target in various human cancers [354]. The mostly observed point mutation of Braf in cancer is valine at position 600 from N terminus changed to glutamine acid residue (V600E) [355]. It constitutes 90% of Braf mutations and this mutation was found in 27%-70% of malignant melanomas, 36%-53% of papillary thyroid cancers and 5%-22% of colorectal cancers and ~30% of serous ovarian in human [356]. Most of the researches in Braf focus on melanoma. In cell culture, Raf regulates MEK which regulates ERK, which regulates cell proliferation, migration and apoptosis [357]. The mutated Braf (V600E, originally documented as V599E) has a 500-fold higher kinase activity compared to the normal Braf [358]. However, the activation of ERK has also been shown by the heterodimer of Craf/Braf which involve a different pathway for normal and mutated Braf [359, 360]. Although the patient response of Braf

inhibitor in clinical trial is exciting, the resistance of Braf mutant inhibition has been observed in clinical trials [361-363]. The mechanism of Braf resistance has been actively carried out and various mechanisms have been uncovered [364-368].

1.5.2 Brief Introduction of mTOR in Cancer

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase, which belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) protein family. Rapamycin is an anti-fungal macrolide found in Streptomyces hygroscopicus bacteria [369]. Rapamycin binds to 12 kDa FK506-binding protein (FKBP12) and the domain that interacts with rapamycin is called FKBP12-rapamycin binding domain (FRB). Rapamycin inhibits mTOR and it was found that it can induce cell cycle arrest which suggest that mTOR is important in regulating cell growth and proliferation [370, 371]. Since then, researchers focus on the function of mTOR and it was also found to regulate metabolism, survival, protein and lipid synthesis and autophagy [372]. mTOR interacts with other proteins to form two functionally different complexes called mTOR complex 1 (mTORC1) and 2 (mTORC2). Both mTOR complexes contain the mTOR kinase, DEPTOR, mammalian lethal with sec-13 protein 8 (mLST8) [373], DEP domain containing mTOR-interacting protein (DEPTOR) [374] and Tti1/Tel2 complexes [375]. The unique protein in mTORC1 are the regulatory-associated protein of mammalian target of rapamycin (raptor) [376] and proline-rich Akt substrate 40 kDa (pras40) [377] while mTORC2 has rapmycin-insensitive companion of mTOR (rictor) [378], stress-activated map kinas-interacting protein 1 (mSin1) [379] and protein observed with rictor 1 and 2 (protor 1/2) [380].

mTORC1 is better characterized and is sensitive to rapamycin. It is downstream of the PI3k-Akt pathway which is dysregulated in a lot of cancers and most of them are related to phosphatase and tensin homolog (PTEN) mutation. PTEN inhibits Akt signaling by dephosphorylating the Akt stimulating phosphatidylinositol (3,4,5)-triphosphate (PIP3) [381]. Akt is a regulator of metabolism, survival, apoptosis, growth and proliferation which plays an important role in tumor survival and drug resistance [382, 383]. Upregulation of Akt could lead the phosphorylation of tuberous sclerosis complex 2 (TSC2) which is the negative regulator of mTORC1 [384]. TSC1/2 regulates Ras

homolog enriched in brain (Rheb) which has GTPase activity and Rheb can interact and activates mTORC1 [385]. mTORC1 phosphorylates and activates S6K which regulates cell size, protein translation and cell proliferation [386].

mTORC2 is originally thought to be rapmycin insensitive however, prolonged treatment of rapamycin reduces mTORC2 signaling in some cells by suppressing the mTOCR2 assembly [387]; the reason is still unknown at the moment. mTORC2 phosphorylates and activates Akt [388].

1.6   Objective of the Study

siRNA is an effective method to downregulate specific gene expression, however, the delivery of siRNA is the stumbling block for its clinical application. The "nano-needle" property of CNT offers a new possibility for drug delivery as well as siRNA delivery. The degree and the site of modification are important factors for determining the transfection capacity of the resulting CNT. The reported CNT which exhibit the "nano-needle" property was functionalized by 1,3-dipolar cycloaddition of azomethine ylides, 1 in 100 carbons were functionalized throughout the CNT [389]. In contrast to the acid functionalization of CNT, only the side wall and opening of CNT were functionalized, the side wall with an intact wall can be functionalized.

However, covalent functionalization of CNT is difficult and non-covalent functionalization is easier to be carried out and it was demonstrated by Kam [295], who used DSPE-PEG to disperse to CNT non-covalently to deliver siRNA. This CNT exhibited high transfection capacity in T cells *in vitro* which is difficult to be transfected. However, here are several limitations on this design. First of all, the siRNA has to be further modified, which would increase the cost of manufacturing. Secondly, the exact amount of siRNA loading has to be determined every time after the conjugation. Thirdly, the CNT will bear net negative charge due to the attachment of siRNA on the surface which reduces the transfection capacity. Moreover, if siRNA is not bound to a surface or electrostatically condensed, they might still be accessible for RNase or DNase. The degree of degradation is unknown and siRNA degradation was not investigated in the publication. It is possible that it is very useful *in vitro* but the siRNA are susceptible to

degradation by nucleases *in vivo* before they reach the target cells or tissue. To avoid the possible interaction between the siRNA and RNases, conjugating cationic polymer to DSPE-PEG can be carried out. There are some commercially available cationic polymers like PLL, PEI, poly(arginine), etc. Among them, PEI is commercially available and it has been widely studied for nucleic acid delivery. It is known that it can deliver pDNA and siRNA and induces endosomal escape. Researchers used PEI to disperse CNT [390] and therefore, PEI alone can be used instead of using DSPE-PEG as the component to disperse CNT. However, PEI alone is not efficient to deliver siRNA, modification is necessary to mediate higher gene silencing. These two different approaches of polymer modification for CNT dispersion for siRNA delivery in various applications constitute the following chapters of the thesis. They will be divided into three chapters and the rationale of the experimental design is given as follow:

In chapter 2, the work focused on utilizing DSPE-PEG for CNT dispersion. DSPE-PEG was connected to PEI and the resulting polymer was used to disperse CNT. This approach is more conservative because the DSPE is able to stably disperse CNT. It is a proof of concept experiment for using PEI to disperse CNT non covalently for siRNA delivery. Optimal ratios between PEI to PEG was determined at the same time.

In chapter 3, modified PEI was used for CNT dispersion. Previously, succinic acid modified PEI (PEI-SA) was shown to mediate transfection as well as reducing the toxicity. However, if the degree of succination is higher than 20%, it cannot mediate gene silencing. It is because the PEI-SA cannot form a stable complex with siRNA. In considering that CNT might reduce PEI binding to siRNA, a lower percentage of succination is more desirable than over functionalization of the PEI. This CNT was examined for topical siRNA delivery because it has been reported that CNT can mediate enhanced transdermal drug delivery. Braf is usually mutated and activated in human melanoma which is believed to be one of the most important genetic aberration in cancer. A mouse cutaneous melanoma model was used for verifying the transdermal siRNA delivery as well as therapeutic efficacy of PEI-SA/CNT with Braf siRNA for reducing tumor progression.

In chapter 4, the PEI-SA was further modified for CNT dispersion and for tumor-targeted delivery. PEI and CNT offer no steric stabilization to the CNT/siRNA complex. The positively charged CNT is very likely to aggregate in the media of negatively charged serum and cleared by the RES during systematic delivery. PEG was conjugated to PEI-SA for increasing the colloidal stability of the CNT/siRNA complex. PEG was also attached to a targeting ligand to the other end of the PEG to enhance the target specificity. Folic acid was chosen as the targeting ligand because folate receptors are overexpressed in various cancers. It is known that mTOR is associated with S6K, which is a gene responsible for cell proliferation. A mouse cutaneous melanoma model was used for verifying the systematic siRNA delivery as well as therapeutic efficacy of the folic acid conjugated CNT with mTOR siRNA for reducing tumor progression.

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Chapter 2

2 DSPE-PEG-PEI Non-covalently Functionalized Single-Walled Carbon Nanotubes for siRNA Delivery *in vitro* and *in vivo*

2.1 Summary

Small interfering RNA (siRNA) can specifically down regulate the expression of a specific gene. It has been shown that modified carbon nanotubes (CNT) protect siRNA and facilitate its entry into cells, however, easy and efficient functionalization of CNT is more desirable. Non-covalent functionalization of CNT can be easily carried out and it has been shown that this approach can be used to deliver siRNA effectively.

Single walled carbon nanotubes (SWCNT) were functionalized by non-covalent association with a lipopolymer DSPE-PEG. Three different ratios of polyethylenimine (PEI) to 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG) were synthesized and the products were used to disperse CNT to form DSPE-PEG-PEI/CNT (DGI/C). DGI/C was used for siRNA delivery *in vitro* and *in vivo*.

Three DGI as well as DGI/C were characterized. The structural, biophysical, and biological properties of DGI/C and their complexes formed with siRNA were investigated. The cytotoxicities of the three DGI/C were low, while gene silencing with DGI/C/siRNA complexes was demonstrated *in vitro* with B16-F10 cells. In addition, we found significant uptake of siRNA as well as gene silencing in liver by intravenous administration.

A novel non-covalent functionalization of CNT was developed, which efficiently delivered siRNA *in vitro* and *in vivo*. The new delivery method has provided a new possibility for siRNA delivery, which could provide insight for the development of non-covalently functionalized CNT for siRNA therapy.

2.2   Introduction

CNT have been used for gene delivery [1-11] and it has been reported that CNT can gain into cells as "nano-needles" by a diffusion-like mechanism [12-15]. The type and dimensions of CNT and the way in which CNT are functionalized determine the mechanism of cellular entry [16, 17]. However, pristine CNT (p-CNT) are not soluble in most solvent and so various modifications or functionalizations have been performed to increase the solubility of CNT in common solvents [18]. CNT can be functionalized covalently and non-covalently for higher solubility [19, 20]. Despite the fact that CNT can be functionalized such that they can gain into cells effectively, they can only carry certain drugs or genes effectively. CNT can bind to single strand DNA [21], but the choice of sequence is limited as the binding is dominated by the interaction of hydrophobic bases of DNA and hydrophobic surfaces of CNT. In order to use CNT for drug or gene delivery, they must be functionalized specifically for the desired application.

siRNA is a powerful tool for research and it is an attractive method to treating disease, specifically those with known targets However, the application in the clinic is still limited, largely due to the difficulty in delivery [22-25]. It has been reported that siRNA can be covalently attached to CNT but this approach limits the siRNA carrying capacity. Also, siRNA must be modified before it is covalently attached to the CNT, which might increase the complexity of manufacture. Non-covalent binding of siRNA to CNT is preferable and stabilization of siRNA with cationic charge is a simple way for CNT to carry a greater amount of siRNA. Previously, it has been reported that CNT can be dispersed with DSPE-PEG non-covalently by the virtue of the hydrophobic lipid tail of DSPE [10, 26, 27]. PEI is a cationic polymer that has been used extensively for nucleic acid delivery. It can condense siRNA and facilitate endosomal escape [28]. It has been shown that CNT covalently conjugated with PEI can increase transfection efficiency [2].

In this study, to combine the advantageous transfection properties of PEI and the properties of DSPE-PEG which enable non-covalent functionalization and dispersion of CNT. DSPE-PEG-PEI (DGI) was prepared at different ratios of DSPE-PEG to PEI. DGI was used to non-covalently functionalize CNT. The *in vitro* siRNA delivery capacities and cytotoxicity were determined.

2.3 Materials and Methods

2.3.1 Chemicals

Purified single-walled carbon nanotubes were purchased from Nano-C (Batch PT1112-60, MA, USA). Polyethylenmine (PEI, M_w 15476, M_n 7893), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and N-Hydroxysuccinimide (NHS) were purchased from Sigma Aldrich (St. Louis, MO). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-COOH) was purchased from Avanti polar lipids, Inc. (Alabaster, AL). Dimethyl sulfoxide (DMSO) was purchased from Caledon Laboratory Chemicals, Inc. (Georgetown, Ontario, CA).

2.3.2 siRNAs

Double-stranded siRNAs Silencer® Cy™3 Labeled GAPDH siRNA (siGAPDH) was obtained from Invitrogen (Burlington, ON, Canada). Luciferase GL2 Duplex was used as scramble siRNA (siScramble) which was obtained from Thermo Scientific (Ottawa, ON, Canada).

2.3.3 Nuclear Magnetic Resonance (^{1}H-NMR)

Polymer was dissolved in D_2O (99%, Sigma) and was transferred to NMR tube. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with INOVA 600 spectrometer (Agilent, Santa Clara, CA) (600MHz). The spectra were recorded at room temperature and the D_2O peak (4.7 ppm) was used as reference.

2.3.4 Synthesis of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-poly(ethylene glycol)-poly(ethylenimine) (DGI)

For DGI 5 and 9, 200 μL (1.75 μmol) of DSPE-PEG-COOH (2.5 mg/mL in chloroform) was added to a round bottom flask and it was dried with compressed air. Then 5 mL DMF and 10 mg (52 μmol) of EDC was added. The mixture was stirred for 15 minutes and then desired amount of PEI solution (23 mg, 2.3 μmol for DGI 5; and 42 mg 4.2 μmol for DGI 9) in MES buffer (0.1 M, pH 5.5) was added. The reaction was allowed to proceed overnight and then the reaction mixture was dialyzed overnight using a 50 kDa

molecular weight cut-off (MWCO) regenerated cellulose membrane (Spectrum Laboratories, Rancho Domingo, USA) against deionized water for 48 hours. The product was then lyophilized for 2 days. For DGI 18, half the amount (2.5 mg; 0.9 µmol) but the same procedure of DSPE-PEG-COOH was added to the round bottom flask. Then 5 mL DMF, 27.0 mg (26.6 µmol) of EDC and 2.80 mg (24.3 µmol) of NHS was added. The mixture was stirred for 15 minutes and then 19.3 mg (1.93 µmol) of PEI solution was added. The reaction was allowed to proceed overnight and then the reaction mixture was dialyzed overnight using a 50 kDa MWCO regenerated cellulose against deionized water for 48 hours. The product was then lyophilized for 2 days and a white wax like solid was recovered (27.2 mg; 96% for DGI 5; 52.8 mg; 111% for DGI 9, 26.1 mg; 26.1 mg; 118% for DGI 18). The ^{1}H-NMR (D_2O, 600MHz) for DGI 5: δ 0.90 (m, 6H, -C$\underline{H}_3$ in stearoyl tail), δ 1.13-1.30 (m, 21H, -C$\underline{H}_2$- in stearoyl tail), δ 2.43-3.50 (m, 914H, -NRC$\underline{H}_2$C$\underline{H}_2$- in PEI), δ 3.68 (s, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG); DGI 9: δ 0.91 (m, 8H, -C$\underline{H}_3$ in stearoyl tail), δ 1.18-1.30 (m, 26H, -C$\underline{H}_2$- in stearoyl tail), δ 2.50-3.50 (m, 1634H, -NRC$\underline{H}_2$C$\underline{H}_2$- in PEI), δ 3.68 (s, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG); DGI 18: δ 0.91 (m, 20H, -C$\underline{H}_3$ in stearoyl tail), δ 1.25-1.30 (m, 29H, -C$\underline{H}_2$- in stearoyl tail), δ 2.40-3.50 (m, 3150H, -NRC$\underline{H}_2$C$\underline{H}_2$- in PEI), δ 3.68 (s, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG). The integral of the methyl group and methylene group of stearoyl are inaccurate in these ^{1}H-NMR because D_2O cannot disperse the lipid tail well. SEC: DGI 5: M_n = 13440, M_w = 21904, PDI = 1.63; DGI 9: M_n = 13245, M_w = 21256, PDI = 1.60; DGI 18: M_n = 14645, M_w = 24249, PDI = 1.66.

2.3.5 Size Exclusion Chromatography (SEC) of DGI

The liquid chromatography system was equipped with a Waters Separations Module 2695 (Waters, Mississauga, ON), a Refractive Index Detector (Waters 2414) and three PLaquagel-OH 40 8µm (300x7.5mm) columns (Polymer Laboratories, Waters) connected in series and to a PLaquagel-OH 8 µm guard column. 0.2 M ammonium acetate/acetic acid (pH 5.3) was eluted at 1 mL/min at room temperature for 35 min/ run. Samples were prepared at a concentration of 10 mg/mL in millique water, filtered through 0.2 µm Supor membrane filters and injected with a 100 µl volume loop. The calibration curve was obtained from PEO/PEG standards and the molecular weight was

calculated by Empower 3 software (Waters). The following standard molecular weights were used: 615, 1010, 3930, 12140, 20000, 31380, 71700, 106500.

2.3.6 Non-covalent Functionalization of SWCNT by DGI

5 mg of DGI was weighted in a tube and 20 mL of deionized water was added to dissolve the polymer. Then 5 mg of CNT was added into the polymer solution. The solution was sonicated for 1 hour at 60 degree Celsius. The undissolved CNT were removed by vacuum filtration with 0.22 μm Nylon filter. Then the unbounded polymer was removed by Amicon (MWCO 100 kDa). The concentrated DGI/CNT (DGI/C) was lyophilized, grey solid was recovered, the recovery of DGI/C 5 was 23.8%, DGI/C 9 was 25.0% and DGI/C 18 was 23.4% which was calculated by the following equation:

$$Percentage\ of\ recovery = \frac{weight\ of\ DGI/C\ recovered}{weight\ of\ CNT + weight\ of\ DGI} \times 100\%$$

2.3.7 Transmission Electronic Microscope (TEM)

DGI/C was dissolved in water and dropped onto a copper grid. After 20 minutes, the solution was removed and the grid was dried under air. The sample was analyzed by a Philips CM 10 Transmission Electron Microscope (Philips, Eindhoven, the Netherlands) operating at 80 kV with a 40 μm aperture.

2.3.8 Gel Shift Assay

Equal volume of 0.5 μg of siRNA and desired amount of DGI/C were mixed and incubated for 30 minutes. Then the complexes were undergone electrophoresis at 100 mV with 1.5% agarose gel and EtBr in TAE buffer. After 20 minutes, the gel was removed, visualized and the picture was recorded with FluroChem M (Protein simple, Santa Clara, CA).

2.3.9 Zeta Potential

DGI/C/siRNA complexes was made by mixing equal volume of siScramble (5 μg) and desired amount of DGI/C, then the solution was incubated for 30 minutes. The solution was then transferred to a disposable capillary cell and was analyzed with Malvern

Zetasizer Nano ZS (Malvern, Worcestershire, UK). He-Ne laser (633nm) with an energy output of 10 mW with automatic laser attenuation was used for measurements. The mean and standard derivation of zeta potentials were calculated by 2 measurements of the average of 10 runs using automatic algorithm.

2.3.10 *In vitro* Gene Silencing in B16-F10

B16-F10 cells (ATCC, Manassas, VA) were seeded in a 12-well plate with density of $1.2x10^5$ cells/well in 1 mL culture media 24 hours before transfection. DGI/C/siRNA complexes were made by mixing equal volume of siRNA and 5 times of DGI/C (w/w), the solution was incubated for 30 minutes. DGI/C/siRNA solution was then added to serum containing media to have a final concentration of 2 µg/mL of siRNA. Lipofectamine 2000 (Invitrogen) was used as positive control according to manufacturer's protocol. 24 hours after transfection, Trizol method (Life technologies) was used for RNA isolation and cDNA were synthesized. The mRNA expressions of GAPDH of samples were quantified with quantitative real time polymerases chain reaction (qRT-PCR) using rRNA expression of 18S as control. The GAPDH and 18S primers and the qRT-PCR TaqMan® Assays buffer were obtained from Life Technologies Inc. (Burlington, ON) and the reactions were done in duplicate with Stratagene MX 3005p QRT-PCR systems (Mississauga, ON). The reaction condition was 10 min at 95^oC, then 40 cycles on 30 s at 95^oC, 1 min at 58^oC and 1 min at 72^oC.

2.3.11 Cytotoxicity in B16-F10

24 hours before transfection, B16-F10 cells were seeded in a 24-well plate with density of $5x10^4$ cells/well in 500 µL culture media. The media were replaced with culture media with desired amount of DGI/C. The cells were then returned to incubation for 24 hours. Media were collected and the cells were typsinized, collected and re-suspended in PBS with 2% FBS and 5 µg/mL 7AAD. The cells were then analyzed with flow cytometry. Percentage of cell death was calculated by the 7AAD positive cells in the treated cells minus the 7AAD positive cells in the untreated cells which were considered as the background cell death.

2.3.12 *In vivo* siRNA Delivery and Gene Silencing

6-8 weeks CD-1 mice (Charles River, Canada) were injected with 200 µg of Cy-3 labelled siGAPDH or siScramble with 200 µg of DGI/C 9 by tail vein and untreated mice were used as negative control. 24 hours after injection, the mice were sacrificed and the organs were taken and frozen in -80 ^{o}C in OCT or in a tube for later use. Crycosectioning was done on the organs and they were observed under fluorescence microscope (Olympus BX51, Olympus Canada Inc., ON, Canada). RNA was isolated with Trizol method. cDNA was synthesized and qRT-PCR was used to quantify the mRNA expression of GAPDH of the samples were analyzed and β-Actin was used as internal control. The reaction condition was 10 min at 95°C, followed by 40 cycles with 30 s at 95°C, 1 min at 62°C and 1 min at 72°C. The primers for GAPDH and β-Actin were:

GAPDH: 5'-GGGGTGAGGCCGGTGCTGAGTAT-3' (forward), 5'-CATTGGGGTAGGAACACGGAAGG-3' (reverse).

β-Actin, 5'-AGGGAAATCGTGCGTGACATCAAA-3' (forward) and 5'-ACTCATCGTACTCCTGCTTGCTGA-3' (reverse).

All animals were housed under pathogen-free conditions. All experiments were done in accordance to the *Guide for the Care and Use on Animals Committee Guidelines*. The animal protocol was approved by the Animal Use Subcommittee (AUS) at Western University.

2.4   Result

2.4.1 Synthesis and Characterization of DGI and DGI/C

DGI was synthesized by activating the carboxyl group in DSPE-PEG-COOH to react with the amine of PEI (Fig. 2.1). The polymers were characterized with ^{1}H-NMR and SEC. ^{1}H-NMR (Fig. 2.2) showed that the peak of PEI (2.5-3.0 ppm) and the peak of PEG (3.68 ppm). The amide peak of PEI (3.0-3.5 ppm) confirmed the successful conjugation of PEI to DSPE-PEG. There different ratios of PEI to DSPE-PEG were synthesized and the ratios were calculated by the follow equation:

$$Ratios\ of\ PEI{:}PEG = \frac{\text{Integral of 1H}-\text{NMR peak of PEI/43 (FW of PEI monomer)}}{\text{Integral of 1H}-\text{NMR peak of PEG/44 (FW of PEG monomer)}}$$

The calculated molecular weight by SEC of DGI 5, DGI 9 and DGI 18 are very similar which is ranging from 13 kDa to 15 kDa for number averaged molecular weight (M_n) and 21 kDa to 24 kDa for weight averaged molecular weight (M_w). Unreacted PEI or DSPE-PEG was not observed.

The details were summarized in (Table 2.1).

R= -H, -CH_2CH_2NHR, -$CH_2CH_2NR_2$
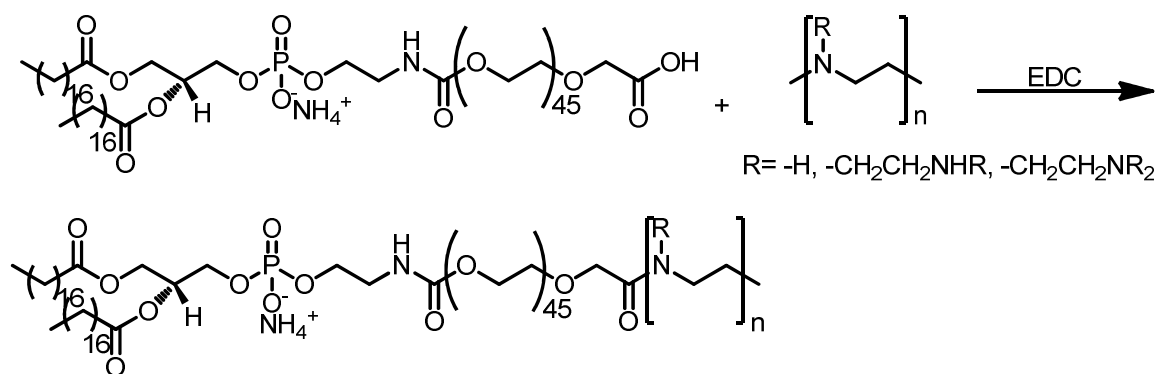
Figure 2.1 Scheme of DGI synthesis

Reaction scheme of DGI. DSPE-PEG-COOH was activated with EDC and then PEI was added for the conjugation. DGI was isolated by dialysis followed by lyophilization.
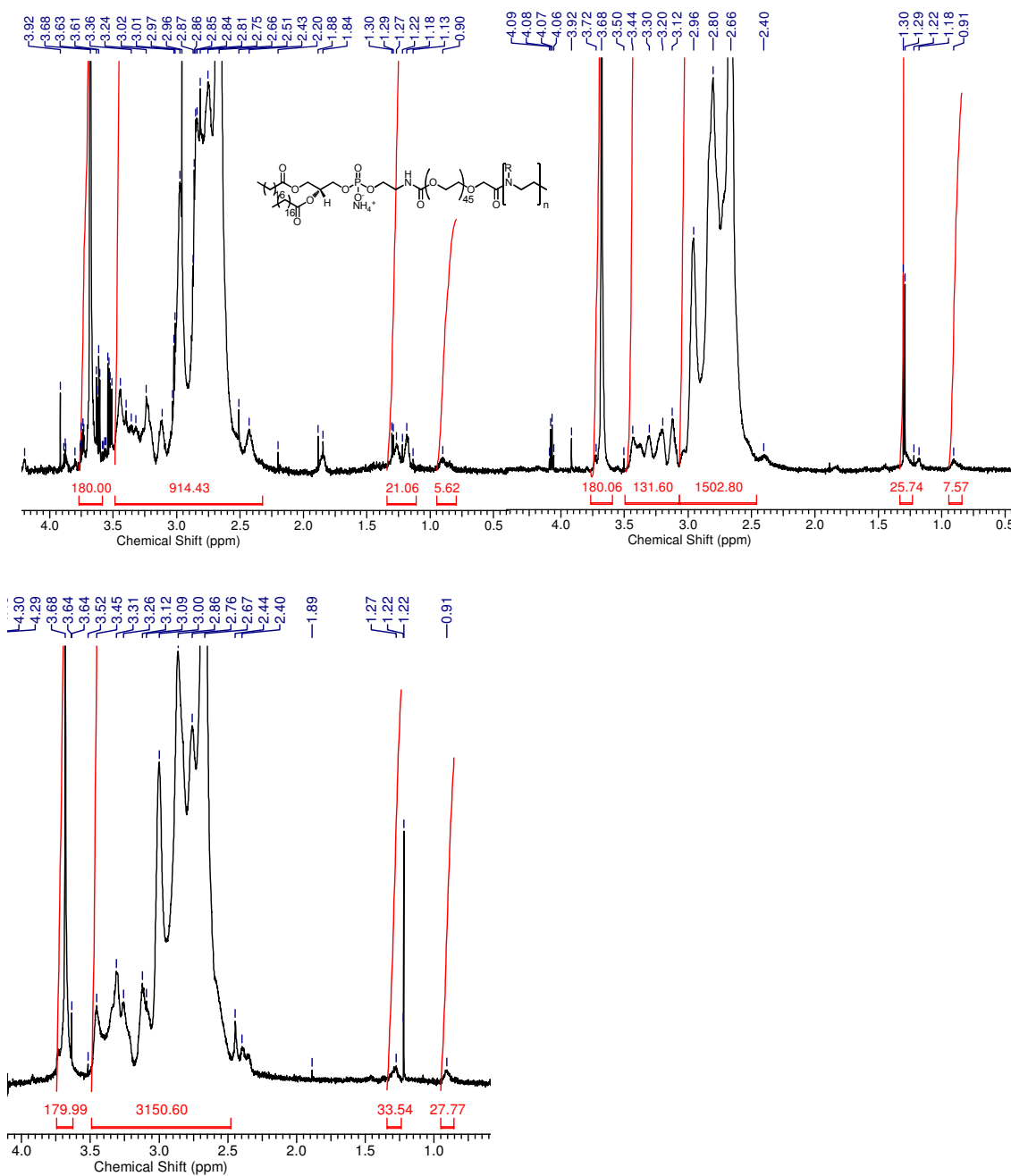
Figure 2.2 ^{1}H-NMR of DGI

^{1}H-NMR of DGI. DGI 5 from the top, DGI 9 in the middle and DGI 18 at the bottom. The polymers were dissolved in D_2O and the acquisition delay was 5 second.
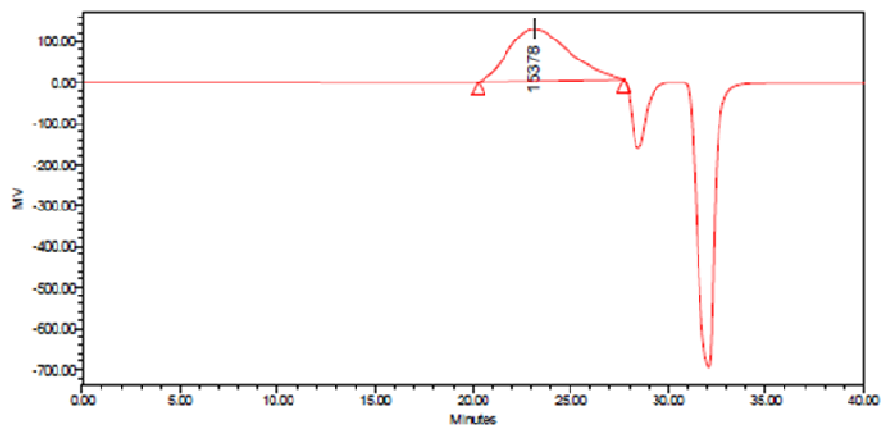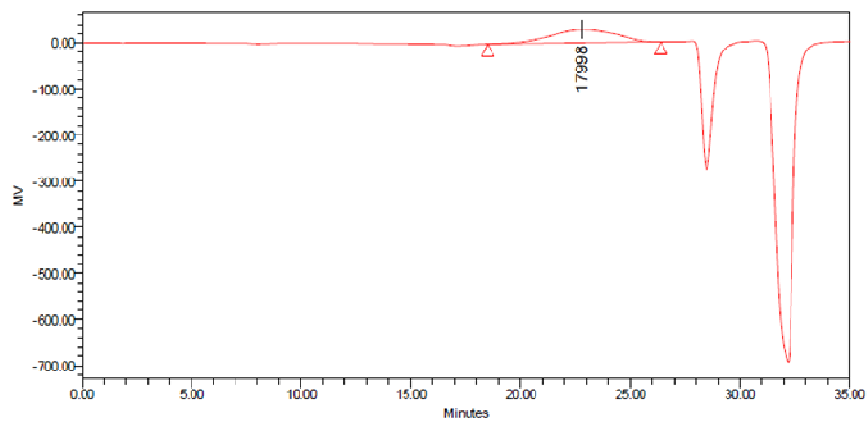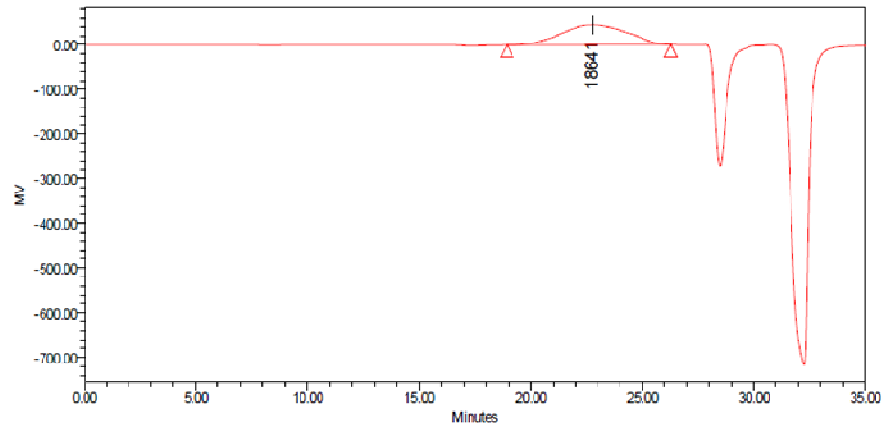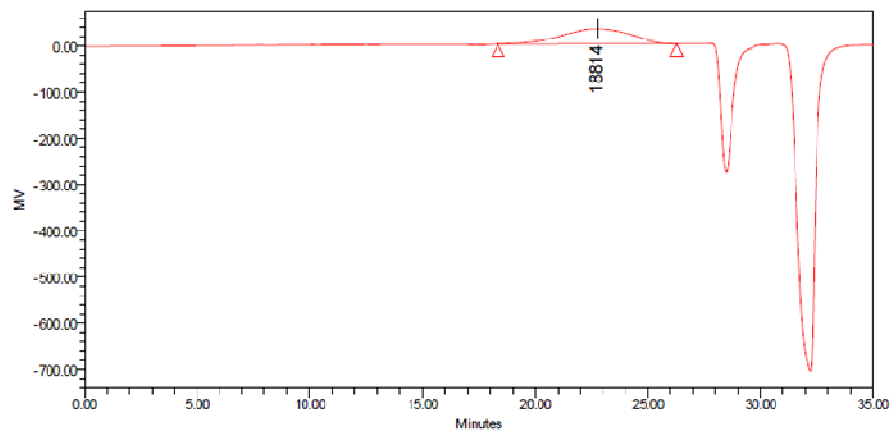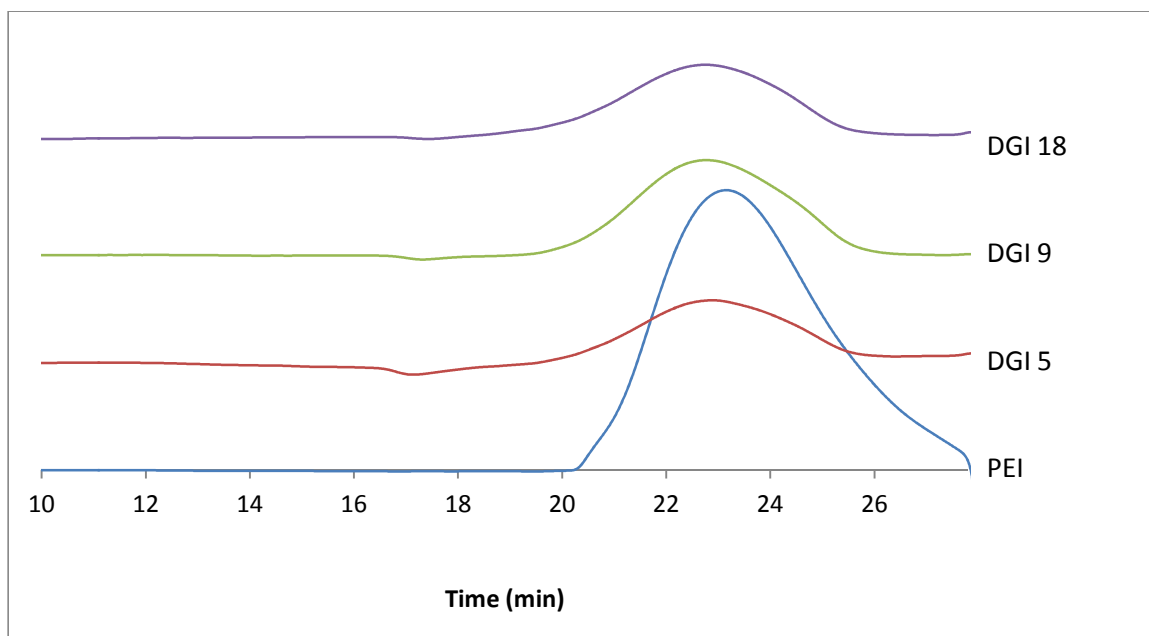
Figure 2.3 SEC of DGI

SEC of PEI and three DGI. PEI on the top, followed by DGI 5, DGI 9 and DGI 18. The bottommost figure is the overlay of all chromatograms. The polymers were eluted by a 0.2 M acetic acid/ammonium acetate (pH 5.3) buffer system with PL aquagel-OH column and were analyzed by RID.

	PEI:PEG ratio (feed)	PEI:PEG ratio (1H-NMR)	M_n	M_w	PDI (M_n/M_w)
DGI 5	6.6:1	5.0:1	13440	21904	1.63
DGI 9	12.1:1	8.9:1	13245	21256	1.60
DGI 18[a]	11.1:1	17.1:1	14645	24249	1.66

Table 2.1 Composition of DSPE-PEG-PEI in weight ratio calculated by ^{1}H-NMR and molecular weight by SEC

[a]DGI 18 was made by EDC/NHS coupling.

Non-covalent functionalization is an effective and simple way to obtain water soluble CNT. In order to obtain a water soluble CNT which is able to carry siRNA by electrostatic interaction, DGI functionalized CNT (DGI/C) was made with reference to Liu's method [10]. TEM (Fig. 2.4) showed that DGI/C is singly dispersed and the length is around 200-1000 nm. The siRNA binding capacity of DGI/C was demonstrated by gel shift assay. The free siRNA migrates along the gel while the migration of siRNA bound to DGI/C would be slowed down or totally stopped. Also, the siRNA cannot bind to ethidium bromide effectively and the fluorescence intensity of the siRNA would be reduced. The result showed that for DGI/C 5, the amount of DGI/C required to of siRNA is 1:1 (w/w) while DGI/C 9 and 18 are at 1:1 (Fig. 2.5).

DGI/C 5   **DGI/C 9**   **DGI/C 18**
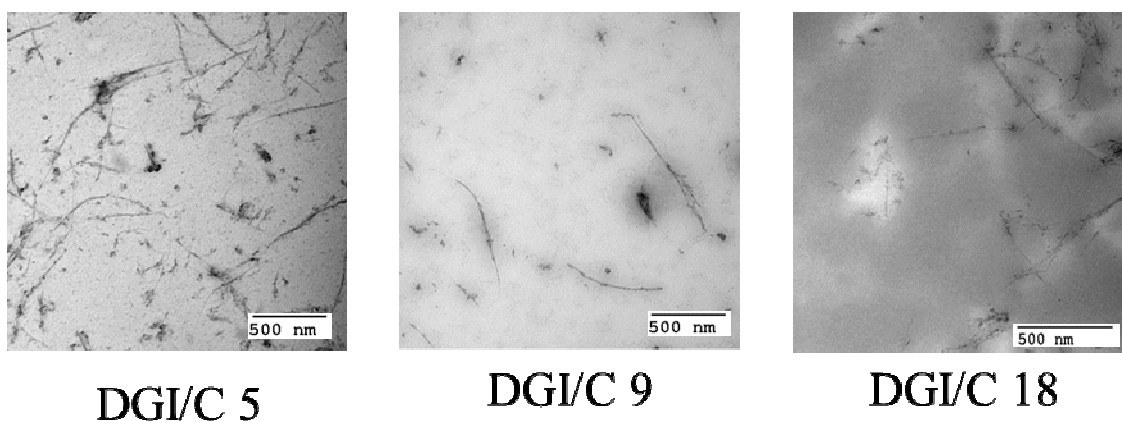
Figure 2.4 TEM of DSPE-PEG-PEI/CNT (DGI/C)

TEM micrograph of DGI/C. Minimum amount of DGI/C solution was added to the copper grid and was air-dried. Then the DGI/C was observed with TEM. The length of the SWCNT was found to be around 200-1000 nm.
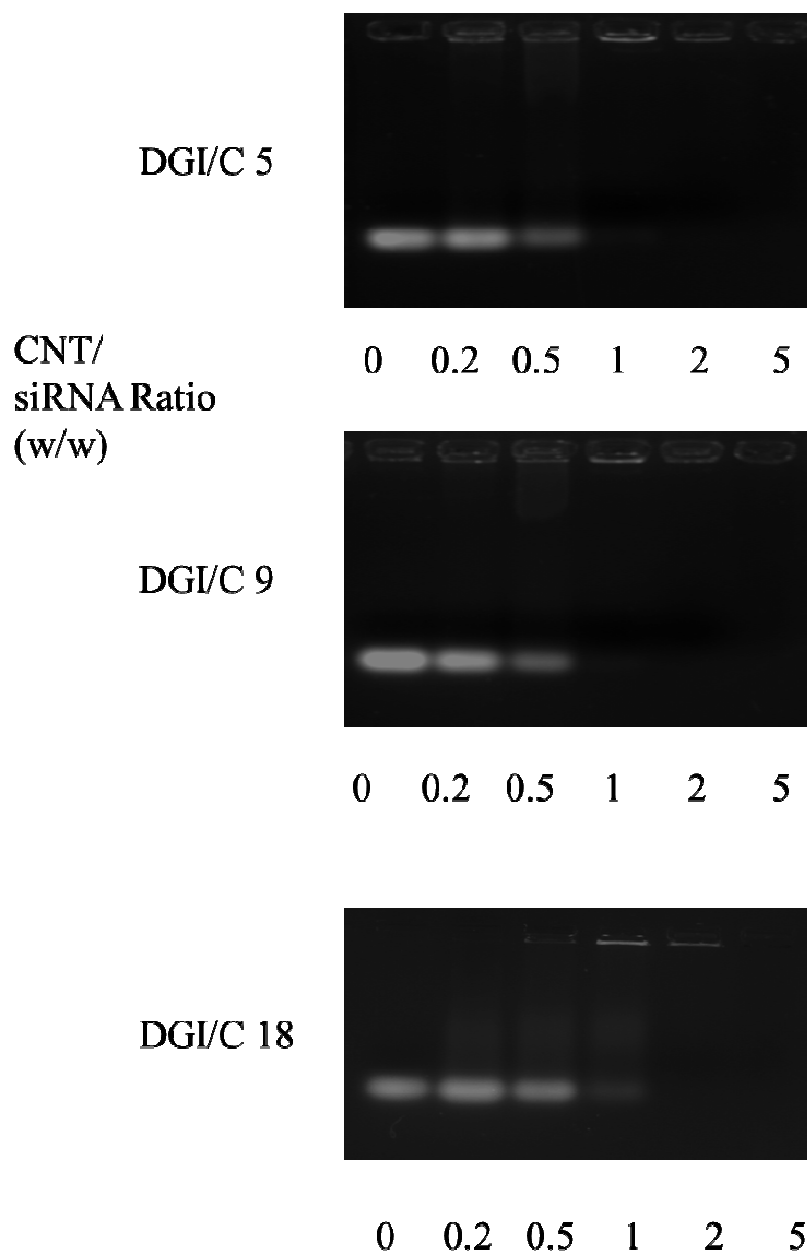
Figure 2.5 Gel shift assay of DGI/C/siRNA

DGI/C/siScramble complexes are made by mixing equal volume of DGI/C and siScramble. DGI/C was diluted to various concentrations while siScramble concentration is fixed. The complexes were incubated for 30 minutes at room temperature and the samples were loaded into agarose gel in TAE buffer (1.5%, w/v) with EtBr. The complexes were underwent electrophoresis for 30 minutes. Then the gel was taken out for

illumination under UV. The weight ratio of DGI/C 5 and DGI/C 9 inhibit the siRNA migration were at ratio 1:1 and DGI/C 18 inhibit the siRNA migration at ratio 2:1.

The stabilities of DGI/C and siRNA complexes were studied by determining the zeta potential. Electrostatic repulsion of the complexes maintains the stability of the complexes and thus, the higher magnitude of the zeta potential, the more stable the complex is. The result (Fig. 2.6) showed that the DGI/C/siRNA complexes have a positive zeta potential and they are high such that it is an indicator of good colloidal stability. Interestingly, the zeta potentials are similar for different CNT to siRNA ratios which were ranged from 47-42 mV for DGI/C 5, 53-48 mV for DGI/C 9 and 37-33 mV for DGI/C 18. All of the zeta potential is positive because of the incorporation of PEI to the CNT surface.
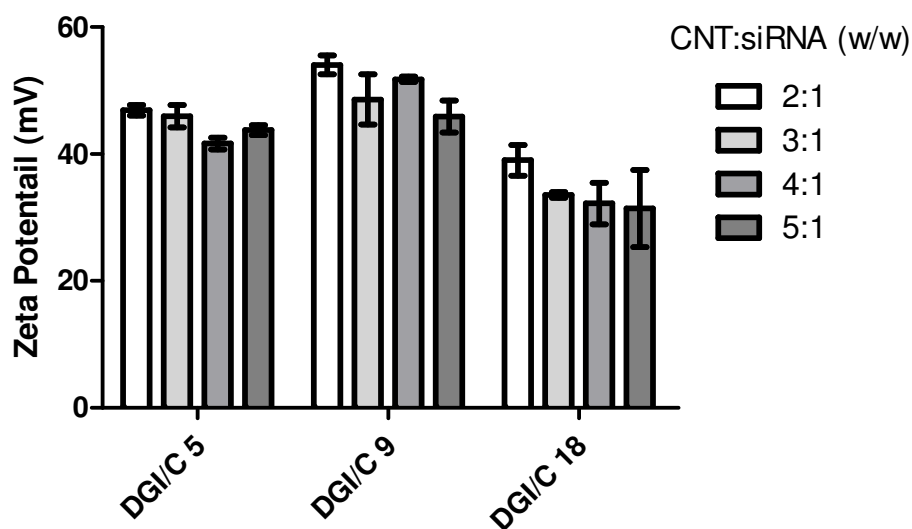
Figure 2.6 Zeta potential of DGI/C/siRNA

DGI/C/siScramble complexes were prepared using the same method as in gel shift assay. The sample was then diluted to 1 mL in deionized water and then transferred to a disposable cell. The experiment was done with 10 runs with duplicate and the error bar is standard deviation. Four CNT : siRNA ratio (w/w) were tested.

2.4.2 *In vitro* Transfection and Cytotoxicity of DGI/C on B16-F10

It has been showed that PEI conjugated CNT could increase the transfection efficiency of PEI. To reveal the siRNA delivering capacities of three different DGI/C, we set out to test the gene silencing in B16-F10 and GADPH was used as the target gene in a 24 hour period. For DGI/C 5, the gene silencing was 75%, the gene silencing of DGI/C 9 was 80% and DGI/C 18 was 60% compared to untreated cells (Fig. 2.7).

To investigate the cytotoxicity of DGI/C, various amount of DGI/C were used to treat B16-F10 cells for 24 hours and the dead cells were stained with 7AAD. All of the DGI/C has low cytotoxicity up to 5 µg/mL. DGI/C 9 has a negligible cytotoxicity up to 10 µg/mL. The percentage of cell death was 46% when the concentration increased to 20 µg/mL and continued to increase to 70% for 40 µg/mL. The cytotoxicities of DGI/C 5 and DGI/C 18 are similar. The cell death is around 10% at a concentration of 10 µg/mL and the cell death increased gradually from 30% to 70% for 20 µg/mL to 40 µg/mL. No significant difference between all DGI/C (Fig. 2.8)
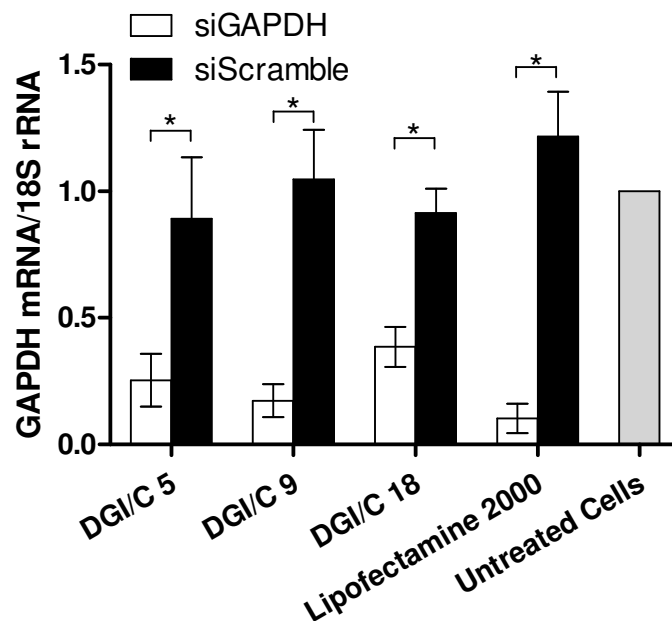
Figure 2.7 *In vitro* gene silencing by DGI/C in B16

B16-F10 cells were transfected with DGI/C and siGAPDH or scramble siRNA. 24 hours after transfection, the RNA was isolated with Trizol method and cDNA was synthesized. The gene expression was done by quantitative RT-PCR. Transfections were done in serum containing media. n=3, Error bar=SD. Asterisk indicated p<0.05 by student's t-tests.
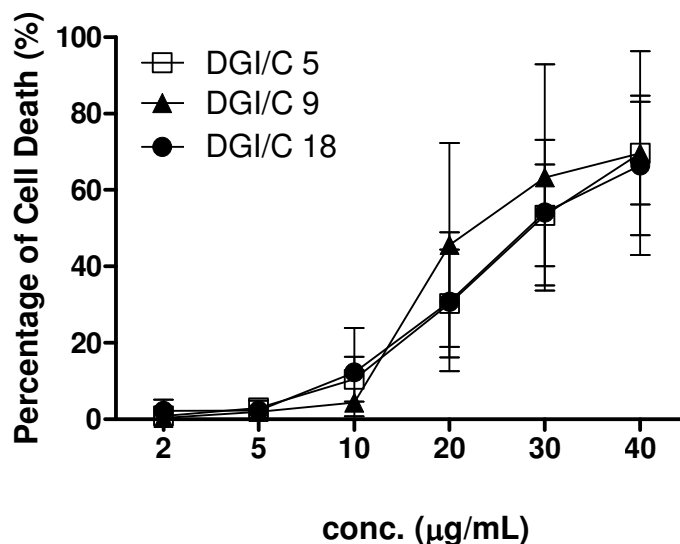
Figure 2.8 Cytotoxicity of DGI/C in B16-F10

B16-F10 cells were treated with various amount of DGI/C in serum containing media. 24 hours after treatment, the cells were typsinized and collected for 7AAD staining followed by flow cytometry analysis. Percentage of cell death was calculated by the 7AAD positive cells in the treated cells minus the 7AAD positive cells in the untreated cells. n=4, Error bar=SD

2.4.3 *In vivo* siRNA Delivery and Gene Silencing of DGI/C

DGI/C 5 has the best gene silencing capacity for *in vitro* experiment. To reveal the potential of using DGI/C 5 for *in vivo* siRNA delivery, DGI/C 5 and Cy-3 labelled siRNA was injected intravenously into CD-1 mice. Fluorescence of siRNA was found mostly in liver and spleen (Fig. 2.9). There were also fluorescence found in lung and kidney. qRT-PCR result showed that gene down-regulation in liver was over 80%. No gene down-regulation was observed for other organs (Fig. 2.10).
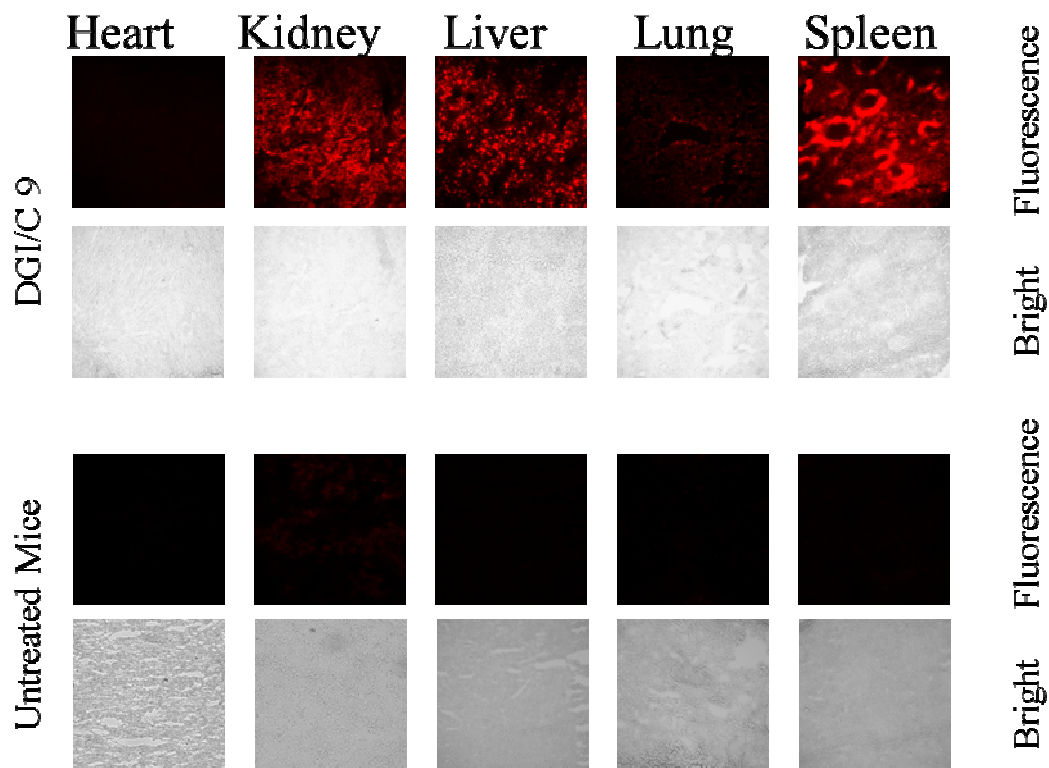
Figure 2.9 *In vivo* siRNA distribution in mice by intravenous injection

DGI/C and siGAPDH was injected into CD-1 mouse. 24 hours after the injection. The mice were sacrificed and the organs were taken out for cryosectioning. The samples were then observed under fluorescence microscope and representative picture was shown. Untreated mice were used as negative control.

a   Heart

b   Kidney

c   Liver

d   Lung
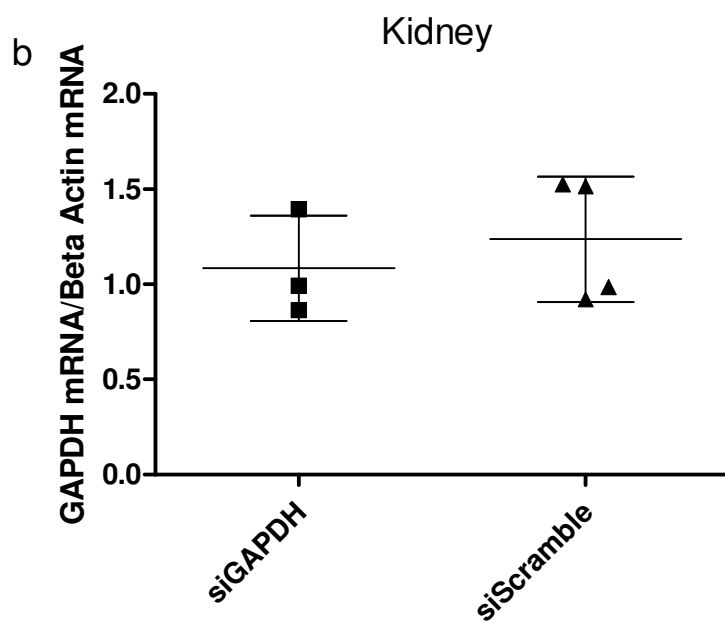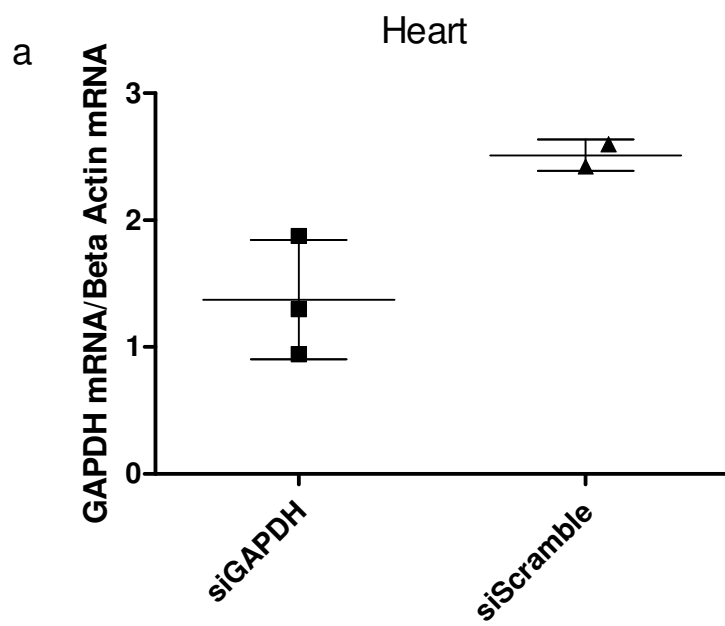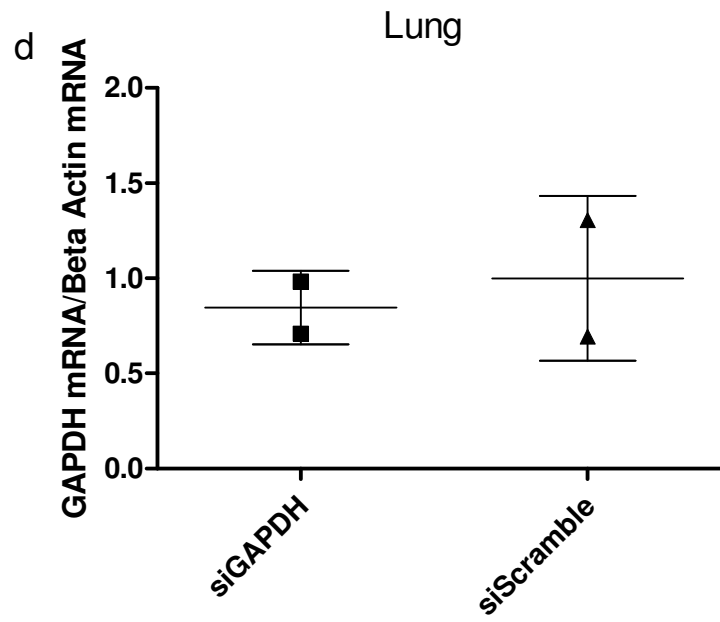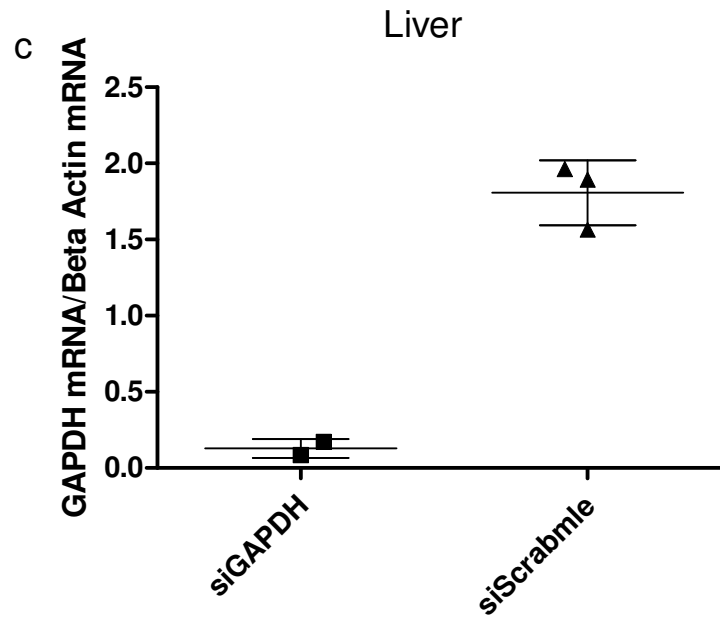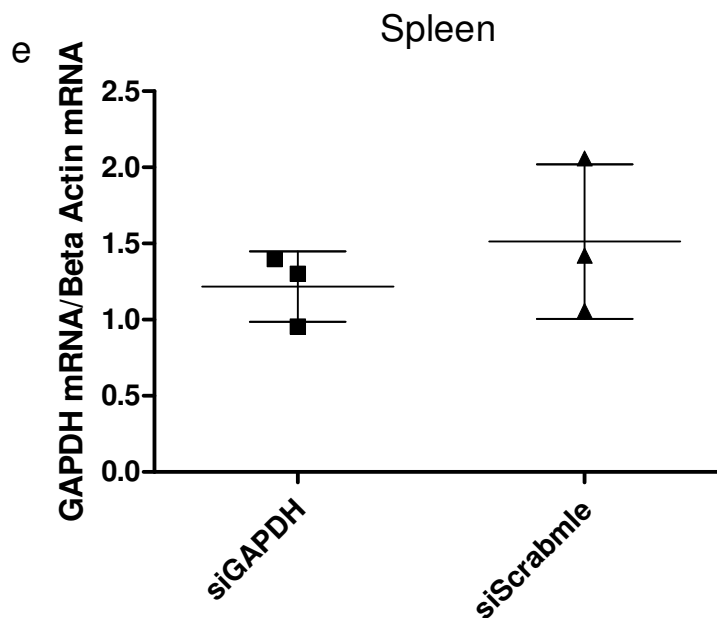
Figure 2.10 *In vivo* **gene silencing in five major organs**

DGI/C and siGAPDH or siScramble were injected into CD-1 mice, mice were sacrificed 24 hours after injection. Organs heart (a), kidney (b), liver (c), lung (d) and spleen (e) were homogenized and the RNA was isolated with Trizol method and qRT-PCR was used to quantify the mRNA expression. n=2 to 4, error bar=SD.

2.5   Discussion

Novel non-covalently functionalized SWCNT were developed for siRNA delivery *in vitro* and *in vivo*. CNT have been used for siRNA delivery [5, 9]. However, covalent functionalization like acid oxidation might damage the CNT structure [30]. On the contrary, non-covalent method is relatively easier to be carried out, also, the integrity of CNT can be preserved.

One of the most efficient methods for non-covalent functionalization is using surfactants [31]. By the virtue of hydrophobic interaction of the aliphatic tail of the surfactants and the surface of carbon nanotubes, they can be dispersed in water for further application.

DSPE-PEG is one of the most promising non-covalent CNT functionalization. It has been used for siRNA delivery and it would induce significant gene down-regulation [10, 26, 27]. However, the siRNA was covalently attached to the lipid and therefore, the release of siRNA would be inefficient unless disulphide or other kinds of triggered release was used for siRNA detachment. Also, the amount of the siRNA has to be determined after non-covalent dispersion of CNT which makes its application complicated. Furthermore, siRNA has to be modified for attaching to the lipid which would increase the cost of siRNA. Therefore, an effective siRNA loading and unloading, with an easy to manipulate siRNA loading amount and universal siRNA attachment/complexation method is more desirable. Electrostatic complexation is an effective way to complex any nucleic acid which is anionic. Also, the release of siRNA can be induced by the interaction of mRNA or anionic protein in the cells. Furthermore, once the ratios of cationic species and siRNA were determined such that there is no free siRNA in the complexes, the amount of siRNA added would be the amount of siRNA in the complexes. Therefore, we set out to use DSPE-PEG to connect to a polycation such that it can condense and deliver siRNA effectively.

DSPE-PEG was conjugated to poly-L-lysine to form DSPE-PEG-PLL (DGL) and it was used to form DSPE-PEG-PLL/CNT (DGL/C) for siRNA delivery (Appendix iii). However, DGL/C cannot deliver siRNA effectively. This indicates that the DGL/C gains into cell via endocytotic mechanism. If the CNT can gain entry into cells by diffusion like mechanism, localization of CNT into cells should be observed. We set out to use another polycation which could deliver siRNA better than PLL because it could enhance endosomal escape.

PEI is a polycation and it is effective in delivering pDNA [32]. However, unmodified PEI is inefficient in delivering siRNA [33, 34]. The covalent attachment of DSPE-PEG to PEI would enhance the delivery but the degree of modification has to be optimized. Therefore, we set out to conjugate DSPE-PEG to PEI (Fig. 1). It is interesting that the yield of the reaction is higher than 100%. It is mostly because PEI is a hygroscopic polymer and the water removal is still incomplete after 2 days lyophilization. ^{1}H-NMR showed the successful conjugation of DSPE-PEG to PEI. The chemical shift of the

ethylene peaks near to amide (δ 3.12-3.44) confirmed the conjugation. Three different ratios of PEI to DSPE-PEG 18, 9 and 5 were synthesized and ratios of PEI to DSPE-PEG were calculated based on the integral of the proton of PEI to PEG. The integral of the stearoyl tail of three DGI are inaccurate due to the hydrophobicity of the aliphatic chain. A more accurate integral could be obtained if it was dissolved in a more soluble solvent. However, the methanol peak overlapped with the PEI peak and it is impossible to integrate and compare the amount of PEI and PEG. SEC was also used to determine the molecular weight of the polymer (table 2.1). Interestingly, the molecular weights determined from SEC are not consistent with the proton NMR result. It is highly possible that due to the hydrophobic nature of the lipid, the elution time is affected because of the hydrophobic interaction between the polymer and stationary phrase and thus, the calculated molecular weights do not reflect the actual molecular weight of the polymer which was determined by the elution time of the polymer. Also, the calibration standard is PEG which is not a polycation. It is well-known that polycationic polymers would interact with the stationary phase. The hydrophobic tail of DSPE future complicated the interaction between the polymer and the column. However, a more accurate method is not available and conventional mass characterization technique is not useful. Most of the characterizations of PEI by mass spectrometric method were done with low molecular weight PEI (2000 Da) [35, 36], whereas higher molecular weight PEI were usually analyzed by viscometric [37] or chromatographic method [38-40] with static light scattering due to the polycationic nature of PEI.

A 50k MWCO dialysis membrane was used instead of lower MWCO, it is because the hydrophobicity of the stearoyl tail would prevent DSEP-PEG conjugated polymer to diffuse out of the dialysis membrane. As determined by the SEC, only one component was eluted out. An interesting observation is that the polydispersity of the polymers are similar to each other (~1.6) but they are smaller than the original PEI (which is 2.5 according to the manufacturer). It is highly possible that the lower molecular weight PEI and non-conjugated PEI were removed by dialysis and thus the molecular weight was narrowed down. After the dispersion of DGI to CNT and forming DGI/C, we observed the DGI/C under TEM. TEM showed that DGI/C has a length of 200-1000 nm which is shorter than with the claim of the manufacturer (800 nm to 1000 nm). Therefore, the

CNT were shortened by this method. This finding is reasonable as reported previously, sonicated CNT have a shorter length [41].

Next, we tested the capacity of DGI/C to condense siRNA electrostatically. Gel shift assay was used to assess the siRNA binding capacity of DGI/C. The free siRNA migrates along the gel while the migration of siRNA bound to DGI/C would be slowed down or totally inhibited. In addition, the siRNA cannot bind to ethidium bromide effectively and the fluorescence intensity of the siRNA with ethidium bromide would be reduced. The result showed that for DGI/C 5 and DGI/C 9, the amount of DGI/C required to inhibit migration of siRNA is 1:1 (w/w) while DGI/C 18 is at 2:1. It is counter-intuitive because there is more PEI content and which should bind more siRNA. However, as demonstrated by the other researchers [42], the binding of siRNA to PEI is weaker but modified PEI can facilitate the binding of siRNA to PEI. Oskuee et al. reported the same trend in gel shift assay [43]. Therefore, the relatively low degree modification of DGI/C 18 has worse siRNA binding capacity compared to DGI/C 9 and DGI/C 5. Colloid stability of the DGI/C and siRNA complexes are important for siRNA delivery, and therefore, zeta potentials of DGI/C/siRNA complexes were determined. It is interesting that the zeta potential of the DGI/C complexes are similar for all the tested ratios. There is a lack of obvious trend even if there is increases of CNT to siRNA ratio, the zeta potential of the DGI/C complexes are similar for all the tested ratios. They have zeta potentials around 40 mV which can be considered as stable because the charge repulsion between complexes inhibits the chance of the complexes to aggregate.

To reveal the siRNA delivering capacities of three DGI/C, we set out to test the gene silencing in B16-F10 cells. All of the DGI/C/siGAPDH is able to induce gene silencing. The gene silencing capacity of DGI/C 9 is the best among all compared to the siScramble control as well as to untreated cells. DGI/C 18 is less effective in inducing gene silencing which is believed to be the inefficient protection of siRNA in serum containing media. When the weight ratio of PEI and PEG was reduced to 9:1, the siRNA could be protected more effectively and DGI/C 5 was showed to have similar siRNA protection efficiency as DGI/C 9. Another possible explanation is that the zeta potential of DGI/C is the highest whereas DGI/C 18 is the lowest. For *in vitro* cell transfection, it has been reported that

the zeta potential plays an important role in the transfection [44]. The cellular uptake of DGI/C 9 was determined by flow cytometry (Appendix iv). The result showed that the cellular entry mechanism is energy dependent. It is possible that after the CNT was modified by PEI, it gains entry into the cells by endocytotic pathway as reported by Liu et al [2]. This implies that the trend of siRNA delivery of DGI/C is very similar to modified PEI.

The cytotoxicity of DGI/C 5 and DGI/C 18 are very similar. The cytotoxicity of DGI/C 9 is low (below 20 ug/mL). The PEI content of DGI/C 18 is the highest and it should be the most toxic if the toxicity is induced mostly by PEI. Therefore, the toxicity of DGI/C is not only related to PEI. It is also possible that the cytotoxicity is related to the zeta potential.

Lastly, we tested the *in vivo* siRNA delivering capacity of DGI/C 9 with Cy3-labelled siGAPDH. It was carried out previously in our lab, highest fluorescence can be observed 2 to 4 hours after tail vein injection of liposomes. Most of the red fluorescence was found in liver and spleen 4 hours after injection. It is not surprising because the reticuloendothelial system (RES) usually take up most of the particles delivered into the body intravenously. 24 hours was chosen for gene silencing detection in mRNA level [45]. Gene down regulation by siRNA was found in the liver, the organ which exhibited the most fluorescence. No gene down-regulation was found in other organs. The GAPDH mRNA of heart of the mice injected with siScramble was found to be overexpressed compared to mice injected with siGAPDH, the siRNA or DGI/C may stimulate the expression of GAPDH in heart. This result inferred that localization of siRNA in the organs do not necessary lead to gene silencing. It is possible that the DGI/C/siRNA cannot gain into some tissue effectively, or the siRNA was degraded in the endosome or lysosome. As a result, gene silencing cannot be observed in spleen or kidney. Also, DGI/C may interact with some cellular components and affect the gene expression of some organs.

2.6   Conclusion

A novel and efficient non-covalently functionalized CNT for siRNA delivery was developed. The polymers as well as the CNT were characterized and these CNT successfully deliver siRNA which mediate significant gene silencing both *in vitro* and *in vivo*. The cytotoxicity of the CNT is low in the concentration for *in vitro* transfection. This research provides insight for the further development of CNT-based siRNA delivery system.

2.7   Reference

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Chapter 3

3 Topical siRNA Delivery with a Novel Non-covalently Functionalized Single-Walled Carbon Nanotube for Melanoma Therapy

3.1 Summary

RNA interference (RNAi) can specifically regulate gene expression, but efficient delivery of small interfering RNA (siRNA) *in vivo* is difficult while it has been shown that modified carbon nanotubes (CNT) protect siRNA, facilitate entry into cells and enhance transdermal drugs delivery. Single walled carbon nanotubes (SWCNT) were functionalized non-covalently with succinated polyethyleimine (PEI-SA). In this study, the water soluble CNT, PEI-SA/CNT (IS/C) were isolated and characterized, the gene silencing induced by IS/C/siRNA complexes was achieved *in vitro* in B16-F10 cells. *In vivo* delivery was topically applied to shaved mouse skin, as well as topically to a C57BL/6 mouse melanoma model. We found significant uptake of Cy3-labeled siRNA specific to Braf (siBraf) and gene silencing in the tumor tissue. Treatment with IS/C/siBraf resulted in attenuation of tumor growth over a 25-day period. This new delivery method has provided a new possibility for future siRNA delivery and therapy, which provides insight for the potential application and development of CNT-based siRNA delivery.

3.2 Introduction

RNAi through siRNA is powerful tool for research and is an attractive method for treating disease specifically with a known target. However, the application of this technology in the clinic is still limited. The bottleneck of the application is the lack of effective delivery methods for siRNA [1-3]. Topical application of therapeutics is an attractive strategy for treating cutaneous pathological conditions. This is because of its non-invasiveness, ease of self-administration, selective targeting to pathological location, lower dosage and lower systematic toxicity for achieving the therapeutic effect [4, 5]. However, the skin acts as the hurdle for topical delivery. It is well known that the skin

serves as the first line of defense, preventing most physical and chemical insult as well as biological invasions. Topical therapeutics is also excluded by the epidermis, while the stratum corneum (SC) is the stumbling block for transdermal delivery [4, 5]. To overcome this barrier, various transdermal strategies have been developed [6]. Recently, CNT have been used for transdermal drug delivery [7-9].

CNT have been explored for various biomedical applications [10-12]. Pristine CNT (p-CNT) are not soluble in most solvents and therefore various modifications or functionalizations have been performed to increase the solubility of CNT in common solvents [13]. CNT have also been functionalized to impart solubility in water [14]. CNT can be functionalized covalently as well as non-covalently, and these approaches have been reviewed extensively [15, 16]. CNT have been used for nucleic acid delivery [17-19] as well as siRNA delivery [20-27]. It has been reported that CNT can gain entry into cells effectively and one of the most promising features is that they can act as "nano-needles" which penetrate cells via a diffusion-like mechanism. The type and dimensions of CNT as well as the manner in which they are functionalized determine the mechanism of cellular entry [28, 29].

Despite the fact that CNT can enter cells effectively, they cannot carry siRNA effectively without modification. Covalent attachment of siRNA to CNT enables siRNA delivery [27, 30] though siRNA carrying capacity is limited. In addition, siRNA has to be modified before covalent attachment to the CNT, which might increase complexity for manufacturing and storage. Non-covalent binding of siRNA to CNT is preferable and stabilization of siRNA with cationic charge is a simple way to carry a large payload of siRNA. It has been reported that poly(ethylenimine) (PEI) is a good polymer candidate for nucleic acid condensation and endosomal escape [31], but it is not very efficient in delivering siRNA [32]. Modified PEI, however, has been shown to deliver siRNA more effectively and it exhibits lower toxicity than the unmodified PEI [33]. Various PEI-CNT were synthesized and were superior to PEI alone for nucleic acid delivery [18, 22] while CNT can be functionalized non-covalently by PEI. Non-covalent functionalization of CNT with modified PEI may lead to a highly efficient siRNA delivery vector. Recently, modified CNT for transdermal drug delivery has been reported [7].

Cutaneous melanoma is a highly invasive carcinoma which is developed from melanocytes [34]. Early treatment of the disease is beneficial [35] and topical applications of siRNA specifically target the gene responsible for proliferation might be beneficial in reducing tumor progression and metastasis in a mice model [36].

In this study, we developed a method, in which non-covalently functionalized CNT were designed for topical siRNA delivery. We chose PEI-SA and SWCNT to combine the siRNA delivering capacity of PEI and the transdermal capacity of CNT. The synthesized polymers and functionalized CNT were characterized. Overall, the feasibility of using CNT for transdermal siRNA delivery was tested on a murine melanoma model.

3.3   Materials and Methods

3.3.1 Chemicals

Purified single-walled carbon nanotubes were purchased from Nano-C (Batch PT1112-60, MA, USA). Polyethylenmine (PEI, M_w 15476, M_n 7893) was purchased from Sigma Aldrich (St. Louis, MO), and succinic anhydride was purchased from Alfa Aesar (MA, USA). Dimethyl sulfoxide (DMSO) was purchased from Caledon Laboratory Chemicals, Inc. (Georgetown, Ontario, CA) and glycerol was purchased from VWR International, Inc. (Edmonton, Alberta, CA).

3.3.2 siRNAs

Double-stranded siRNAs Silencer® Cy™3 Labeled GAPDH siRNA (siGAPDH) was obtained from Invitrogen (Burlington, ON, Canada). Luciferase GL2 Duplex used as scramble siRNA (siScramble) and Braf siRNA (siBraf) were obtained from Thermo Scientific (Ottawa, ON, Canada). The sequence of siBraf was: GCU UAC UGG AGA GGA GUU ACA.

3.3.3 Cell Culture

B16-F10 cells were obtained from ATCC (Manassas, VA). Cells were cultured with DMEM (Gibco, Life technologies, Burlington, ON, Canada) with 10% FBS (Gibco) at 37^oC in humidified atmosphere.

3.3.4 Animals

CD-1 mice and C57BL/6 mice were obtained from Charles River (Canada). All animals were housed under pathogen-free conditions. All experiments were done in accordance to the *Guide for the Care and Use on Animals Committee Guidelines*. The animal protocol was approved by the Animal Use Subcommittee (AUS) at Western University.

3.3.5 Nuclear Magnetic Resonance (NMR)

Polymer was dissolved in D_2O (99%, Sigma) and was transferred to an NMR tube. [1]H nuclear magnetic resonance (NMR) spectra were obtained with INOVA 600 spectrometer (Agilent, Santa Clara, CA) (600MHz). The spectra were recorded at room temperature and the D_2O peak (4.7 ppm) was used as reference.

3.3.6 Synthesis of Succinated Polyethylenimine (PEI-SA)

PEI-SA was synthesized based on Zintchenko's method [33]. Briefly, 0.60 g (0.06 mmol) of PEI was dissolved in 10 mL of water. NaCl (0.25 g) was added and the pH was adjusted to 5 by the addition of 12 M HCl. 72.2 mg (721 µmol) of succinic anhydride was then dissolved in 10 mL of DMSO and was added drop-wise into the PEI solution. The reaction mixture was stirred at room temperature overnight and then was dialyzed using a 15 kDa molecular weight cut-off (MWCO) Spectra regenerated cellulose membrane (Spectrum Laboratories, Rancho Domingo, USA) against 0.25 M NaCl solution for 4 hours followed by 44 hours with deionized water. The water changed 3 times per day. The product was then lyophilized and a yellow solid was recovered (0.77g; 114%). ^{1}H-NMR (D_2O, 600MHz): δ2.4 (s, 4H of succinic acid), δ2.77-3.56 (m, 4H of polyethylenimine). 5.3% of the amine in PEI were succinated. Percentage of succinic acid modification was calculated by:

$$Percentage\ of\ succination = \frac{1H\ peak\ of\ succinic\ acid}{1H\ peak\ of\ PEI} \times 100\%$$

3.3.7 Non-covalent Functionalization of SWCNT by PEI-SA

5 mg of PEI-SA was weighed in a tube and 20 mL of deionized water was added to dissolve the polymer. Then, 5 mg of CNT was added into the polymer solution. The

solution was sonicated for 1 hour. The undissolved CNT were removed by vacuum filtration with a 0.22 μm Nylon filter. Then, the unbound polymer was removed by using an ultra-15 centrifugal filter units (100 kDa MWCO, Amicon, Billerica, MA). The concentrated PEI-SA/CNT (IS/C) was lyophilized and a black solid was recovered (28.2%). Percentage of recovery was calculated as follow:

$$Percentage\ of\ recovery = \frac{weight\ of\ IS/C\ recovered}{weight\ of\ CNT + weight\ of\ PEI - SA} \times 100\%$$

3.3.8 Transmission Electronic Microscope (TEM)

IS/C was dissolved in water and dropped onto a copper grid. After 20 minutes, the solution was removed and the grid was dried under air. The sample was analyzed by a Philips CM 10 Transmission Electron Microscope (Philips, Eindhoven, the Netherlands) operating at 80 kV with a 40 μm aperture.

3.3.9 Gel Shift Assay

Equal volumes containing 0.5 μg of siRNA and the desired amount of IS/C were mixed and incubated for 30 minutes. The resulting complexes were electrophoresed at 100 mV using 1.5% agarose gel and EtBr in TAE buffer. After 20 minutes, the gel was removed, visualized under UV lamp and the picture was taken with an Olympus C8080 digital camera (Olympus, Center Valley, PA).

3.3.10 Zeta Potential

IS/C/siRNA complexes were prepared by mixing equal volumes containing siScramble (5 μg) and desired amount of IS/C, then the resulting solution was incubated for 30 minutes. The solution was then transferred to a disposable capillary cell and was analyzed using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK) with He-Ne laser (633nm). An energy output of 10 mW with automatic laser attenuation was used for measurements. The mean and standard derivation of zeta potentials were calculated with average of 10 runs using automatic algorithm.

3.3.11 *In vitro* Gene Silencing in B16-F10

B16-F10 cells were seeded in a 12-well plate with density of 1.2×10^5 cells/well in 1 mL culture media 24 hours before transfection. IS/C/siRNA complexes were made by mixing equal volumes of siRNA and 5 times IS/C (w/w), then the solution was incubated for 30 minutes. Then the IS/C/siRNA solution was transferred to serum containing media in cell culture to have a final concentration of 2 µg/mL of siRNA. 24 hours after transfection, the RNA was extracted using the Trizol method and cDNA was synthesized. The mRNA expressions of Braf in the samples were quantified with qRT-PCR in a Stratagene MX 3005p QRT-PCR systems (Mississauga, ON) using β-Actin as a reference. The reaction condition was 10 min at 95°C, followed by 40 cycles with 30 s at 95°C, 1 min at 62°C and 1 min at 72°C. The primers for Braf and β-Actin were:

Braf, 5'-CAATTGGCTGGGACACGGACAT-3' (forward) and 5'-TTGACAACGGAAACCCTGGAAAAG-3' (reverse);

β-Actin, 5'-AGGGAAATCGTGCGTGACATCAAA-3' (forward) and 5'-ACTCATCGTACTCCTGCTTGCTGA-3' (reverse).

3.3.12 Cytotoxicity in B16-F10

24 hours before transfection, B16-F10 cells were seeded in a 24-well plate with density of 5×10^4 cells/well in 500 µL culture media. The media were replaced with culture media with desired amount of IS/C and PEI. The cells were then returned to incubation for 24 hours. Media were collected and the cells were typsinized, collected and re-suspended in PSB with 2% FBS and 5 µg/mL 7AAD. The cells were then analyzed with flow cytometry. Percentage of cell death was calculated by the 7AAD positive cells in the treated cells minus the 7AAD positive cells in the untreated cells which were considered as the background cell death.

3.3.13 Proliferation Assay for B16-F10 cells

B16-F10 cells were seeded in a 6-well plate with density of 2.4×10^5 cells/well in 2 mL culture media. 1 µg/mL of Braf siRNA was used for silencing B16-F10 cells with 2 µL/µg Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). 24 hours after

transfection, the cells were trypsinized and reseeded into a 96 well plate (500 cells per well). The cells were then incubated for 96 hours and media were removed. 20 mg/mL of MTT (Calbiochem, Gibbstown, NJ) in serum free media was added and incubated for 3 hours. The media were removed and DMSO was added to dissolve the crystals. The absorbance at 570 nm was recorded using a Tecan Infinite M1000 PRO (Manndorf, Switzerland).

3.3.14   Topical siRNA Delivery and *in vivo* Gene Silencing IS/C

CD-1 mice were anaesthetized and shaved. In the no incubation treatment, 6 μg of Cy3-labelled siGAPDH was mixed with glycerol (50% in final solution v/v), DMSO (10% in final solution v/v) and nuclease free water. The desired amount of IS/C was then mixed with the siRNA solution and the mixture was applied onto the mice's skin immediately. For IS/C/siRNA with incubation, equal volumes of Cy3-labelled siGAPDH (6 μg) and desired amount of IS/C was mixed and incubated for 30 minutes. After the IS/C/siRNA solutions were mixed with glycerol (50% in final solution v/v), DMSO (10% in final solution v/v) and nuclease free water before the solution was immediately applied to the mice's skin. The area of application is around 0.25 cm^2 (0.5 cm x 0.5 cm). The mice were sacrificed 4 hours after the application and the skin was cleaned with water and then removed for cryosectioning and staining with H&E. The penetration of Cy-3 labeled siRNA into the skin was observed by fluorescent microscopy.

To determine the efficacy of gene silencing, the same no incubation method was carried out expect that the mice were sacrificed 24 hours after the application instead of 4 hours. The skin was used for RNA extraction by Trizol method with the aid of a homogenizer. cDNA was synthesized and the mRNA levels of GAPDH was quantified by qRT-PCR using β-Actin as reference. qRT-PCR was carried out using the same procedure described above and the sequence of the GAPDH primers were: GAPDH: 5'-GGGGTGAGGCCGGTGCTGAGTAT-3' (forward), 5'-CATTGGGGTAGGAACACGGAAGG-3' (reverse).

3.3.15 Topical siRNA Delivery and Gene Silencing in Tumor in Melanoma Bearing Mice

Melanoma bearing mice were generated by inoculating $2x10^5$ B16-F10 cells intra-dermally into C57BL/6. Seven days after tumor inoculation, mice were anesthetized and IS/C/siRNA without incubation was applied onto the tumor. To test for siRNA penetration, the tumor was removed twenty four hours after siRNA application and frozen in OCT for cryosectioning. Images were obtained using a fluorescence microscope (Olympus BX51, Olympus Canada Inc., ON, Canada)

The same procedure was applied for topical siRNA delivery but the tumor was treated for 48 hours. RNA was isolated using the Trizol method for qRT-PCR and protein was extracted for western blotting.

3.3.16 Treatment of Melanoma Bearing Mice Using IS/C and Braf siRNA

Melanoma bearing mice were generated by inoculating $2x10^5$ B16-F10 cells intra-dermally into C57BL/6. Three days after tumor inoculation, mice were anesthetized and randomized according to the observed size of the appearance of black pigment. IS/C (12 μg), siRNA (6 μg, siBraf or siScramble), glycerol (50% in final solution v/v), DMSO (10% in final solution v/v) were mixed without incubation and was applied onto the tumor every other day. The tumor sizes were measured with caliper. Twenty five days after tumor inoculation, the mice were sacrificed and tumors were removed. The tumor weights were measured with a 2-digit electronic balance.

3.3.17 Statistics

Data were expressed as mean± standard error or standard deviation of the mean and the results were analyzed a by one way ANOVA followed by Dunnett's test as post-test, student's t-test or two way ANOVA followed by Bonferroni's test as post-test. $p < 0.05$ was considered statistically significant. All the data analysis was performed in GraphPad Prism.

3.4   Results

3.4.1 Synthesis and Characterization of PEI-SA and PEI-SA/CNT (IS/C)

It has been shown that branched PEI is not effective in delivering siRNA due to its strong complexation with the siRNA. In order to increase the siRNA protection, PEI-SA was synthesized (Fig. 3.1) [33]. In initial work, both 5% and 10% modification of the amines was investigated and it was found that 5% amine modification was better for CNT dispersion. Therefore, subsequent work focused on the preparation and study of this polymer. We found that the percentage of succination was more consistent if the reaction was performed overnight instead of for 3 hours. Following removal of unreacted succinic acid by dialysis, ^{1}H NMR spectroscopy of the product (Fig. 3.2) showed that peaks corresponding to the methylene protons of PEI (2.7-3.6 ppm) and the proton of succinic acid were present (2.49 ppm), confirming the successful modification of PEI with succinic acid.

$$R=-CH_2CH_2NR_2; \ -CH_2CH_2NHR; \ -CH_2CH_2NH_2$$
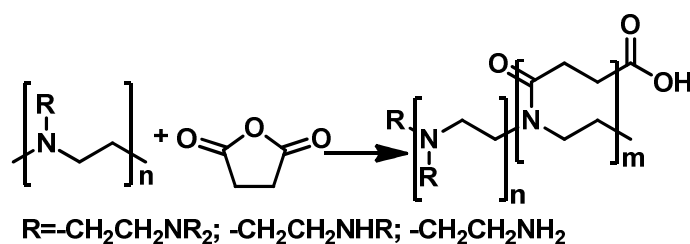
Figure 3.1 Scheme of synthesis of PEI-SA

Reaction scheme of PEI-SA. PEI was reacted with succinic anhydride overnight to form polyethylenimine-succinic acid (PEI-SA). PEI-SA was isolated by dialysis followed by lyophilization.
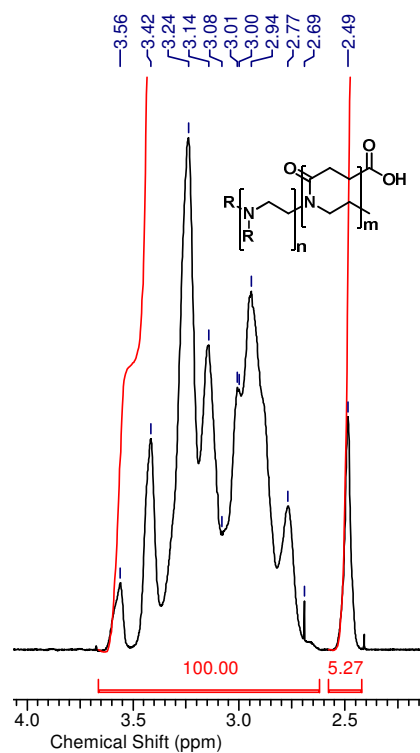
Figure 3.2 ^{1}H-NMR of PEI-SA

^{1}H-NMR of PEI-SA the polymer was dissolved in D_2O and the acquisition delay was 5 second.

Non-covalent functionalization is an effective and simple way to obtain water soluble CNT [37]. In order to obtain a water soluble CNT which is able to carry siRNA, we functionalized CNT using PEI-SA (IS/C) [27]. TEM (Fig. 3.3) showed that IS/C was singly dispersed with a length ranging from 200 nm to 1 μm.
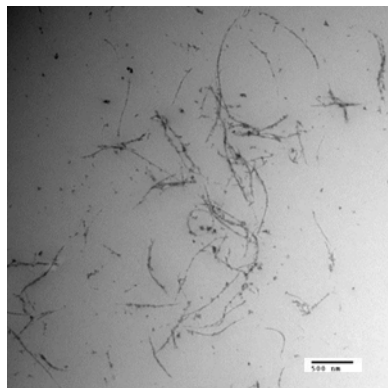
Figure 3.3 TEM micrograph of IS/C

Minimum amount of IS/C solution was added to the copper grid and was air-dried. The length of the SWCNT ranges from 200 nm to 1 µm.

The siRNA binding capacity of IS/C was demonstrated by gel shift assay. The free siRNA migrates along the gel while the migration of siRNA bound to IS/C is slowed down or totally stopped. In addition, the bound siRNA cannot bind to ethidium bromide effectively and thus the fluorescence intensity of the bound siRNA would be reduced. The results showed that the amount of IS/C required to neutralize the charge of siRNA is 2:1 (w/w) as unbound siRNA was not observed at this ratio (Fig. 3.4).
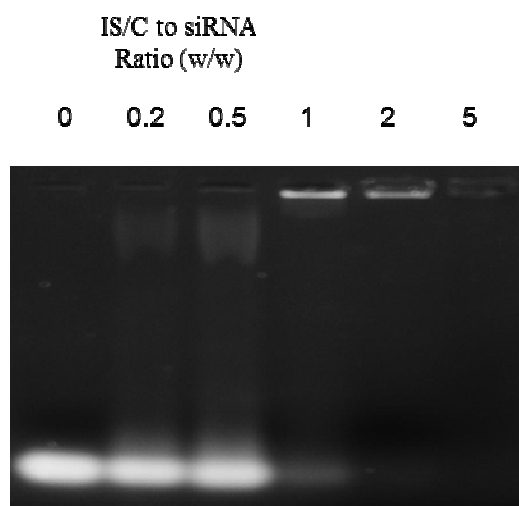
Figure 3.4 Gel shift assay of siRNA with IS/C

IS/C/siScramble complexes are made by mixing equal volume of IS/C and siScramble. IS/C was diluted to various concentrations while siScramble concentration is fixed. The complexes were incubated for 30 minutes at room temperature and the samples were loaded into agarose gel in TAE buffer (1.5%, w/v) with EtBr. The complexes were subjected to electrophoresis for 30 minutes. Then the gel was taken out for illumination under UV. The weight ratio of siRNA condensed by IS/C is 2:1.

The stability of IS/C and siRNA complexes was studied using zeta potential measurements. The higher the magnitude of the zeta potential, the more stable the complexes would be due to electrostatic repulsion. The result showed that the IS/C/siRNA complexes have a strongly positive zeta potential, which indicate good colloidal stability (Fig. 3.5).
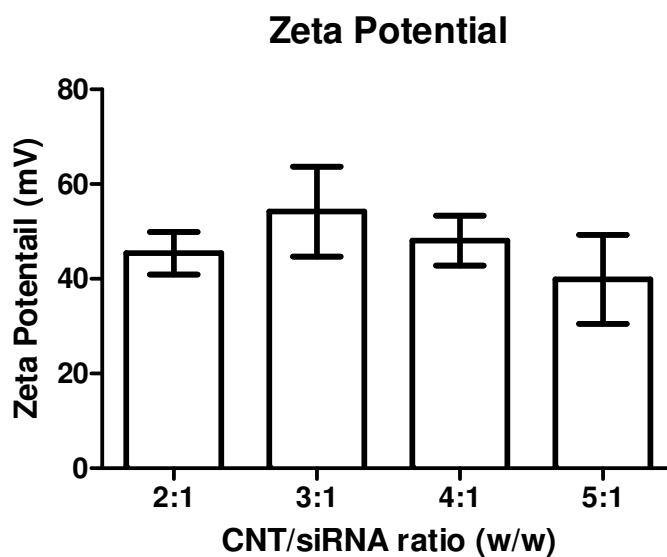
Figure 3.5 Zeta potential of IS/C/siRNA

The sample was dispersed in deionized water. The experiment was done with 10 runs and the error bar is zeta deviation.

3.4.2 *In vitro* Transfection and Cytotoxicity of IS/C on B16-F10

It has been shown that PEI conjugated with CNT could increase the transfection efficiency of PEI [22, 38]. To reveal the potential of IS/C in siRNA delivery, we examined the efficacy of gene silencing using siBraf delivered by IS/C in B16-F10 cells. Compared to untreated cells (Fig 3.6), the Braf expression of cells transfected with IS/C and siScramble have no significant difference. The cells transfected with IS/C and siBraf have significant difference between siScramble treated and untreated cells. The percentage of Braf down regulation was found to be 80% by qRT-PCR. The cytotoxicity of IS/C was determined in B16-F10 and PEI 10k (M_n) was used as a comparison. IS/C has a lower cytotoxicity compared to PEI in the tested concentrations (Fig 3.7).
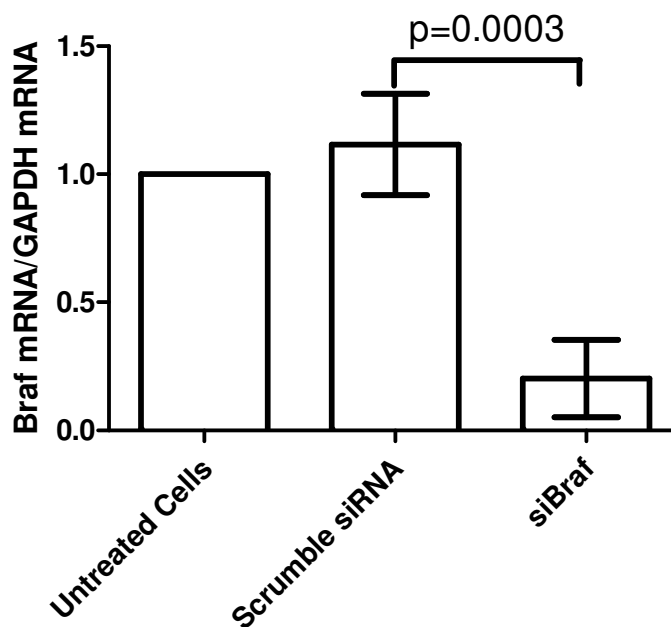
Figure 3.6 *In vitro* gene silencing using IS/C

B16-F10 cells were transfected with IS/C and siBraf or scramble siRNA. 24 hours after transfection, the RNA was isolated with Trizol method and cDNA was synthesized. The gene expression was done by quantitative RT-PCR. Transfections were done in serum containing media. Error bar=SD, n=4.
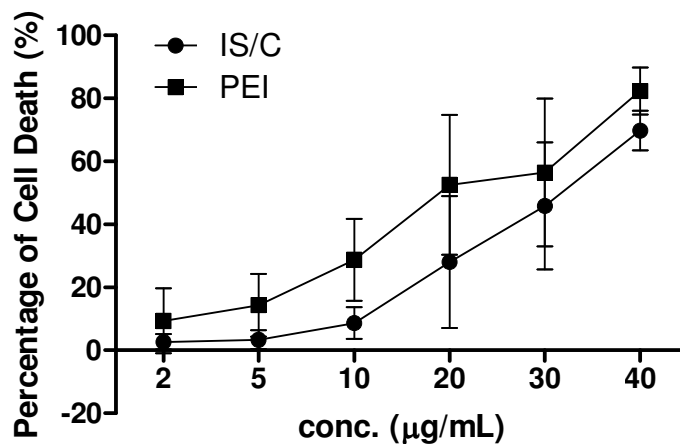
Figure 3.7 Cytotoxicity of IS/C

B16-F10 cells were treated with various amounts of IS/C and PEI in serum containing media. 24 hours after treatment, the cells were trypsinized and collected for 7AAD staining followed by flow cytometry analysis. Percentage of cell death was calculated by the 7AAD positive cells in the treated cells minus the 7AAD positive cells in the untreated cells. n=3, Error bar=SD

3.4.3 Cell Proliferation of Braf siRNA treated B16-F10 cells

Next, we examined whether the knockdown of Braf by siRNA affects the cell proliferation, it is a kinase which could phosphorylate MEK [39], it is a critical molecule in the MAPK pathway and which leads to tumor growth [40], may influence B16-F10 melanoma cell proliferation. MTT interacts with CNT and therefore it is not a reliable cytotoxic assay for the cell proliferation [41], we tested cell proliferation of B16-F10 after gene silencing of Braf using Lipofectamine 2000 instead of using IS/C (Fig. 3.8). Compared to untreated cells and cells transfected with scramble siRNA, siBraf reduced

the proliferation of B16-F10 cells by almost 60%. Taken together, these data suggest that IS/C is effective in delivery of siRNA. Induction of Braf gene silencing in B16-F10 cells results in inhibition of tumor cell proliferation *in vitro*.
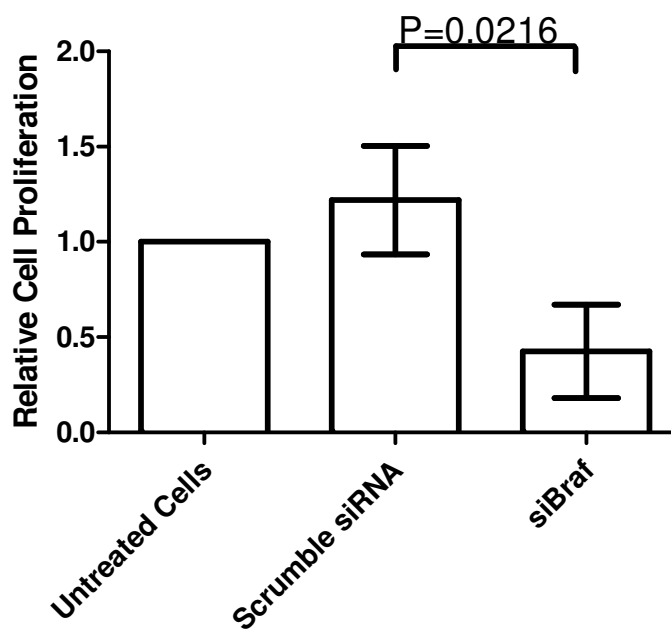
Figure 3.8 Cell proliferation of B16-F10 by MTT

B16-F10 cells were transfected with siBraf and lipofectamine 2000 and after 24 hours, cells were trypsinized and 500 cells were seeded into 96 wells plates. The cells were then incubated for additional 96hr. Then media were replaced with serum free media with 10% MTT and the cells were incubated for 3 more hours. Media were removed and DMSO was added to wells and the absorbance at 570 nm was recorded. n=6; error bar=SD (* indicates p<0.05 by two tail student's t-test)

3.4.4 Topical siRNA Delivery and Gene Silencing to CD-1 Mice Skin with IS/C

CNT have been used for transdermal drug delivery. However, they have not been used for transdermal siRNA delivery. In order to investigate the potential of using IS/C to deliver siRNA into skin, IS/C and siRNA was topically applied on CD-1 mice. Untreated mouse skin was used as negative control (Fig. 3.9a) and Cy-3 labeled siRNA was used as the background control (Fig. 3.9b). Recently developed by our lab, Lipofectamine 2000 with 10% DMSO and 50% glycerol was used as a positive control [42] (Fig. 3.9c) PEI-SA with 10% DMSO and 50% glycerol was used as a comparison (Fig 3.9d&e). Fluorescence was observed 4 hours after the application was chosen with reference to other researchers in topical siRNA delivery [43]. siRNA was not incubated with PEI-SA. Interestingly, PEI-SA does not enhance the penetration of siRNA into skin regardless of the ratio tested (2:1 and 5:1). IS/C with different IS/C to siRNA ratios were tested with 10% DMSO and 50% glycerol. IS/C were incubated with siRNA (Fig. 3.9f-h) and no incubation was used as comparison (Fig. 3.9i-k) for transdermal delivery. For the incubated IS/C/siRNA, as the ratio of IS/C increases, the siRNA penetration into the skin reduced. For IS/C without siRNA incubation, 3 of the tested ratios have similar depth of siRNA penetration into the skin. The result demonstrated that polymer alone cannot effectively deliver siRNA topically while IS/C is able to deliver siRNA. Therefore, CNT are crucial to facilitate the transdermal siRNA delivery. The gene silencing of IS/C with siGAPDH and siScrabmle on skin were also carried out. The result showed around 50% of GAPDH was down regulated (Fig. 3.10).
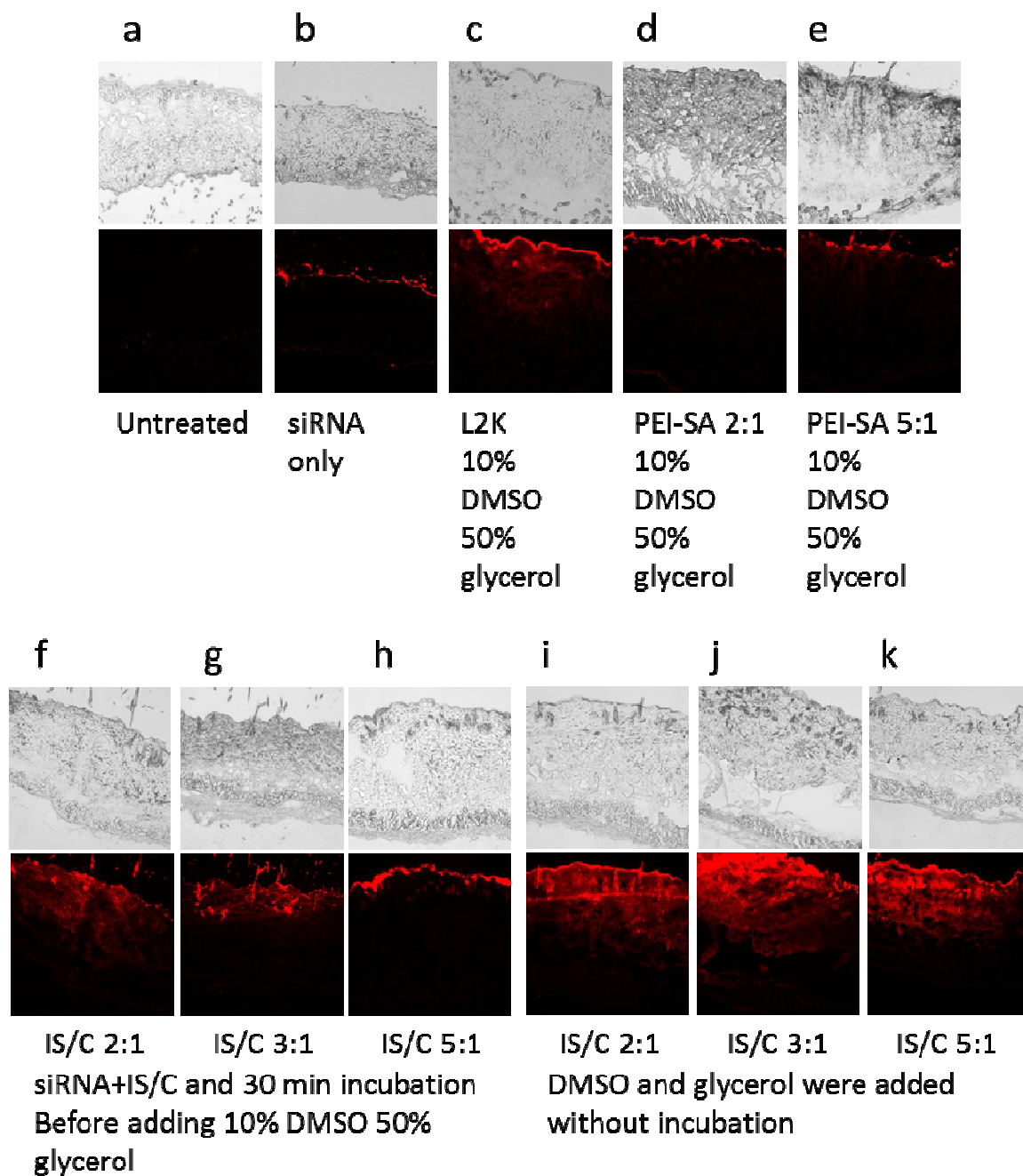
Figure 3.9 Topical siRNA delivery by IS/C in CD-1 mice

The mice were anesthetized and shaved. siRNA was labeled with Cy-3 fluorescence dye, the siRNA solutions were applied onto the skin. The mice were sacrificed 4 hours after siRNA solution application. The skin samples were removed and cryosectioned. The photos were taken with florescence microscope. (a) Untreated skin as negative control;

(b) siRNA only as Cy-3 labeled siRNA only control; (c) lipofectatmine 2000 (L2K) as positive control. PEI-SA with a 5:1 (d) and 2:1 (e) ratios were used to compare with the one without CNT. IS/C with different ratios and with different preparation method were compared. siRNA and IS/C were incubated for 30 minutes and then mixed with DMSO and glycerol solution for (f), (g) and (h) (IS/C to siRNA ratio 2:1, 3:1 and 5:1) while siRNA were mixed with DMSO and glycerol solution. Then IS/C was added to the solution and immediately applied to the mice skin for (i) (j) and (k) (IS/C to siRNA ratio 2:1, 3:1 and 5:1). 10% DMSO and 50% glycerol was added from (c) to (k) as transdermal enhancer. Compared to polymer alone, CNT is important for topical siRNA delivery. Incubation of IS/C and siRNA reduced the skin penetration of siRNA.
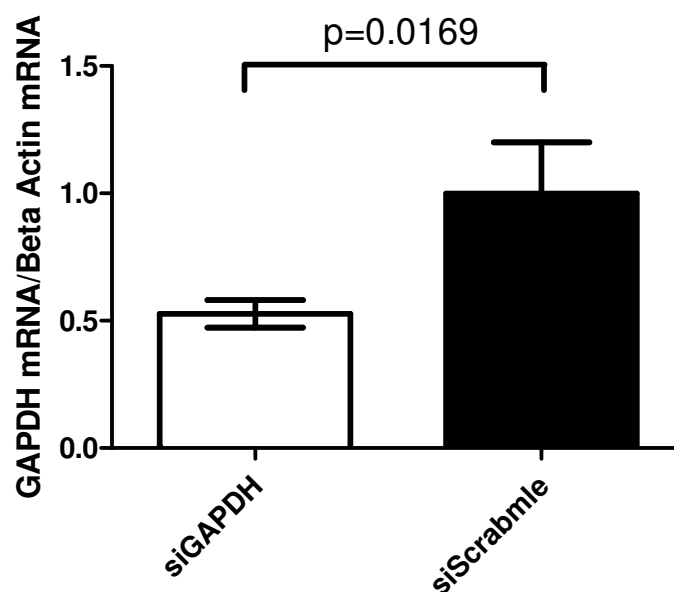
Figure 3.10 Topical gene silencing with IS/C and siGAPDH

The gene silencing on skin was quantified by qRT-PCR. mRNA expression of GAPDH was normalized with the mRNA expression of β-Actin. n=3, error bar=SEM

3.4.5 Gene silencing in melanoma using IS/C

To investigate the feasibility of using IS/C to deliver siRNA into melanoma, we generated a murine melanoma model by inoculating B16-F10 cells into C57BL/6 mice. After tumors formed, we applied IS/C-siRNA once locally to the tumor-bearing mice. The siRNA penetration into the tumor was observable (Fig. 3.11).

The qRT-PCR result showed that the gene down regulation for the siBraf was over 70%, while the siScramble and untreated tumors had no significant difference in Braf expression (Fig. 3.12). Western blot of the tumor samples also showed similar results (Fig. 3.13). Taken together, these results suggested that topical application of IS/C with siBraf is capable of down-regulating Braf in tumors in terms of both RNA level and protein level.
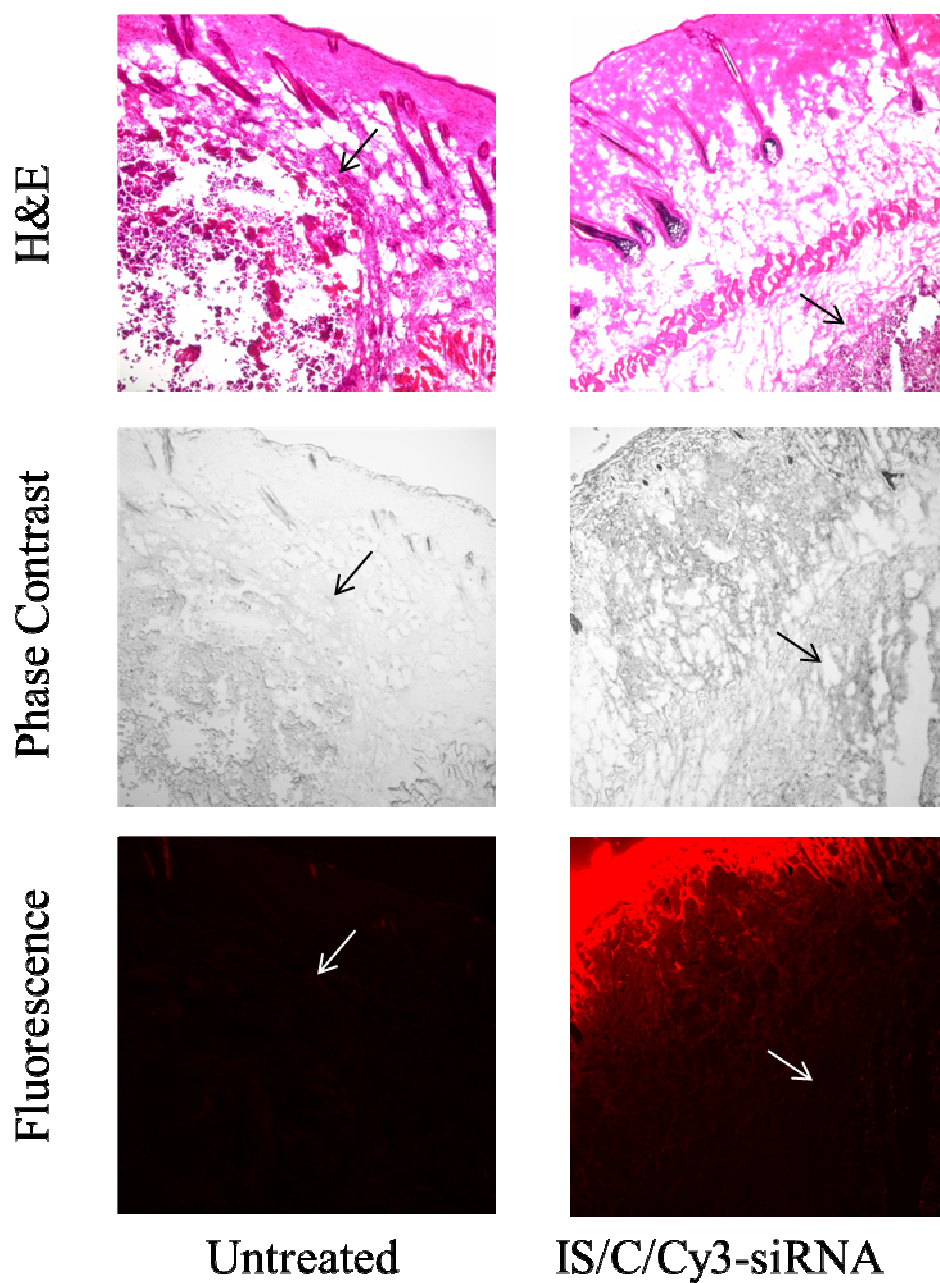
Figure 3.11 Topical delivery of siRNA to tumor

Tumor bearing mouse was applied with ISC/Cy3-labeled siRNA complexes. 24 hours after the application, the tumor was removed and frozen in OCT. It was cryosectioned and the photos were taken with fluorescence microscope. Photo on the left is an untreated tumor sample while photo on the right is the topically treated tumor. The arrow indicated the tumorous tissue (large nucleus, reduced cytoplasm).
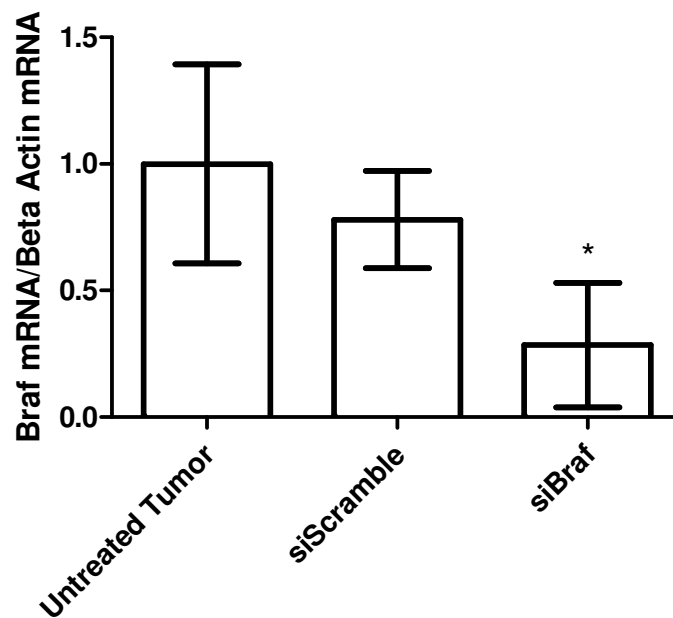
Figure 3.12 Gene silencing of topically treated tumor with IS/C/siBraf by qRT-PCR

Tumor bearing mice were treated with IS/C/siBraf, 2 days later, the mice were sacrificed and the tumor samples were removed. The tumor samples were homogenized and the RNA were isolated by Trizol method for qRT-PCR. n=6; Error bar=SEM (* indicates $p < 0.05$ by one way ANOVA, followed by Dunnett's test as posttest)

a)

Braf   85 kDa

GAPDH   42 kDa

b)
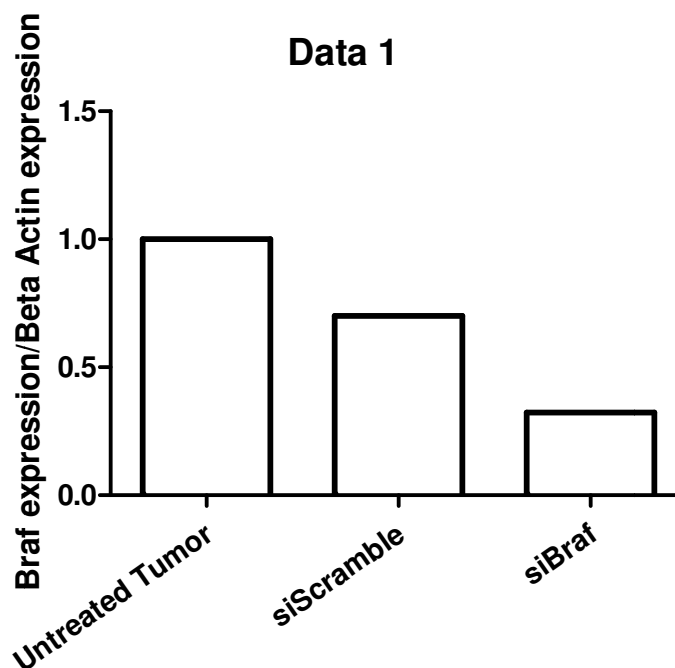
Figure 3.13 Gene silencing of topically treated tumor with IS/C/siBraf by western blot

Tumor bearing mice were treated with IS/C/siRNA, 2 days later, the mice were sacrificed and the tumor samples were removed. (a) The tumor samples were homogenized and the protein was isolated with RIPA buffer for western blot. Representative image was showed. (b) Quantification of the protein band intensity of the western blot by photoshop.

3.4.6 Treatment of Melanoma Through Topical siRNA Delivery of Braf siRNA using IS/C

Braf is an important gene in the MAPK pathway, which is responsible for regulating cell growth and proliferation. To test the feasibility whether topically knocking down of Braf may inhibit melanoma progression, we treated melanoma-bearing mice with IS/C and Braf siRNA. The tumor size is significantly different for the IS/C/siBraf from tumor treated with IS/C/scrambled siRNA. The tumor growth was inhibited significantly but the tumors did not regress. There is no significant difference between untreated tumor and tumor treated with IS/C/scrambled siRNA (Fig. 3.13). A similar trend was observed in the average tumor weight when the mice were sacrificed on day 25. The final tumor weight was not significantly different between untreated mice and IS/C/scrambled siRNA treated mice while the IS/C/siBraf treated tumor was dramatically reduced (Fig. 3.14). These data imply that IS/C/siBraf is able to reduce the tumor progression by Braf down-regulation.
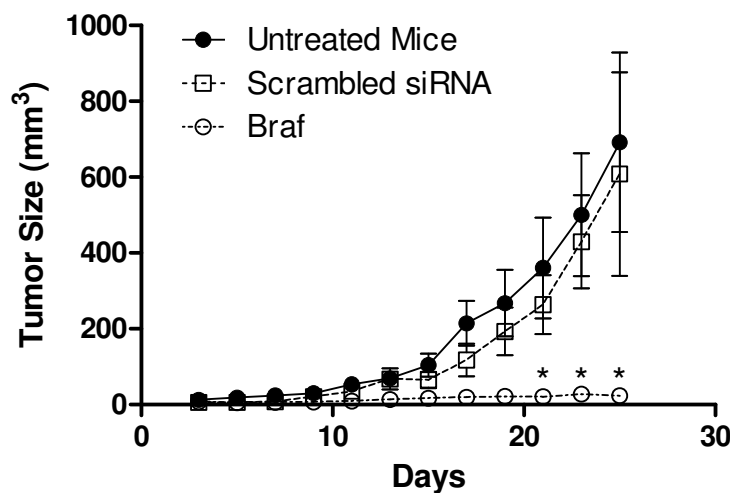
Figure 3.14 Tumor size of tumor bearing mice topically treated with IS/C/siBraf

3 days after the mice were inoculated with tumor, the mice were topically applied with IS/C/siRNA solution for every 2 days. The tumor size of mice was estimated by the

length and width of the tumor by a caliber in nearest 0.5 mm. The sizes were calculated with $\pi(LxW^2)/6$. 2-way ANOVA with Bonferroni's test as post-test showed that significant difference between the scramble control and the IS/C/siBraf group. Significant difference of tumor volume begin from 21st day (p<0.05), to 23rd day (p<0.001) and 25th day (p<0.001). n=6 for untreated mice and siScramble, n=7 for siBraf; error bar=SEM
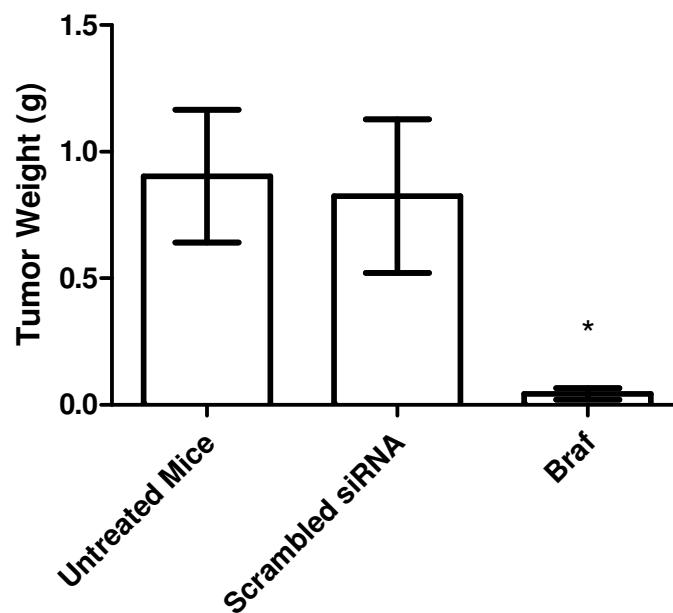
Figure 3.15 Tumor weight of tumor bearing mice topically treated with IS/C/siBraf

3 days after the mice were inoculated with tumor, the mice were topically applied with IS/C/siRNA solution for every 2 days. The tumor weight of mice was removed and weighted on the day when the mice were sacrificed (Day 25). n=6 for untreated mice and siScramble, n=7 for siBraf; error bar=SEM (Asterisk indicated p<0.05, samples analyzed by 1-way ANOVA followed by Dunnett's test as post-test by comparing with siScramble group)

3.5   Discussion

Transdermal delivery of therapeutics has many advantages over oral or systematic

delivery. There are fewer digestive enzymes, the pH is not extreme, and the treatment is both non-invasive and can be self-administered. If it is a topical pathological condition, topical delivery is more efficient due to higher bioavailability because of the proximity. This can also result in lower systematic toxicity. However, the epidermis of skin, especially the SC, prevents most of the diffusion of exogenous chemicals. Even though the siRNA passed the SC barrier, it cannot gain entry into the cells effectively because the cell membrane is another barrier. Both siRNA and cell membranes are anionic and therefore tend to repel each other [44, 45]. Thus, an efficient transdermal siRNA delivery vector is required. Transdermal siRNA delivery has been carried out and some success has been attained [43, 46]. CNT have been shown to enhance transdermal drug delivery [7, 9].

Cutaneous melanoma is a carcinoma developed on skin, and in human cutaneous melanoma, Braf is usually overexpressed and mutated which is well-studied that it is responsible for cell proliferation [47]. Braf inhibitor has been used in clinical trials, but resistance to it has been observed [48], therefore an alternative approach for Braf inhibition for treating or reducing the tumor progression is needed. siRNA is a good candidate because it induces post transcriptional gene silencing which would not circumvent drug resistance. Braf silencing could be the alternative approach to reduce tumor progression.

Vertically aligned CNT can act as nano-spear for drug delivery [49] but the safety of insoluble CNT is an issue because its accumulation and retention can be toxic and induce undesirable immune responses [50]. Therefore, we functionalized the CNT to make them water soluble and biocompatible. Our design was to disperse the CNT with a water soluble polycation PEI to test if the CNT and the siRNA delivering capacity of succinated PEI are compatible such that a good transdermal siRNA delivery can be achieved.

Novel non-covalently functionalized SWCNT were developed for siRNA delivery *in vitro* and topically *in vivo*. CNT have been used for siRNA delivery [22, 26] as well as topical drug delivery [7-9], however, they have not been used for topical delivery for siRNA. The SC is the stumbling block for topical therapeutic agent delivery due to its

hydrophobic nature. CNT have a high aspect ratio and they are hydrophobic, thus it might enhance the penetration of macromolecules.

We succinated the amine of PEI to make PEI-SA and interestingly, the yield is higher than 100%. It is highly possible that PEI is a hygroscopic polymer and water cannot be totally removed by 2 days lyophilization. PEI-SA was characterized using ^{1}H NMR spectroscopy and it was found that 5% of the amines were modified with succinic acid. The CNT were dispersed with PEI-SA by a sonication method and the water soluble CNT were recovered by removing the insoluble CNT and the unbound polymer by ultrafiltration. IS/C is singly dispersed CNT with length ranging from 200 nm-1000 nm. The CNT were shortened by sonication. IS/C condense the siRNA at a ratio of 2:1 (w/w). Moreover, its complex with siRNA has a stable zeta potential in the tested ratios. It is important to have a high magnitude of zeta potential so the siRNA is well protected as well as being stable as water soluble suspension. IS/C successfully delivered siRNA *in vitro* into B16-F10 cells and the Braf expression of were reduced by 80%. The cytotoxicity of IS/C is lower than PEI alone. It is possible that for IS/C, the cytotoxicity is induced mostly because of the polymer.

The topical delivery of siRNA with IS/C was demonstrated by observing the fluorescence of Cy-3 labelled siRNA 4 hours after topical siRNA application. The duration was optimized by a previous study using siRNA with glycerol and DMSO in our lab, the fluorescence between 1 to 4 hours is similar while the fluorescence attenuated for longer duration. Interestingly, siRNA cannot penetrate into the skin with PEI-SA alone, while IS/C is very effective in delivering siRNA into the skin. It is probably due to the size of CNT such that the siRNA wrapped on top of CNT can effectively enter into the dermis. Another notable observation is that the IS/C incubated with siRNA has less penetration compared to IS/C added to siRNA without incubation. Although both of them deliver siRNA into the skin, no incubation is better than incubated IS/C/siRNA. The reason for the reduced transdermal capacity after incubation is unknown. It is possible that the IS/C act as transdermal enhancer or the initial formation of IS/C/siRNA complexes has a higher transdermal activity. For later experiments, we used a IS/C: siRNA ratio of 2:1 with 10% DMSO and 50% glycerol without incubation. Both DMSO and glycerol act as

transdermal enhancer, however, DMSO and glycerol alone cannot achieve efficient siRNA skin penetration. IS/C, on the other hand, can deliver siRNA into the skin and into the tumor. There is a significant gene down regulation after IS/C/siRNA was applied to both skin and tumor. It is interesting that the percentage of gene down regulation in tumors was higher than the skin, which is probably because tumor cells take up the IS/C/siRNA complexes more actively compared to cells in the dermis.

Topical siRNA delivery into cutaneous melanoma for tumor growth inhibition in a mice model was previously demonstrated by Tran *et al* [36]. However, they used sonoporation for enhanced transdermal activity. We set out to demonstrate the same concept by using Braf siRNA and carbon nanotubes without sonoporation. It is known that Braf is not mutated in B16-F10 cells [51], but in humans, the cell proliferation of wild type Braf plays a role in Craf activation [52]. The cell proliferation experiment also demonstrated that Braf downregulation in B16-F10 cells reduced the proliferation compared to the control. A similar trend was observed for the tumor size of the IS/C/siBraf treated mice. The inhibition was significant compared to the scramble control as well as untreated tumor. In the observed period (25 days), the tumors of the IS/C/siBraf treated group had not progressed significantly compared to control groups. As a proof of concept experiment on utilizing CNT for siRNA delivery, our experimental was designed to maximize the effect of siRNA topical therapy and to try to exploit the most promising feature of topical delivery, the ease of administration and a more frequent schedule of reagent administration. The promising feature of this siRNA delivery system is that the IS/C could enhance the siRNA delivery without using instruments for skin permeation enhancement. One possible advancement upon this delivery system could be the development of a transdermal patch instead of using applied siRNA solution. A continuous release of drug with the physical protection of the patch could be beneficial for long lasting gene down regulation, which would also circumvent the trouble of frequent applications.

Cutaneous melanoma is a highly invasive disease. The patients' delays as well as diagnostic delay are important factors for impeding early diagnosis and treatment [53]. To compensate the delay, it would be ideal if there were a topical drug delivery system to

suppress the growth of melanoma or even induce tumor regression if immediate surgical removal is not possible. The patients can apply the drug themselves easily and tumor growth can be slowed down immediately, improving patient the survival. Based on our animal experiments, this topical siRNA delivery system might be able to carry out the above mentioned function and provides a possible alternative to current melanoma treatment. Further experiment on the transdermal behavior in an artificial human skin is needed for the testing the therapeutic potential of this delivery system. Although in the clinical settings, most of the early stage melanoma was treated by surgical removal, it would be beneficial to control the growth of late stage melanoma. The topical application of siRNA targeting normal Braf is a local delivery and the possible side effect could be minimal, however, the inhibition of normal Braf would affect the Braf function of healthy tissue. The possible improvement on the therapy in future experiments to circumvent the side effect would be using a combination of mutated Braf (V600E for example) and MEK/ERK silencing [54], Akt3 [36] or mTOR silencing [55] for reducing tumor progression. Another possibility to utilize this transdermal siRNA delivery system is for melanoma prevention. The concept was demonstrated by Chung et al. with an inhibitor [S,S'-1,4-phenylenebis(1,2-ethanediyl)bis-isoselenourea] (PBISe) [56]. Furthermore, this topical siRNA delivery system can potentially be used for treating skin diseases such as dermatitis.

3.6   Conclusion

A novel non-covalently functionalized SWCNT with IS/C for topical siRNA delivery was developed. The polymer for dispersing the CNT and the IS/C were characterized. The capacity for delivering siRNA *in vitro* and topically *in vivo* was demonstrated. The potential of utilizing this CNT for RNAi therapy was further tested with Braf siRNA on a melanoma model. Significant tumor progression reduction was observed in a 25 day interval. Thus, this novel topical siRNA delivery system has a potential to be used as tumor progression inhibition in a clinical setting.

3.7   Reference

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Chapter 4

4   Targeted siRNA Delivery with a Folic Acid Conjugated Single-Walled Carbon Nanotube for Cancer Therapy

4.1   Summary

RNA interference (RNAi) can specifically regulate the gene expression, but efficient and targeted delivery of small interfering RNA (siRNA) *in vivo* to pathological cells or tissue is difficult. It has been shown that modified carbon nanotubes (CNT) protect siRNA and facilitate its entry into cells. Folate receptor (FR) is overexpressed in cancer and it has a high binding constant with folic acid.

Single walled carbon nanotubes (SWCNT) were functionalized by non-covalent association with a folic acid conjugated polyethylenimine (PEI). PEI was modified with polyethylene glycol (PEG) and succinic acid. This product was used to disperse CNT and water soluble CNT were isolated for siRNA delivery. *In vivo* siRNA delivery was done by intravenous injection to melanoma bearing mice and mTOR siRNA (si-mTOR) was used to test in vivo gene silencing and anti-cancer therapy since mTOR is usually overexpressed in cancer.

The polymers as well as the CNT with folic acid (FGIS/C) and without folic acid (GIS/C) were characterized. The structural, biophysical, and biological properties of FGIS/C and GIS/C and their complexes formed with siRNA were investigated. We found significant uptake of siRNA as well as gene silencing in tumor by FGIS/C. Treatment with FGIS/C/si-mTOR resulted in attenuation of tumor growth in a murine melanoma model.

In conclusion, a novel functionalized targeted CNT was developed for cancer siRNA delivery, which siRNA was delivered *in vivo* to a murine melanoma model. The new delivery method has provided a possibility for cancer treatment, which could provide insight into the potential application and development of CNT-based antisense-based therapy.

4.2   Introduction

RNAi was discovered in 1998 [1] and it can be induced by siRNA. It is a powerful tool and is an attractive method for research. It can be used for treating disease specificity with a known target, however, the clinical application of this technology is still limited. Systematic siRNA delivery to pathological cells or tissue is still ineffective and it remains as the bottleneck of the application [2-6].

CNT were discovered in 1991 [7] and various modifications or functionalizations have been utilized to increase the solubility of CNT because pristine CNT (p-CNT) are not soluble in most common solvents [8]. Water soluble CNT have been functionalized [9-11] and some of them have been explored for various biomedical applications [12-14]. CNT have been used for nucleic acid delivery [15-17], such as small siRNA delivery [18-29]. It has been reported that CNT can gain entry into cells by a diffusion-like mechanism, in which the CNT penetrate into cells like "nano-needles". The cellular entry mechanism of CNT are not limited to diffusion-like mechanism but depend on the type and the manner in which they are functionalized and also the dimensions of CNT [30, 31].

mTOR is an important gene/protein in the downstream of PI3K/AKT pathway, which is responsible for regulating cell growth and proliferation and its inhibitors have been used for treating cancer in clinical trials [32]. FR are usually overexpressed in cancer [33, 34] and it can increase the specific binding of the folic acid (FA) conjugated components to cells which express high level of FR [35]. B16 generated melanoma expresses FA in an *in vivo* mice model [36]. FA has been attached to CNT for drug delivery [37]. Previously, we reported a non-covalently functionalized CNT with succinated PEI (IS/C) for topical siRNA delivery. To further exploit this siRNA delivery system for delivering siRNA to cancer, we set out to modify it with PEG and FA such that PEG can reduce the toxicity, increase the serum stability and have a higher target specificity of the CNT/siRNA complexes. The modified polymer and the CNT were characterized. This feasibility of using this targeted siRNA delivery system to reduce cancer progression was examined on a murine melanoma model.

4.3   Materials and Methods

4.3.1 Chemicals

Single-walled carbon nanotoubes were purchased from Nano-C (Batch PT1112-60, MA, USA) and succinic anhydride was purchased from Alfa Aesar (MA, USA). Polyethylenimine (PEI, M_w 15476, M_n 7893), polyethylene glycol (PEG, M_n 2000), polyethylene glycol methyl ether (mPEG, M_n 2000), folic acid (FA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and N-Hydroxysuccinimide (NHS) were purchased from Sigma Aldrich (St. Louis, MO). Solvents were purchased from Caledon (Georgetown, Canada) and other chemicals for synthesis were purchased and used without purification from Sigma Aldrich unless otherwise specified.

4.3.2 siRNAs

Luciferase GL2 Duplex used as scramble siRNA (siScramble) and mTOR siRNA (si-mTOR) were obtained from Cell Signaling Technology, Inc. (catalogue number 6332, Danvers, MA).

4.3.3 Cell Culture

B16-F10 and KB cells were obtained from ATCC (Manassas, VA). B16-F10 cells and KB cells were cultured with DMEM (Gibco, Life technologies, Burlington, ON, Canada) with 10% FBS (Gibco) at 37^oC and humidified atmosphere.

4.3.4 Animals

C57BL/6 mice were obtained from Charles River (Canada). All animals were housed under pathogen-free conditions. All experiments were done in accordance to the *Guide for the Care and Use on Animals Committee Guidelines*. The animal protocol was approved by the Animal Use Subcommittee (AUS) at Western University.

4.3.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

Polymer was dissolved in D_2O (99%, Sigma) or $CDCl_3$ (99%, Sigma) and was transferred to an NMR tube. ^{1}H nuclear magnetic resonance (NMR) spectra were obtained with

INOVA 600 spectrometer (Agilent, Santa Clara, CA) (600MHz). The spectra were recorded at room temperature and the D_2O peak (4.7 ppm) was used as reference.

4.3.6 Size Exclusion Chromatography (SEC)

The liquid chromatography system was equipped with a Waters Separations Module 2695 (Waters, Mississauga, ON), a Refractive Index Detector (Waters 2414) and three PLaquagel-OH 40 8µm (300x7.5mm) columns (Polymer Laboratories, Waters) connected in series and to a PLaquagel-OH 8 µm guard column. 0.2 M ammonium acetate/acetic acid (pH 5.3) was eluted at 1 mL/min at room temperature for 35 min/ run. Samples were prepared at a concentration of 10 mg/mL in millique water, filtered through 0.2 µm Supor membrane filters and injected with a 100 µl volume loop. The calibration curve was obtained from PEO/PEG standards and the molecular weight was calculated by Empower 3 software (Waters). The following standard molecular weights were used: 615, 1010, 3930, 12140, 20000, 31380, 71700, 106500.

4.3.7 Synthesis of Polymers

α,ω-diamino poly(ethylene glycol) (PEG-NH$_2$) (**1b**) was synthesized based on Elbert's method [38] and α-carboxyl-ω-methyloxy poly(ethylene glycol) (mPEG-COOH) (**2b**) was synthesized based on Zalipsky's method [39].

4.3.7.1   Synthesis of α,ω-diamino poly(ethylene glycol) (PEG-NH$_2$)

PEG-NH$_2$ was synthesized based on Elbert's method [38] using α,ω-dihydroxyl poly(ethylene glycol) (M_n 2000, Sigma Aldrich). ^{1}H-NMR (CDCl$_3$, 600MHz): δ 2.89 (t, J=5.3 Hz, 4H,-OCH$_2$C$\underline{H}_2$NH$_2$ for both ends), δ 3.53-3.77 (m, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG), 84% statistical conversion which is calculated by:

$$\frac{\text{Integral of 1H} - \text{NMR peak of } proton\ next\ to\ amine/4}{\text{Integral of 1H} - \text{NMR peak of PEG}/180} \times 100\%$$

4.3.7.2   Synthesis of Folate Functionalized PEG (FA-PEG)

0.12 g (0.28 mmol) of folic acid, 0.7 g (3.6 mmol) of EDC HCl and 0.5 g (4.3 mmol) of NHS were dissolved in 15 mL of DMSO. Then the mixture was stirred for 15 minutes.

The mixture was then added dropwise into a solution of 0.46 g (0.23 mmol) of PEG-NH$_2$ dissolved in 5 mL DMSO. The reaction mixture was stirred at room temperature overnight and then was dialyzed using a 3.5 kDa molecular weight cut-off (MWCO) Spectra regenerated cellulose membrane (Spectrum Laboratories, Rancho Domingo, USA) against deionized water for 48 hours. A yellowish solid was recovered after lyophilization (0.46 g; 81.4% in mole). ^{1}H-NMR (D$_2$O, 600MHz): δ 2.07-2.40 (m, 4H, -CH$_2$CH$_2$- in glutamic acid of folic acid), 3.18-3.84 (m, 180H, -OCH$_2$CH$_2$- in PEG), δ 6.71-6.87 (m, 2H, ortho protons in the benzene of folic acid), δ 7.59-7.72 (m, 2H, meta protons in the benzene of folic acid), δ 8.66-8.75 (m, 1H, proton in pteridine). Mole ratio of folic acid modification is 0.8:1 (FA : PEG) and was calculated by: (Integral of 1H − NMR peak of *piteridine proton*): (Integral of 1H − NMR peak of PEG/180)

4.3.7.3   Synthesis of Folate Functionalized PEG Succinic Acid (FA-PEG-SuOH)

0.2 g (84 μmol) of FA-PEG was dissolved in 5 mL of DMSO. 0.17 g (1.70 mmol) of succinic anhydride was dissolved in 5 mL of DMSO and was added to the FA-PEG solution. The reaction mixture was stirred at room temperature overnight and then was dialyzed using a 3.5 kDa MWCO regenerated cellulose membrane against deionized water for 48 hours. A yellowish solid was recovered after lyophilization (0.12 g; 58.1% in mole). ^{1}H-NMR (D$_2$O, 600MHz): δ 2.31 (s, 6H, ethylene proton from succinic acid and ethylene proton from glutamic acid in folic acid), δ 2.64 (s, 2H, ethylene proton of succinic acid adjacent to amide), δ 3.32-3.62 (m, 180H, -OCH$_2$CH$_2$- in PEG), δ 6.58-6.81 (m, 2H, ortho protons in the benzene of folic acid), δ 7.48-7.63 (m, 2H, meta protons in the benzene of folic acid), δ 8.56-8.90 (m, 1H, proton in pteridine).

4.3.7.4   Synthesis of Folate Functionalized PEG-PEI (FA-PEG-PEI)

57 mg (22 μmol) FA-PEG-SuOH was dissolved in 5 mL DMSO. 79 mg (41 μmol) of EDC HCl and 7.6 g (66 μmol) of NHS were added. The mixture was stirred for 15 minutes. 0.22 g (220 μmol) PEI was dissolved in DMSO and the activated FA-PEG solution was added dropwise into the PEI solution. The reaction mixture was stirred at room temperature overnight and then was dialyzed using a 15 kDa MWCO regenerated

cellulose membrane (Spectrum Laboratories) against deionized water for 48 hours. A yellowish solid was recovered after lyophilization (0.22 g; 77.8% by weight). ^{1}H-NMR (D$_2$O, 600MHz): δ 2.43-3.42 (m, -NRC$\underline{H}_2$C$\underline{H}_2$- from PEI), δ 3.42-3.67 (m, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG), δ 6.78-6.85 (m, 2H, ortho protons in the benzene of folic acid), δ 7.62-7.70 (m, 2H, meta protons in the benzene of folic acid), δ 8.59-8.65 (m, 1H, proton in pteridine). SEC: M$_n$ = 15471 g/mol, M$_w$ = 36868 g/mol, PDI = 2.38. Weight ratio of PEI to PEG is 4.15 : 1 which is calculated as follow:

$$Ratios\ of\ PEI:PEG = \frac{\text{Integral of 1H}-\text{NMR peak of PEI/43 (FW of PEI monomer)}}{\text{Integral of 1H}-\text{NMR peak of PEG/44 (FW of PEG monomer)}}$$

4.3.7.5 Synthesis of Folate Functionalized Succinated PEG-PEI (FA-PEG-PEI-SA)

0.12 g (9.20 µmol) of FA-PEG-PEI was dissolved in 1 mL of water. NaCl (25 mg) was added and the pH was adjusted to 5 by the addition of 12 M HCl. Then, 11.0 mg (0.11 mmol) of succinic anhydride was dissolved in 1 mL of DMSO and was added drop-wise into the PEI solution. The reaction mixture was stirred at room temperature overnight and then was dialyzed using a 15 kDa MWCO regenerated cellulose membrane against 0.5 M NaCl solution for 4 hours followed by 44 hours with deionized water. The water was changed 3 times per day. A yellowish product was obtained after lyophilization (0.18 g; 72.5% by weight). ^{1}H-NMR (D$_2$O, 600MHz): δ 2.19-3.38 (m, -NRC$\underline{H}_2$C$\underline{H}_2$- from PEI and ethylene proton from succinic acid), δ 3.44-3.67 (m, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG), 6.68-6.81 (m, 2H, ortho protons in the benzene of folic acid), δ 7.57-7.66 (m, 2H, meta protons in the benzene of folic acid), δ 8.57-8.65 (m, 1H, proton in pteridine). Percentage of amine conversion is 7.3% which is calculated by the following equation:

$$\frac{integral\ of\ 1H\ peak\ (PEI+succinic\ acid\ in\ GIS)-integral\ of\ 1H\ (PEI\ peak\ in\ PEG-PEI)}{integral\ of\ 1H\ (PEI+succinic\ acid\ peak\ in\ GIS)} \times 100\%$$

4.3.7.6 Synthesis of α-carboxyl-ω-methyloxy ether poly(ethylene glycol) (mPEG-COOH)

2b was synthesized based on Zalipsky's method [39] using methyloxy ether poly(ethylene glycol) (M$_n$ 2000, Sigma Aldrich). Sodium was used instead of potassium

tert-butoxide. ^{1}H-NMR (CDCl$_3$, 600MHz): δ 3.38 (s, 3H, -OCH$_3$), δ 3.58-3.82 (m, 180H, OC$\underline{H}_2$C$\underline{H}_2$ in PEG), δ 4.15 (s, 2H) 99% conversion.

4.3.7.7 Synthesis of PEG-PEI

50 g (25 μmol) of PEG-COOH was dissolved in 5 mL DMSO, and then 97 mg (0.5 mmol) of EDC HCl and 61 mg (0.53 mmol) of NHS were added. The mixture was stirred for 15 minutes. 0.25 g (25.4 μmol) PEI was dissolved in DMSO and the activated PEG solution was added dropwise into the PEI solution. The reaction mixture was stirred at room temperature overnight and then was dialyzed using a 15 kDa MWCO regenerated cellulose membrane against deionized water for 48 hours. White solid was recovered after lyophilization (0.28 g; 93.2% by weight). ^{1}H-NMR (D$_2$O, 600MHz): δ 2.44-3.48 (m, -NRC$\underline{H}_2$C$\underline{H}_2$- from PEI), δ 3.66-3.70 (m, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG), δ 3.92 (s, 2H, -OC$\underline{H}_2$CO-). SEC: M$_n$ = 17515 g/mol, M$_w$ = 30693 g/mol, PDI = 1.75. Weight ratio of PEI to PEG is 4.15 : 1 which is calculated as follow:

$$Ratios\ of\ PEI{:}PEG = \frac{\text{Integral of 1H−NMR peak of PEI/43 (FW of PEI monomer)}}{\text{Integral of 1H−NMR peak of PEG/44 (FW of PEG monomer)}}$$

4.3.7.8 Synthesis of PEG-PEI-SA

137 mg (14.4 μmol) of PEG-PEI was dissolved in 10 mL H$_2$O. NaCl (0.25 g) was added and the pH was adjusted to 5 by the addition of 12 M HCl. Then, 13 mg (1.3 mmol) of succinic anhydride was dissolved in 10 mL of DMSO and was added drop-wise into the polymer solution. The reaction mixture was stirred at room temperature overnight and then was dialyzed using a 15 kDa MWCO Spectra regenerated cellulose membrane against 0.5 M NaCl solution for 4 hours followed by 44 hours with deionized water. The water changed 3 times per day. The product was then lyophilized and a white solid was recovered (0.18 g; 118% by weight). ^{1}H-NMR (D$_2$O, 600MHz): δ 2.38-3.40 (m, -NRC$\underline{H}_2$C$\underline{H}_2$- from PEI), δ 3.66-3.68 (m, 180H, -OC$\underline{H}_2$C$\underline{H}_2$- in PEG). Percentage of amine conversion is 7.2% which is calculated by:

$$\frac{integral\ of\ 1H\ peak\ (PEI+succinic\ acid\ in\ GIS) - integral\ of\ 1H\ (PEI\ peak\ in\ PEG-PEI)}{integral\ of\ 1H\ (PEI+succinic\ acid\ peak\ in\ GIS)} \times 100\%$$

4.3.7.9 Non-covalent Functionalization of SWCNT by FGIS or GIS

5 mg of FGIS or GIS was weighed in a tube and 20 mL of deionized water was added to dissolve the polymer. Then, 5 mg of CNT was added into the polymer solution. The solution was sonicated for 15 minutes at 60 degree Celsius and then vortexed. This step was repeated 6 times. The undissolved CNT were removed by vacuum filtration with a 0.22 μm Nylon filter. Then, the unbound polymer was removed by using ultra-15 centrifugal filter units (100 kDa MWCO, Amicon, Billerica, MA). The concentrated CNT solution was centrifuged to remove solid residues and then lyophilized and a grey yellowish or a grey solid was recovered (22.1% for FGIS/C and 25.9% for GIS/C). The recovery was calculated as follow:

$$Percentage\ of\ recovery = \frac{weight\ of\ FGIS/C\ or\ GIS/C\ recovered}{weight\ of\ CNT + weight\ of\ FGIS\ or\ GIS} \times 100\%$$

4.3.8 Transmission Electron Microscope (TEM)

FGIS/C or GIS/C was dissolved in water and dropped onto a copper grid. After 20 minutes, the solution was removed and the grid was dried under air. The sample was analyzed by a Philips CM 10 Transmission Electron Microscope (Philips, Eindhoven, the Netherlands) operating at 80 kV with a 40 μm aperture.

4.3.9 Gel Shift Assay

Equal volumes containing 0.5 μg of siRNA and the desired amount of FGIS/C or GIS/C were mixed and incubated for 30 minutes. The resulting complexes were analyzed by electrophoresis at 100 mV using 1.5% agarose gel and EtBr in TAE buffer. After 20 minutes, the gel was removed, visualized and the picture was recorded with FluroChem M (Protein simple, Santa Clara, CA).

4.3.10 Zeta Potential

FGIS/C/siRNA or GIS/C/siRNA complexes were prepared by mixing equal volumes containing siScramble (5 μg) and desired amount of CNT solution, then the resulting solution was incubated for 30 minutes. The solution was then transferred to a disposable capillary cell and was analyzed using a Malvern Zetasizer Nano ZS (Malvern,

Worcestershire, UK, He-Ne laser (633nm)). An energy output of 10 mW with automatic laser attenuation was used for measurements. The mean and standard deviation of zeta potentials were calculated with two measurements of the average of 10 runs using automatic algorithm.

4.3.11   Cellular Uptake of siRNA by KB cells

KB cells were seeded in a 24-well plate with a density of $5x10^4$ cells/well in 0.5 mL respective culture media for folate containing media. The same cell density was used for folate free condition. The cells were washed 3 times with PBS and then resuspended folate free RPMI 1640 (Gibco). Then, the cells were incubated overnight in 37 ^{o}C and humidified atmosphere. FGIS/C/siRNA and GIS/C/siRNA complexes were made by mixing equal volumes of Cy3-labeld si-mTOR and 5 times FGIS/C or GIS/C (w/w), 2 µg/mL of siRNA was used for transfecting the cells. The solution was incubated for 1 hr. Then the FGIS/C/siRNA and GIS/C solutions were transferred to serum containing media. 1 hr after transfection, the cells were washed by PBS, trypsinized and analyzed by flow cytometry.

4.3.12   Cell Proliferation of B16-F10 by MTT

B16-F10 cells were seeded in a 6-well plate with density of $2.4x10^5$ cells/well in 2 mL culture media. 2 µg/mL of lipofectamine 2000 and 1 µg/mL of si-mTOR was used for silencing B16-F10 cells. 24 hours after gene silencing, the cells were trypsinized and reseeded into a 96 well plate (500 cells per well). The cells were then incubated for 96 hours and media were removed. MTT (Calbiochem, Gibbstown, NJ) in serum free media (20 mg/mL) was added and incubated for 3 hours. The media were removed and DMSO was added to dissolve the crystals. The absorbance at 570 nm was recorded using a Tecan Infinite M1000 PRO (Manndorf, Switzerland).

4.3.13   Cytotoxicity of FGIS/C and GIS/C in B16-F10 cells

B16-F10 cells were seeded in a 24-well plate with density of $5x10^4$ cells/well in 500 µL culture media 24 hours before transfection. The media were replaced with culture media containing the desired amount of FGIS/C or GIS/C. The cells were then returned to

incubation for 24 hours. The media were collected and the cells were typsinized, collected and re-suspended in PSB with 2% FBS and 5 µg/mL 7AAD. The cells were then analyzed with flow cytometry. Percentage of cell death was calculated by the 7AAD positive cells in the treated cells minus the 7AAD positive cells in the untreated cells which were considered as the background cell death.

4.3.14 *In vivo* Delivery of siRNA with FGIS/C and GIS/C to Melanoma Bearing Mice

Melanoma bearing mice were generated by inoculating $2x10^5$ B16-F10 cells subcutaneously into C57BL/6 mice. 7 days after tumor inoculation, 100 µg of Cy3-labelled siRNA and 300 µg of FGIS/C or GIS/C were injected into the mice intravenously. 24 hours after injection, the mice were sacrificed and the tumor was removed and frozen in OCT. The tumor was crycosectioned, stained with H&E and observed under fluorescence microscope (Olympus BX51, Olympus Canada Inc., ON, Canada).

4.3.15 Treatment of Melanoma Bearing Mice using FGIS/C and mTOR siRNA

Melanoma bearing mice were generated by inoculating $2x10^5$ B16-F10 cells subcutaneously into C57BL/6 mice. 4 days after tumor inoculation, mice were randomized and 300 µg of FGIS/C or GIS/C and 100 µg siRNA (si-mTOR or siScramble) in 5% glucose were injected into mice. There were 4 groups of mice, untreated mice, FGIS/C/si-mTOR, FGIS/C/siScramble and GIS/C/si-mTOR. The tumor sizes were measured with a caliper every two days and the mice were injected with CNT/siRNA once each week. 20 days after tumor inoculation, the mice were sacrificed and tumors were removed. The tumor weights were measured with a 2-digit electronic balance. Tumor was frozen for later use. Tumor was frozen for later use.

4.3.16 Statistics

Data were expressed as mean± standard error or standard deviation of the mean and the results were analyzed a by one way ANOVA followed by Dunnett's test as post-test, student's t-test or two way ANOVA followed by Bonferroni's test as post-test. $p < 0.05$

was considered statistically significant. All the data analysis was performed in GraphPad Prism.

4.4   Results

4.4.1 Synthesis and Characterization of FA-PEG-PEI-SA (FGIS) and FA-PEG-PEI-SA/CNT (FGIS/C)

The synthesis of FA-PEG-PEI-SA (FGIS) was performed as shown Fig. 4.1. First, hydroxyl-terminated PEG was converted to the amine-terminated PEG by first mesylation and then displacement of the mesylate with ammonia according to a previously reported procedure [38]. The amine functionalized PEG was then reacted with 1 equivalent of folic acid in the presence of the coupling agent DEC to provide FA-PEG. The remaining amine on FA-PEG was then reacted with succinic anhydride to provide FA-PEG-SuOH. It should be noted that as the initial reaction with folic acid provides a statistical mixture of PEG with two amines (~25%), one amine and one folic acid (~50%), and finally two molecules of folic acid (~25%), the resulting FA-PEG-SuOH was also expected to be a statistical mixture containing PEG with two succinic anhydries (~25%), one succinic hydride (~25%)and one folic acid (~50%) and two molecules of folic acid (~25%). FA-PEG-SuOH was then coupled to PEI to provide FGI, and finally this PEI conjugate was reacted with succinic anhydride to provide FGIS. ^{1}H-NMR spectroscopy of FGIS showed the successful modification (Fig. 4.3). The weight ratio between PEI to PEG was determined to be 4.1 and the percentage of succination was 7.3% which was determined based on the integrals of the PEI peaks (δ 2.5-3.5) before succination and after succination because of the overlap of PEI and succinic acid peaks. FGI was analyzed by SEC (Fig. 4.4) and was found to have a M_n of 15471 and M_w of 36868 in comparison to the starting PEI, which had a M_n of 7893 and a M_w of 15476. It is likely that a small degree crosslinking occurred during the conjugation of FA-PEG-SuOH to the PEI, but this should not be detrimental to the CNT binding or transfection capabilities. The detail of the molecular weight was summarized in Table 4.1.

The synthesis of PEG-PEI-SA (GIS) was performed as shown in Fig. 4.2. Methoxylated PEG was converted to the carboxylic acid PEG-terminated PEG by reacting the alcohol

with potassium to form an alkoxide and then reacted with the *t*ert-butyl bromoacetate with reference to a previous report [39]. The PEG acid was then reacted with PEI by EDC coupling to provide PEG-PEI. PEG-PEI was then reacted with succinic acid to provide GIS. ^{1}H-NMR spectroscopy of GIS showed the successful modification (Fig. 4.3). The weight ratio between PEI to PEG was determined to be 4.2 and the percentage of succination was 7.2% which was again, determined based on the integrals of the PEI peaks (δ 2.5-3.5) before succination and after succination because of the overlap of PEI and succinic acid peaks. PEG-PEI was analyzed by SEC (Fig. 4.4) and was found to have a M_n of 17515 and a M_w of 30693 (Table 4.1).
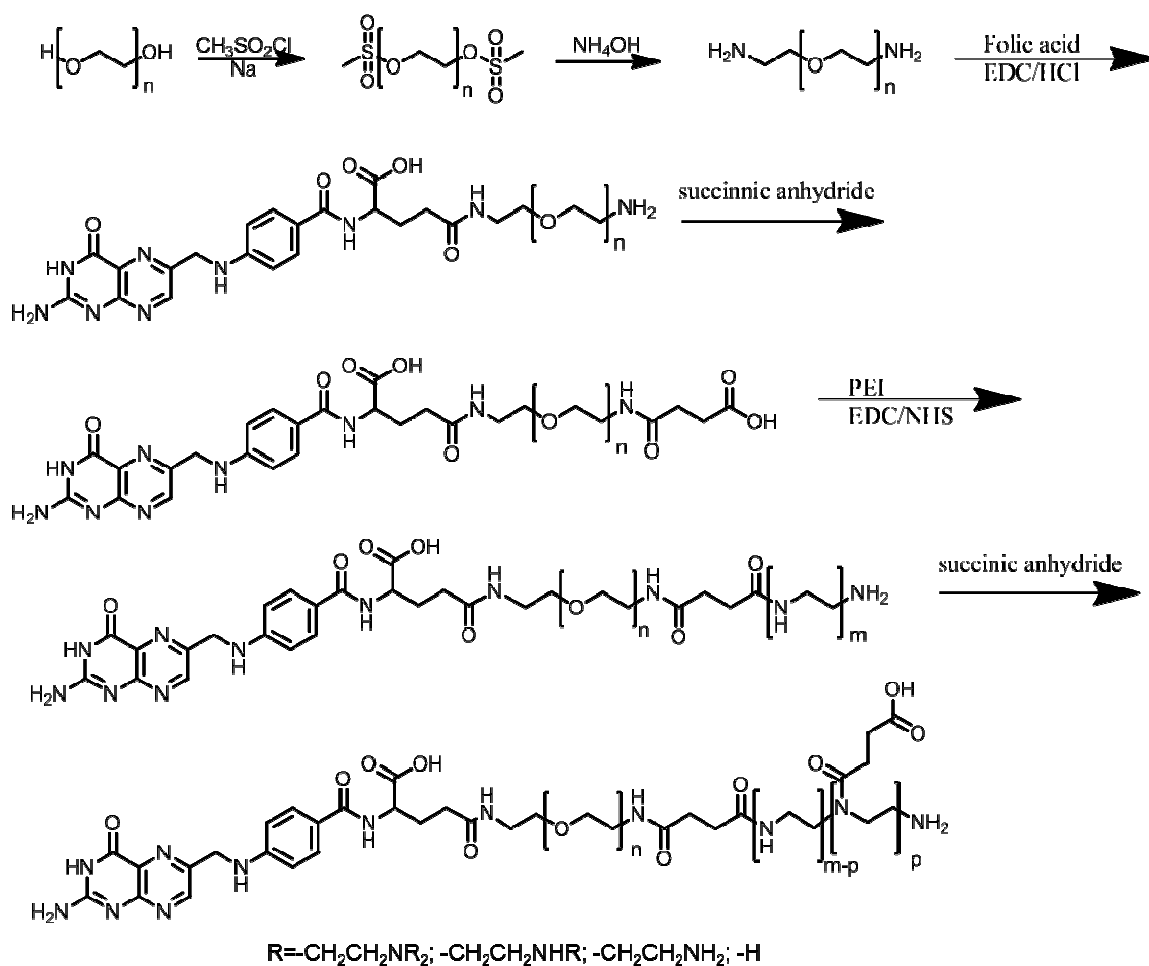

R=-CH$_2$CH$_2$NR$_2$; -CH$_2$CH$_2$NHR; -CH$_2$CH$_2$NH$_2$; -H
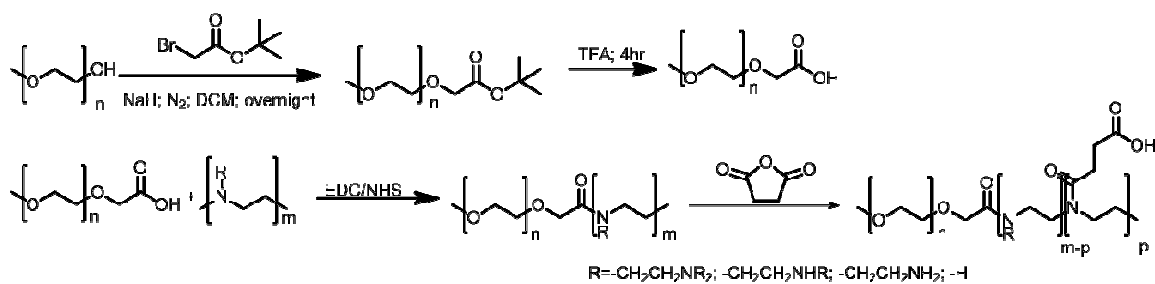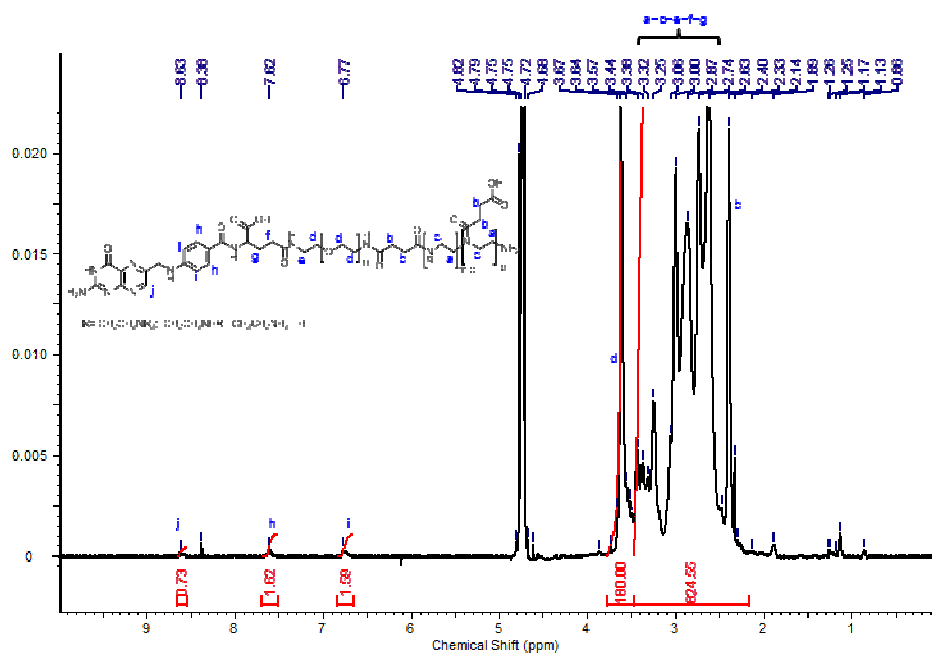
Figure 4.1 Scheme of synthesis of FA-PEG-PEI-SA (FGIS)

Figure 4.2 Scheme of synthesis of PEG-PEI-SA (GIS)
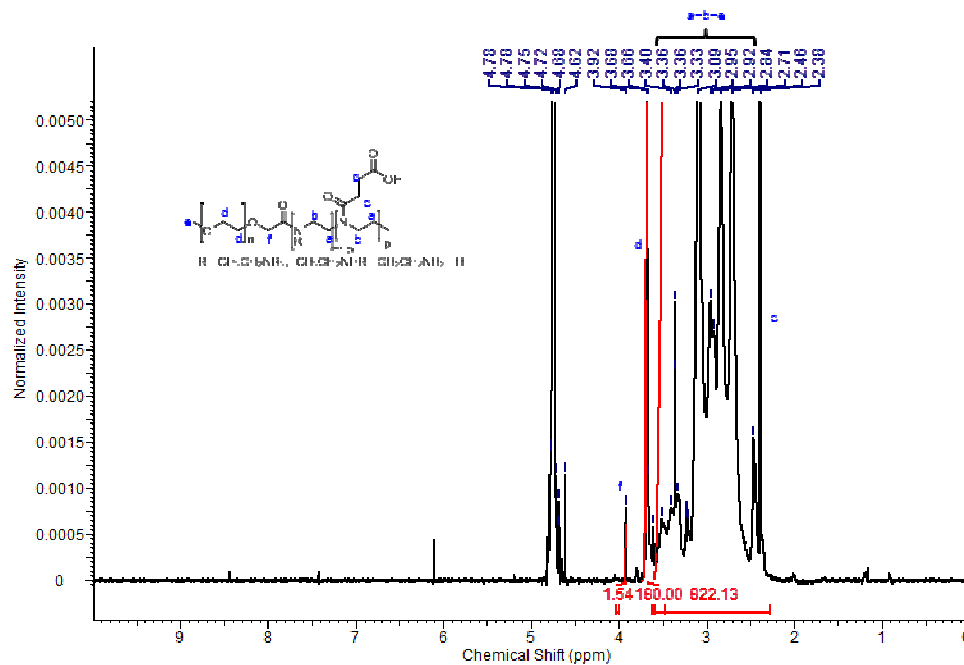
Figure 4.3 ^{1}H-NMR of FGIS and GIS
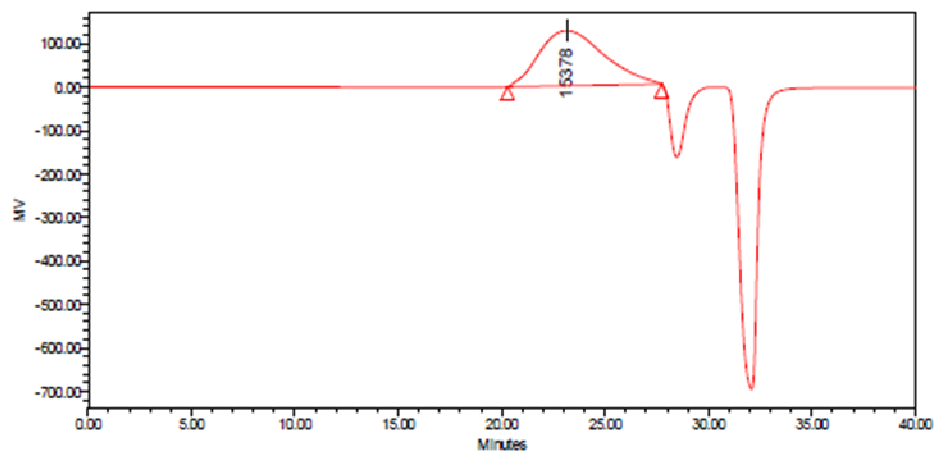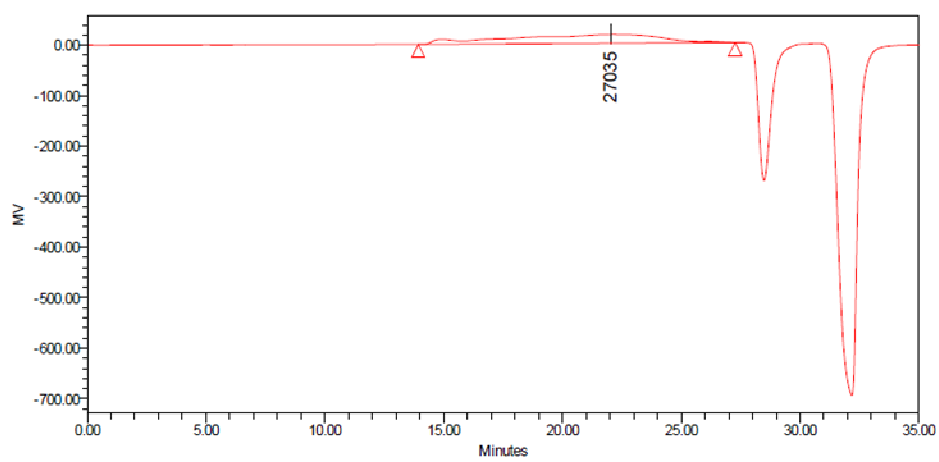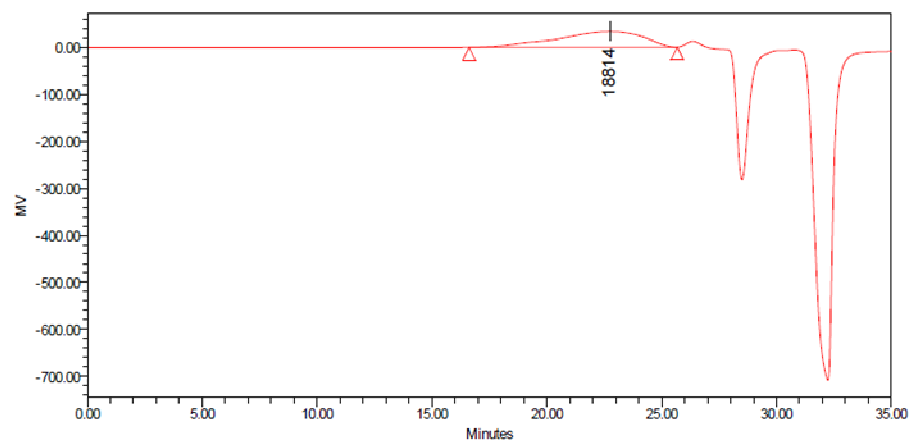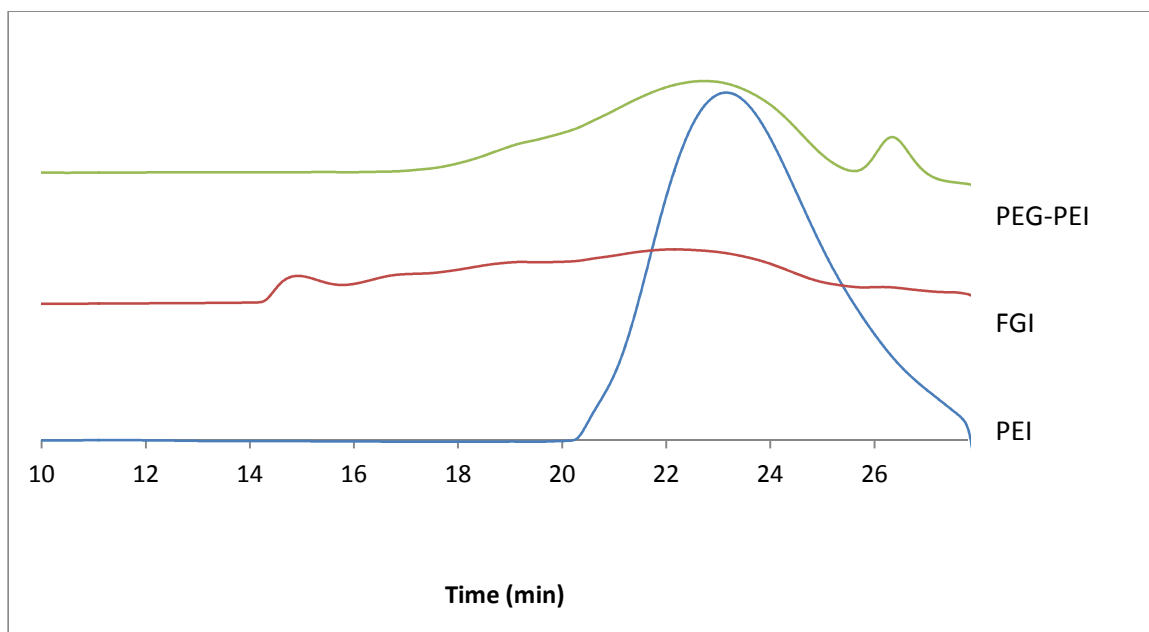
Figure 4.4 SEC of PEI, FGI and PEG-PEI

SEC of PEI, FGI and PEG-PEI. PEI on the top, followed by FGI and PEI-PEI. The bottommost figure is the overlay of all chromatograms. The polymers were eluted by a 0.2 M acetic acid/ammonium acetate (pH 5.3) buffer system with PL aquagel-OH column and were analyzed by RID.

	M_n	M_w	PDI (M_n/M_w)
FGI	15471	36868	2.38
PEG-PEI	17515	30693	1.75

Table 4.1 Calculated molecular weight of FGIS and GIS by SEC

After the non-covalent functionalization of CNT by FGIS and GIS, TEM (Fig. 4.5) showed that FGIS/C and GIS/C were singly dispersed with a length of approximately 500 nm which is shorter than the claim of the manufacturer. CNT were shortened in the process.
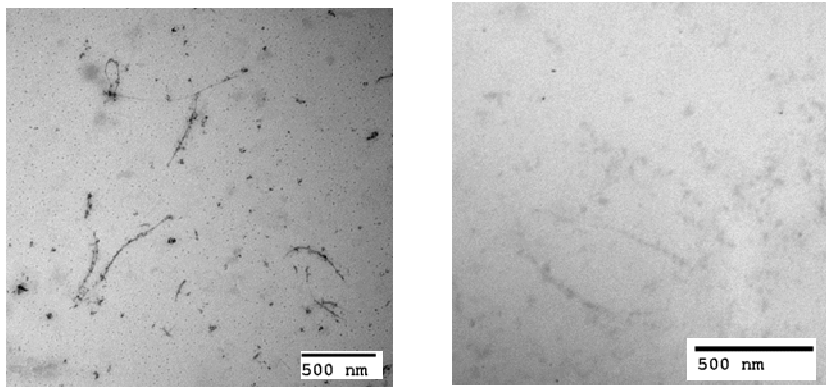
Figure 4.5 TEM micrograph of GIS/C and FGIS/C

Minimum amount of CNT solution was added to the copper grid and incubated for 20 minutes. The excessive solution was then removed, the grid is air-dried and the samples were observed under TEM. The length of the GIS/C (right) and FGIS/C (left) are around 100-600 nm.

The siRNA binding capacity of FGIS/C and GIS/C were demonstrated by gel shift assay. The capacities of the siRNA condensation of both CNT were tested. Free siRNA migrates along the gel while siRNA complexed with CNT will be slowed down or totally ceased. Also, siRNA cannot bind to ethidium bromide effectively after it binds to the cationic CNT and thus the fluorescence intensity of the bound siRNA would be reduced. The results showed that the ratio of FGIS/C required to inhibit the migration of siRNA is 2:1 (w/w) while GIS/C is 1:1 (Fig. 4.6).
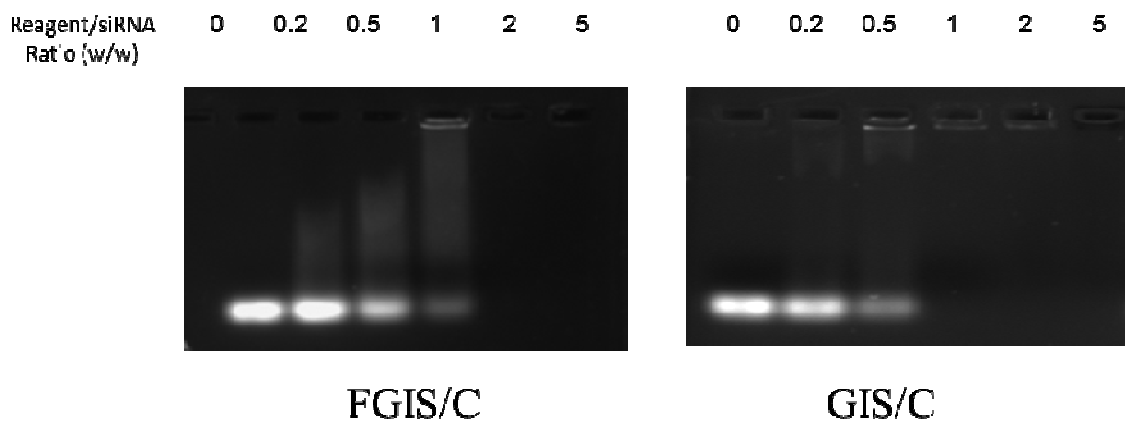
Figure 4.6 Gel shift assay of siRNA with FGIS/C and GIS/C

Electrophoresis was done on the samples and the gel was taken out for visualizing the bands. The weight ratio of siRNA condensed by FGIS/C is 2:1 and by GIS/C is 1:1.

The stability of FGIS/C or GIS/C and siRNA complexes was studied using zeta potential. The higher the magnitude of the zeta potential, the more stable the complexes would be, due to electrostatic repulsion. The result showed that FGIS/C/siRNA has a zeta potential range from 35.8 mV to 48.5 mV while GIS/C/siRNA the zeta potential from 53.9 to 62.9 as GIS/C to siRNA ratio increases. Overall, FGIS/C/siRNA complexes have a lower zeta potential than GIS/C/siRNA complexes probably due to the incorporation of folic acid. Nevertheless, they have high zeta potential which is an indicator of good colloidal stability (Fig. 4.7).
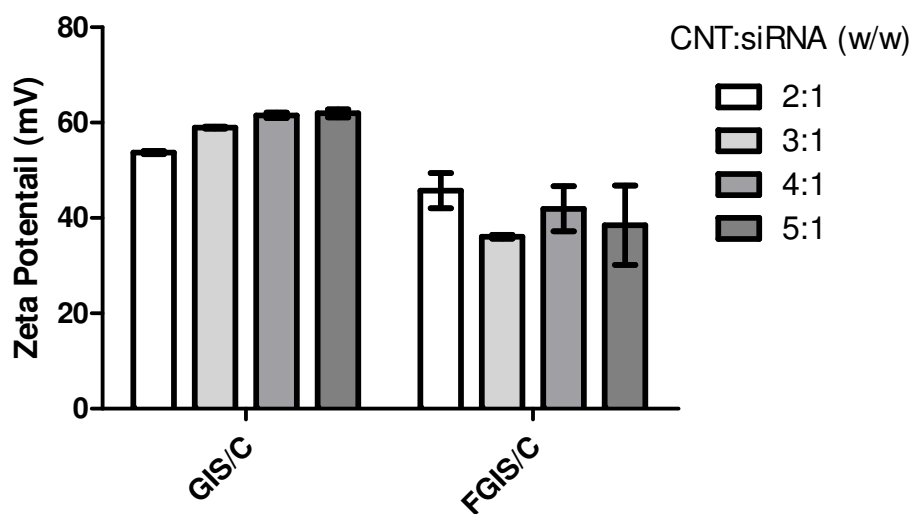
Figure 4.7 Zeta potential of FGIS/C/siRNA and GIS/C/siRNA

The sample was dispersed in deionized water and analyzed with Zetasizer. The experiment was done with 10 runs and the error bar is standard deviation. Four CNT:siRNA (w/w) ratios were tested. n=2

4.4.2 *In vitro* siRNA Delivery with FGIS/C and GIS/C

The cellular uptake difference between folic acid bearing CNT (FGIS/C) and non-folic acid bearing CNT (GIS/C) were compared in KB which is known to express high level of FR. The result showed that there is a slight increase of siRNA uptake after attaching FA (Fig. 4.8).
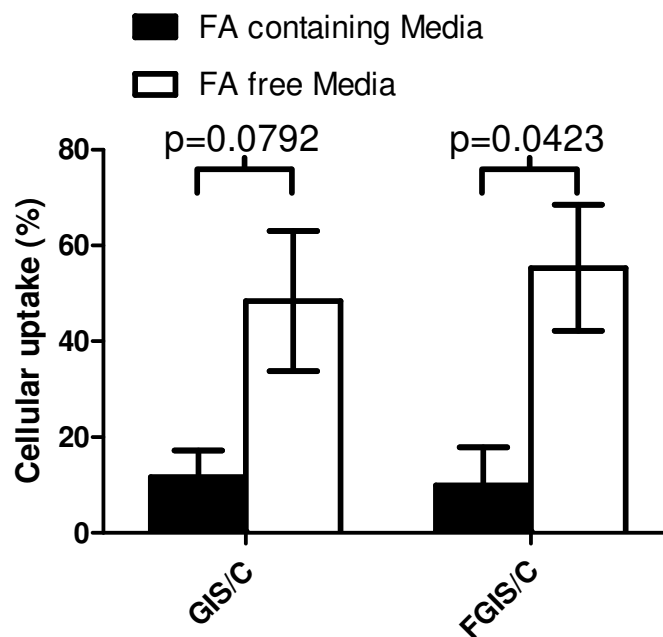
Figure 4.8 Cellular uptake of Cy3-labelled siRNA by KB

KB cells were transfected with Cy3-labelled siGAPDH, and then the cells were returned to incubation for 1 hour. Then cells were washed, typsinized, collected and re-suspended in PBS with 2% FBS. The cells were then analyzed with flow cytometry. n=3; error bar=SEM

4.4.3 *In vitro* Cytotoxicity of FGIS/C and GIS/C

The cytotoxicity of FGIS/C and GIS/C were examined in B16 cells with flow cytometry after 7AAD staining. The cytotoxicity is low up to the concentration of 10 μg/mL (< 10% cell death). Their cytotoxicities are similar up to 30 μg/mL, no significant difference between FGIS/C and GIS/C. Significant differences between FGIS/C or GIS/C to IS/C were found between concentrations 20 μg/mL and 30 μg/mL. (Fig 4.9)
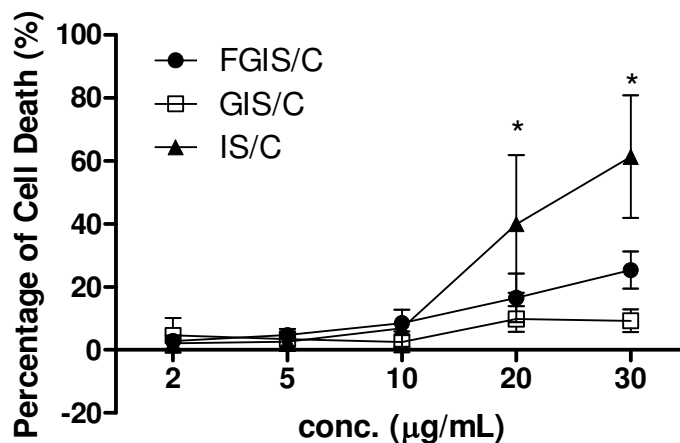
Figure 4.9 Cell proliferation of B16-F10 by MTT

B16-F10 cells were treated with desired amount of FGIS/C or GIS/C and then the cells were returned to incubation for 24 hours. Media were collected and the cells were typsinized, collected and re-suspended in PSB with 2% FBS and 5 μg/mL 7AAD. The cells were then analyzed with flow cytometry. Percentage of cell death was calculated by the 7AAD positive cells in the treated cells minus the 7AAD positive cells in the untreated cells. IS/C data from chapter 3 was used for comparison. Asterisk indicated $p < 0.05$ by two way ANOVA with Bonferroni post-tests. n=3; error bar=SEM

4.4.4 *In vitro* Cell Proliferation of mTOR siRNA

In vitro cell proliferation of mTOR siRNA was examined with lipofectamine 2000. Previously it has been reported that CNT would interact with MTT [40] and so, lipofectamine 2000 was used instead of FGIS/C or GIS/C. Significant reduction of cell proliferation (75%) was found in si-mTOR treated B16 cells. (Fig. 4.10)
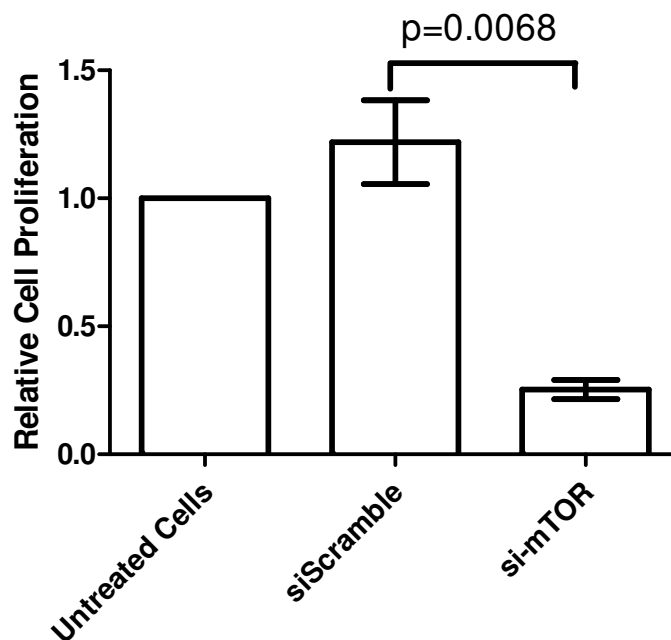
Figure 4.10 Cell proliferation of B16-F10

B16-F10 cells were transfected with si-mTOR and lipofectamine 2000 and after 24 hours, cells were trypsinized and 500 cells were seeded into 96 wells plates. The cells were then incubated for additional 96 hours. Then media were replaced with serum free media with 10% MTT and the cells were incubated for 3 more hours. Media were removed, DMSO was added to wells and the absorbance at 570 nm was recorded. n=6; error bar=SEM (p value was calculated with Student's t-test)

4.4.5 *In vivo* Delivery of siRNA with FGIS/C and GIS/C to Melanoma Bearing Mice

It has been reported that CNT localize around tumor. Also, PEG could increase the circulation half-life of the siRNA vehicle and thus, it is expected that the siRNA are more likely to localize around tumor by enhanced permeability and retention (EPR) effect. To examine whether the conjugation of folic acid is beneficial to the CNT we functionalized. We set out to use FGIS/C and GIS/C to test the difference between the two. It is well-

known that B16-F10 overexpress FR and it was used for tumor inoculation. The differences between FGIS/C and GIS/C in delivering siRNA into tumor were examined by intravenous injection into melanoma bearing mice. The fluorescence picture showed that the localization of siRNA in tumor was more for the FGIS/C/siRNA treated mice than the GIS/C/siRNA treated mice (Fig. 4.11). Therefore, there is evidence that CNT or PEG conjugated delivery vehicle localized in the tumor more than those without CNT or PEG. FA conjugation can further increase the localization of the vehicle to FR expressing tumor. FGIS/C is a more specific siRNA delivery vehicle for tumor compared to GIS/C.

Figure 4.11 *In vivo* uptake of tumor of FGIS/C/siRNA GIS/C/siRNA

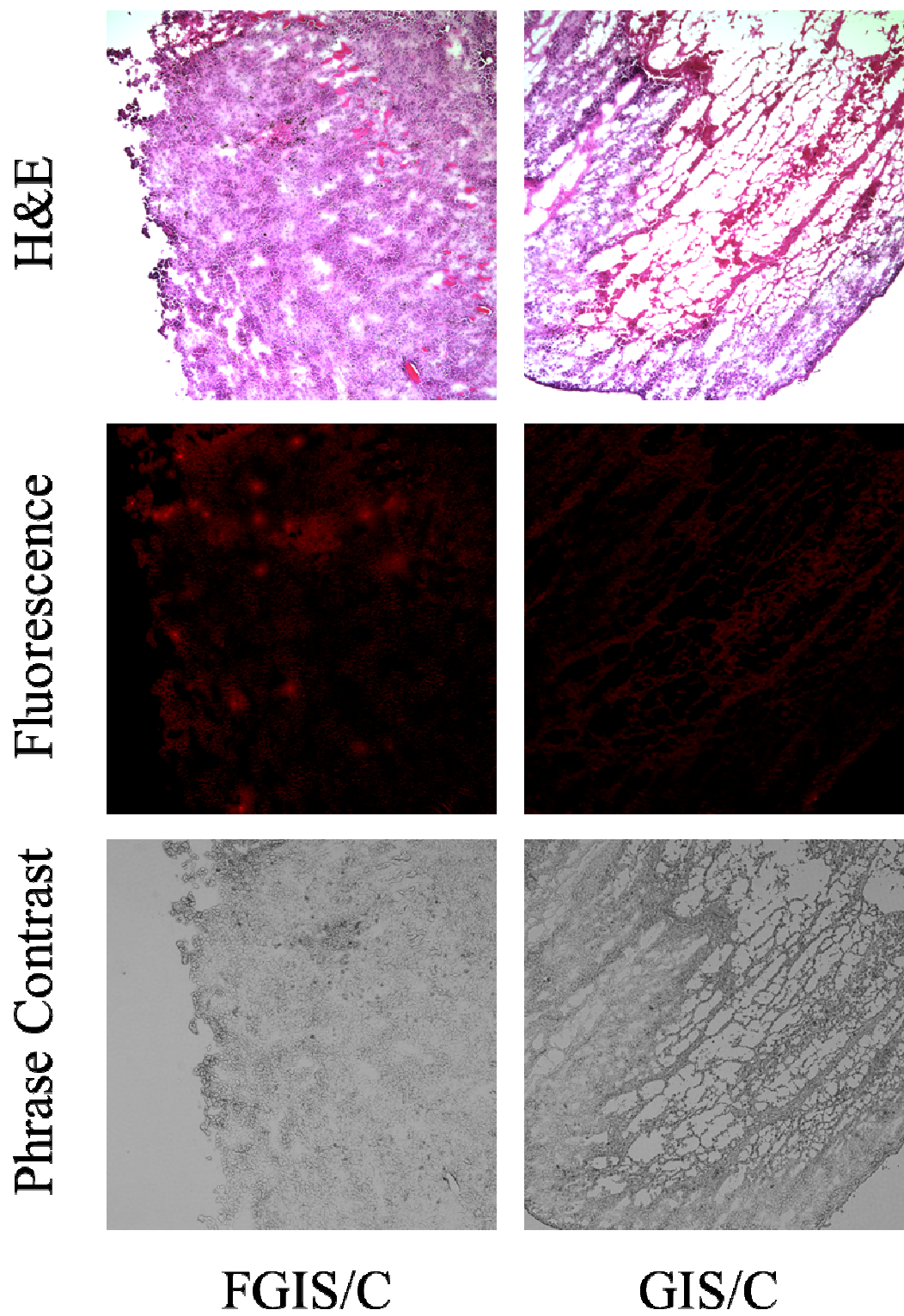2 days before the mice were sacrificed (18th day). 100 µg of Cy3-labelled siRNA and 300 µg of FGIS/C or GIS/C were injected into the mice by tail vein injection. The mice were sacrificed 48 hours after injection and the tumor was removed and frozen in OCT. The tumor was crycosectioned and the samples were observed under fluorescence microscope. H&E staining was done to show the tumor which has an enlarged nucleus and reduced cytoplasm.

4.4.6 Treatment of Melanoma Through Intravenous siRNA Delivery of mTOR siRNA Using FGIS/C

FGIS/C/siRNA localized more in the tumor compared to GIS/C/siRNA as demonstrated, therefore, we set out to test if it is the same in treating tumor with siRNA. mTOR is responsible for cell growth and proliferation. To test the therapeutic effect of using mTOR siRNA to inhibit tumor progression, we treated melanoma-bearing mice with FGIS/C/si-mTOR.

The tumor size is significantly different for the FGIS/C/si-mTOR from tumor treated with FGIS/C/siScramble. The tumor growth was inhibited significantly for the FGIS/C/si-mTOR group. There is no significant difference between untreated tumor and tumor treated with FGIS/C/siScramble (Fig. 4.12), however, there is also no significant difference between FGIS/C/si-mTOR and GIS/C/si-mTOR. A similar trend was observed in the average tumor weight when the mice were sacrificed on day 20. The final tumor weight was not significantly different between untreated mice and FGIS/C/siScramble treated mice while the FGIS/C/si-mTOR and GIS/C/si-mTOR treated group was dramatically reduced compared to scramble control (Fig. 4.13). Significant difference of tumor volume was found from 16th day, to 18th day and 20th day between FGIS/C/mTOR and FGIS/C/siScramble. Again, the tumor weight between FGIS/C/si-mTOR and GIS/C/si-mTOR treated group have no significant difference. However, Western blot data showed that the mTOR down-regulation by FGIS/C/si-mTOR is higher than GIS/C/si-mTOR (Fig. 4.14). The percentage of protein downregulation was 65% for FGIS/C/si-mTOR while it was 18% for GIS/C/si-mTOR (Fig. 4.15). These data imply

that FGIS/C is superior to GIS/C in delivering siRNA *in vivo* and thus the mTOR expression in tumor of FGIS/C/si-mTOR treated mice is lower than that of GIS/C/si-mTOR treated mice, however, both FGIS/C/si-mTOR and GIS/C/si-mTOR reduced the tumor progression to a similar level.
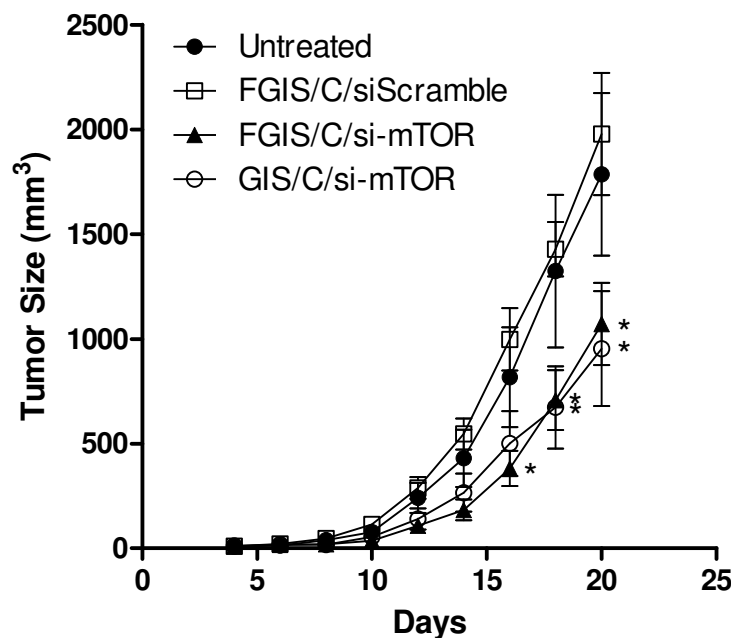
Figure 4.12 Tumor size of tumor bearing mice intravenously injected with FGIS/C, GIS/C and siRNA

4 days after the mice were inoculated with tumor, the mice were injected intravenously with FGIS/C/si-mTOR solution for every 7 days until 20th day for sacrifice. The tumor size of mice was estimated by the length and width of the tumor by a caliber in nearest 0.5 mm. The sizes were calculated with $\pi(LxW^2)/6$. 2-way ANOVA with Bonferroni test as post-test showed that significant difference between the scramble control and the FGIS/C/si-mTOR treated mice. Significant difference of tumor volume begin from 16th day ($p<0.05$), to 18th day ($p<0.01$) and 20th day ($p<0.001$) between FGIS/C/mTOR and FGIS/C/siScramble. n=7 for untreated mice, n=6 for FGIS/C/si-mTOR and FGIS/C/siScramble and n=5 for GIS/C/si-mTOR; error bar=SEM.
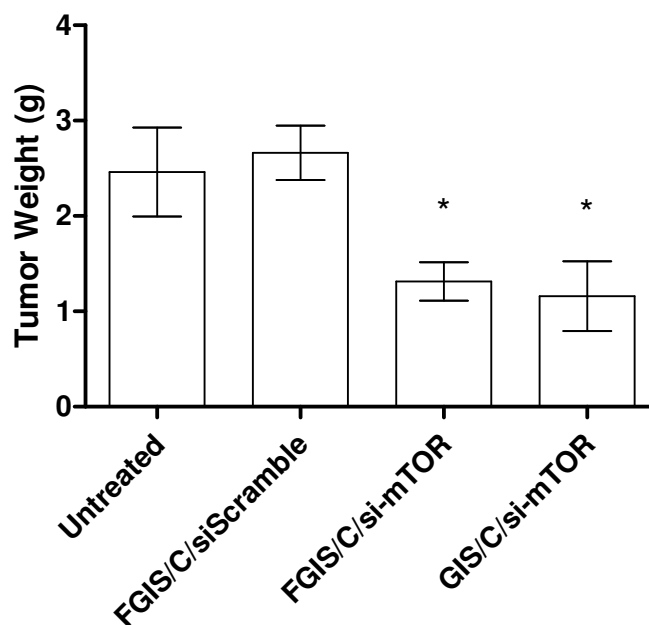
Figure 4.13 Tumor weight of tumor bearing mice intravenously injected with FGIS/C, GIS/C and siRNA

The tumor of mice was removed and weighted on the day when the mice were sacrificed (Day 20). n=7 for untreated mice, n=6 for FGIS/C/si-mTOR and FGIS/C/siScramble and n=5 for GIS/C/si-mTOR; error bar=SEM. (Asterisk indicated $p<0.05$, samples analyzed by 1-way ANOVA followed by Dunnett's test as post-test by comparing with siScramble group)
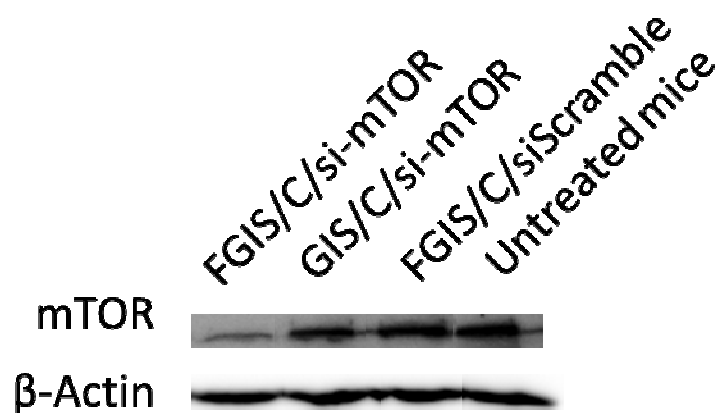
Figure 4.14 Western blot of the tumor samples

The tumor samples were homogenized and the protein was isolated with RIPA buffer for western blot. mTOR was analyzed and beta actin was used as internal control.
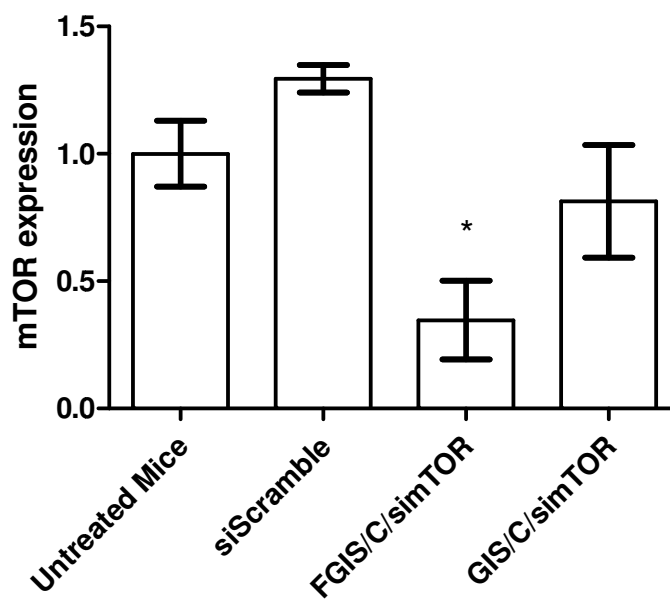
Figure 4.15 Relative mTOR expression on protein level

The band intensity of western blot was quantified with photoshop. n=3 error bar=SEM

(Asterisk indicated p<0.05, samples analyzed by 1-way ANOVA followed by Dunnett's test as post-test by comparing with siScramble group)

4.5   Discussion

Novel non-covalently functionalized SWCNT were developed for siRNA delivery *in vivo*. This novel siRNA delivery system is modified based on our previously reported CNT delivery system with PEG and folic acid. PEG could reduce the toxicity, enhance the colloidal stability and increase the circulation half-life of the conjugated component [41-43]. It is very important for *in vivo* systematic delivery to cancer because the accumulation of it to the tumor will be increased due to EPR effect [44]. Similar observation when CNT were used for drug delivery to tumor [12]. Also, PEGylation reduces the clearance of particles by RES [41].

FA is essential for DNA synthesis as well as the biosynthesis of S-adenosyl methionine, an essential chemical for methylation which is important for various biochemical reactions [34]. Cancer cells usually overexpress FR and which can be exploited for targeted siRNA delivery. Therefore, we set out to attach FA to CNT with PEG. FA can be attached to one end of PEG for cancer cell targeting and the succinated PEI can be attached to the other end for dispersing the CNT as well as condensing siRNA by electrostatic interaction. To reduce the self-crosslinking succinated PEI, succinic anhydride modification on the amine of PEI was done in the final step. FA was attached to PEG-NH$_2$ by EDC/NHS coupling. Dialysis was used as purification in most reactions for removing most of the unreacted folic acid, succinic anhydride, EDC and NHS. Precipitation in diethyl ether method was attempted for PEG-FA but it was not successful probably due to high solubility of PEG in DMSO/diethyl ether mixture. FA-PEG-NH$_2$ was reacted with succinic anhydride to form FA-PEG-SuOH and then reacted with the amine of PEI by EDC/NHS coupling to form FA-PEG-PEI. Then, it was reacted with succinic anhydride to form FA-PEG-PEI-SA. For PEG-PEI-SA (GIS), PEI was attached to mPEG-COOH by EDC/NHS coupling. Then PEG-PEI was reacted with succinic anhydride to form PEG-PEI-SA. The conjugation of folic acid to one end of PEG was done by stoichiometric control with EDC/NHS coupling. The γ-carboxyl group of

glutamic acid in folic acid reacts preferentially with the amine because it has a higher reactivity than the α-carboxyl group [45-47]. Succination was done after the conjugation of PEI to FA-PEG which is to avoid the self-crosslinking of succinated PEI during EDC/NHS coupling with FA-PEG. However, there is still crosslinking observed in the SEC (Fig. 4.4). The small peak at ~15 min indicated there is a high molecular weight species which is probably the crosslinked polymer, however, the percentage is relatively low. The degree of succination is similar for both polymers. CNT with FA, FGIS/C and CNT without FA, GIS/C were made by non-covalent functionalization.

One interesting observation is that, the ratios to condensing the siRNA for GIS/C and FGIS/C are different despite the degree of succination of the polymers are similar. GIS/C has a higher capacity to condense siRNA while FGIS/C has a lower capacity. One of the possible reasons is the conjugation of FA to the PEG. FA has a pKa of 4.65 and it is deprotonated at physiological condition. It is highly possible that the FA reduced the siRNA condensing capacity. Excluding for the amine attached to succinic acid and to PEG, the modified PEI has an average of 16.8 protonable amines for each PEG attached. FA is attached to the other end of PEG which is far away from the PEI/CNT core. FA is close to the surface and therefore, the negatively charge FA affect the overall siRNA binding capacity of the FGIS/C is more significant than the PEI. Also, succinic acid neutralizes the charge on PEI and only a portion of the amines in PEI are protonated at a given pH. A small amount of folic acid induced significant changes on the siRNA binding capacity.

The zeta potentials also indicated similar observation as in gel shift assay, regardless of the ratios, the zeta potential of GIS/C/siRNA is higher than FGIS/C. It is expected that if the siRNA delivery is driven principally by zeta potential, GIS/C should outperform FGIS/C. KB, a cell line which known to express high level of FR was used for testing the difference *in vitro*, there is slight increase of siRNA uptake for FGIS/C, however, there is no statistical significant difference. It is possible that the *in vitro* cellular uptake is dominated by the magnitude of zeta potential. Another possible reason is that the media recommended by ATCC for KB cells are not RPMI 1640 which was the only FA free media we can obtain commercially, the cells behaviors might be changed as well as the

FR expression profile and cellular uptake activity after 1 day incubation. A further examination on the FR expression profile and cellular uptake behaviors should be studied for understanding the cellular uptake behavior.

The importance of a targeting moiety in delivering siRNA was reflected *in vivo* siRNA localization by systematic administration. For *in vitro* system, charged-mediated uptake plays a more important role than receptor-mediated uptake and therefore there is no difference *in vitro*. It is because the complexes are in the well and they will not be eliminated or removed from the system. Also, the concentration of siRNA is higher than *in vivo*. Furthermore, for *in vivo* system, they can be cleared away by the RES, trapped by other organs or excreted from the body. Therefore, we set out to test the targeting capacity of FGIS/C *in vivo*. A recent study proved that systematic mTOR inhibition reduced the tumor growth but weight reduction was not observed [48]. Therefore, systematic mTOR inhibition is not likely to induce intolerable toxicity to the mice. CNT with mTOR siRNA was administrated systematically. There is more localization of siRNA for the mice treated with FGIS/C/si-mTOR compared to GIS/C/si-mTOR. The localization of siRNA leads to reduction in mTOR expression in protein level which was demonstrated by western blotting. Therefore, for *in vivo* delivery to tumor, charged-mediated uptake is less efficient to localize the siRNA effectively into the tumor compared to a more specific receptor-mediated uptake. Even though both PEG and CNT enhanced the localization of the complexes to tumor by EPR effect, attachment of FA is beneficial for siRNA delivery for gene downregulation. A recent *in vivo* doxorubicin delivery study to tumor with multiple wall carbon nanotubes by Mehra et. al. [49] showed slightly improved localization and therapeutic effect. Nevertheless, FGIS/C has a higher capacity to deliver siRNA *in vivo* to the tumor *in vivo* and it induces gene downregulation.

It is interesting that the inhibition of mTOR in protein level is significantly higher for FGIS/C/si-mTOR compared to GIS/C/si-mTOR, however, the tumor growth inhibition of FGIS/C/si-mTOR and GIS/C/si-mTOR do not have significant difference. The tumor growth inihibition of FGIS/C/si-mTOR and GIS/C/si-mTOR were significant higher compared to FGIS/C/siScramble and untreated mice. Therefore, the growth inhibition is

not due to the FGIS/C alone. It is more likely that the downregulation of mTOR inhibited the tumor growth for FGIS/C/si-mTOR. The therapeutic outcome of the FGIS/C and GIS/C are similar and it is possible that a more frequent treatment of mice would reveal the difference between the two. One possible explanation is that the GIS/C/si-mTOR gain into some cells or tissue which might affect the tumor growth, it might localize around the tumor by EPR and it downregulated the mTOR of blood vessel such that the nutrient supply to the tumor is reduced and at the end, the size of the tumor. Another possibility is that there is no difference in the distribution of the siRNA between FGIS/C and GIS/C in the treated tumors as a whole but the localization of siRNA in the tumor cells which express high level of FR. The tumor is a mixture of high FR and low FR expressing cells and therefore, the dominant factor for reducing the tumor progression is zeta potential.

Recent studies found out that Akt/mTOR interact with RAF/EKR pathway and which promotes melanoma growth [50-52]. Multiple gene targeting such as Braf/EKR/mTOR is more promising and it is well known that rapamicin can only inhibit mTORC1 but not mTORC2 [32, 53-55] and resistance to Braf inhibitors has been found [56, 57]. Although there are inhibitors for kinase function inhibition of both mTORC1 and mTORC2 [48], siRNA specifically downregulate a gene and thus all of the protein function, which can guarantee the mTOR function is lost and less likely to develop undesirable side effect or drug resistance. Also, siRNA might not get into all kind of cells such as difficult to transfect cells like T cells or dendritic cells which might reduce the off-targeting side effect.

4.6   Conclusion

A novel and efficient non-covalently functionalized CNT for cancer siRNA delivery was developed. PEI based polymers with folic acid and without folic acid were synthesized and they were used for functionalize CNT. The polymer/CNT composites were characterized and their siRNA delivering capacities were accessed *in vivo* in a murine melanoma model. Tumor progression was suppressed and gene silencing was found *in vivo* on the protein level. However, the tumor size and tumor weight after treatment between FA bearing CNT and CNT without FA has no significant difference.

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Chapter 5

5 General Discussion

5.1 Discussion of CNT as siRNA Delivery Vector

The core research question of this thesis is: is CNT a good candidate for siRNA delivery? This question is based on the validity of the premise: RNAi is a good approach for new generation therapeutics. If the premise is not true, no matter how efficient CNT based siRNA delivery vectors are developed, it is meaningless in providing alternative therapeutics to our current ones. Certainly, the examination of CNT in siRNA delivery alone cannot answer this question and the experimental data in chapter 2-4 might not provide a clear answer. Also, there is a hidden research question based on the first question: what is the benefit of CNT for siRNA delivery or drug delivery? In other words, what are the possible benefits CNT could provide such that it would get thought the barriers in: reaching target cells/tissue, cellular entry, cellular trafficking and finally, siRNA release for inducing the PTGS.

The scope of the studies throughout the thesis focused on utilizing the previously reported knowledge on siRNA delivery system to develop a CNT based vector for biological applications. Two different kinds of polymers were synthesized and one of these was used to attach folic acid which is used as a targeting ligand. Two of the functionalized CNT were used in a cancer model. The thesis chapters are summarized as follow:

- Chapter 2: DGI/C was used for *in vitro* and *in vivo* systematic siRNA delivery

- Chapter 3: IS/C was used for *in vitro* and topical siRNA delivery; a mouse cutaneous melanoma model was used for examining the therapeutic potential of topical application of IS/C was demonstrated with siBraf.

- Chapter 4: FGIS/C was used for systematic siRNA delivery; a mouse cutaneous melanoma model was used for examining the therapeutic potential of systematic administration of FGIS/C was demonstrated with si-mTOR.

In these chapters, we tried to explore the feasibility of utilizing CNT for siRNA delivery. As discussed in chapter 1, delivery of siRNA or nucleic acids is difficult in general, especially *in vivo*. siRNA delivery *in vitro* can be carried out easily using commercially available reagents produced by various company like Invitrogen, Roche, Thermo scientific, Cell Signaling Technology, Santa Cruz, Polyplus and more. Most of the reagents are based on a lipid based recipe. Most of the reagents cannot deliver siRNA *in vivo* efficiently and therefore, an alternative method for siRNA delivery is necessary.

The use of CNT and PEI are the core of the three siRNA delivery system studied in this thesis and their efficacies were examined. PEI modification is necessary for effective siRNA delivery while siRNA modification into sticky siRNA can mediate higher gene silencing [1]. Here, we demonstrated the method to modify the delivery vector instead of the siRNA for the delivery. CNT-based delivery vector can gain entry into the cells effectively but it has to be modified before it can be singly dispersed in water. The method used in this thesis for the CNT dispersion is based on non-covalent method and it was carried out with a sonicator. The efficiency of dispersing the CNT is low (20-30% w/w) when compared to covalent methods. An interesting behavior of CNT is that sonication would shorten the CNT beyond a certain time [2]. Another interesting observation reported was that, the degree of functionalization depends on the power of sonication instead of the duration of sonication [3]. Therefore, it is highly possible that only the shortened CNT can be dispersed. Also, the CNT dispersed is highly dependent on the power of the sonicator. Therefore, the short CNT is easier to be dispersed than the long CNT. In the report of Kam [4], the CNT was 50-300 nm, however, we found that the length of the CNT for DGI/C, IS/C, FGIS/C and GIS/C, the length are around 200-1000 nm. One of the most probable explanation is the power of the sonication in Kam's report was higher than the one reported here. However, the sonication power was not mentioned in their paper. One possible explanation is that the CNT Kam et. al. used has a wider length distribution while the CNT we obtained has a narrower length distribution and thus, the larger CNT was removed by centrifugation while only smaller CNT were preserved.

CNT has been used for nucleic acids delivery. The criterion for an ideal delivery system, as mentioned before, is that the delivery system should act like a "magic bullet" such that it targets the pathological tissue without affecting the host. However, in practice, what we consider is: is the harm to the patient outweighed by the benefit to the patient? The conventional small molecular cytotoxic drugs (for example cisplatin) almost kill all kinds of cells indiscriminately. Theoretically, systematic delivery of untargeted anti-cancer drug is not a good delivery method. However, it is an effective way to reduce the progression of cancer. In other words, the benefit of using the drug outweighed the side effects; it is acceptable in clinical settings as the best available option. Applying the same rationale to CNT prepared in this thesis provides goals. If the gene silencing mediated by the CNT delivered siRNA is not higher than the siRNA alone, it is not a good delivery system because it lacks the functional improvement. Another one is if the gene silencing induced by CNT mediated delivery of siRNA was worse than the commercially available reagents, then it is not the best option as there are no advances in tackling the technical barriers. For the first goal, as we observed from the literature and the results throughout the study, CNT increased the siRNA uptake in cells [4] and enhanced cancer drug delivery [5]. Therefore, it is justified to use CNT for siRNA delivery. For the second goal, the CNT studied throughout these studies were better than some of the commercially available reagents like DGI/C is able to deliver siRNA to the liver and induces notable gene silencing; IS/C is able to deliver siRNA via topical application and FGIS/C and GIS/C are able to deliver siRNA to tumors. Therefore, it is justified to use CNT for siRNA delivery in RNAi therapy.

The interesting "nano-needle" effect was observed and it is interesting in studying the cellular uptake mechanisms, however, for gene/drug delivery, is the non-selective nano-needle important for gene/drug delivery? The primary goal of using CNT is to exploit its "nano-needle" property for a more efficient delivery, especially delivery of siRNA to difficult-to transfect cells [6-8]. Nano-needle is one of many possible mechanisms for utilizing CNT in gene/drug delivery. Regardless of the cellular entry mechanism, the use of CNT for gene/drug delivery is justified based on the angle of enhancing the bioavailability of gene/drug, at least for cancer [9-13]. Another question is whether the non-selective cellular entry is harmful? In other words, would the CNT induce potential

undesirable side effects if non-targeted cells or tissues were affected and how important is it? As discussed briefly in chapter 1, the immunogenicity of CNT was demonstrated but there are a lot of controversial reports on its toxicity. This is not covered intensively in this thesis, it is not the focus of the thesis and a definite conclusion cannot be made. According to the experimental data presented in this thesis, the cytoxicity of the CNT depends on its functionalization. Indeed, the development of chapter 3 and chapter 4 were based on the experience of chapter 2. The cytotoxicity of DGI/C is high and it would be more preferable to have a delivery system with lower toxicity. Therefore, same as the conclusion made in chapter 1, the cytotoxicity largely depends on the functionalization. Also, it is believed that CNT does not naturally exist. Although there are reports on neuron cells and various biological agents that are able to break down CNT [14-21], CNT is not easy to be broken down compared to other polymers. Take poly(lactic acid) for example, it can be broken down by hydrolysis therefore water is able to mediate the degradation of poly(lactic acid). CNT still requires specific enzymes to break it down and all of the reports so far use covalently modified CNT. It is more preferable for a drug/gene delivery system to use a degradable CNT instead of non-degradable CNT. However, it was also reported that CNT can be eliminated from the circulation [22, 23]. Therefore, the use of eliminable CNT instead of degradable CNT is more preferable because of the intrinsic difficulty of CNT degradation. With the already developed eliminable CNT, we are optimistic about the toxicity issue for utilizing CNT as a systematic delivery vector.

Another criterion to consider is the type of siRNA it carries. For example, if the carried siRNA is used to stop the proliferation of the cells, it may be harmful if it went to reproductive organs. Another example is mTOR, mTOR is a well-studied gene which is important for T cell differentiation [24-26], therefore its systematic down regulation should be avoided unless it is the goal of the therapy. Alternatively, if the siRNA was to stop the function of specific cells such that other cells didn't express that molecule like V600E Braf, it is harmless for non-selective delivery. Therefore, the selectivity and specificity can also be fine-tuned by the type of siRNA delivered. The distribution of CNT based delivery system found by other researchers was similar to what was found and reported in chapter 2. We can see that the uptake mostly related to the size of the

complexes, which, most of them ended up localizing in the liver and spleen. Therefore, extra care should be taken for the use of siRNA which might alter the function of the liver and spleen.

In chapter 3, IS/C was used for transdermal siRNA delivery. It is so far the first topical siRNA delivery using CNT. Based on the promising results in chapter 3, the mechanism of the transdermal behavior should further be characterized and tested in artificial human skin. Also, the acute and chronic immune response of skin to IS/C should also be characterized, which can be done *in vitro* using artificial human skin or a porcine ear skin model by measuring the release of cytokine. The transdermal behavior can be tested with Franz diffusion cell filled with PBS at pH 7.4 with temperature maintained at 37 $^{\circ}$C. The transdermal capacity can then be done by observing the Cy3-labelled siRNA with fluorescence microscopy. Mice are not a good model for studying human skin transdermal activity because of the different thicknesses in SC between mouse and human as well as the diffusion coefficient [27].

In chapter 4, FGIS/C was used for systematic siRNA delivery. It is quite interesting that there is no significant difference between the FGIS/C and GIS/C in *in vitro* siRNA uptake for KB cells. One of the possible reasons is although the cell culture media of KB cells were replaced by folate free media, the level of overexpression is still "low" such that the difference cannot be seen in KB cells. As discussed in chapter 4, the suitability of culture media might play a role, which might account for the difference. Further study on the cells FA expression profile and behavior is needed to understand the difference. Nevertheless, *in vitro* modelling is inadequate for mimicking *in vivo* environments and the zeta potential, as well, the size of the complexes plays a more important role than the others [28]. For the *in vivo* experiment, it would be great if the injection can be done more frequently to see if there is a better therapeutic outcome, especially if it is possible to see the difference between FGIS/C/si-mTOR and GIS/C/si-mTOR. It is well known that gene silencing could last for around 7 days in actively dividing cells [29]. Therefore, mTOR in tumors is not always down-regulated. A higher dose or more frequent treatment can be used. Based on the existing data of siRNA localization as well as western blotting, we believed that FGIS/C exhibit better tumor targeting ability while GIS/C is as effective

based on other unknown factors. Certainly, if the localization of CNT to the tumor was principally driven by the zeta potential of the complexes, the trend reported here, including the localization of siRNA and down regulation of mTOR protein level should be the same.

5.2   Future Prospect of CNT in siRNA Delivery

In this thesis, the novel non-covalent functionalization of CNT enables us to examine various hypotheses as well as revealing the potential of utilizing CNT for siRNA delivery. Based on some of the observations of the CNT studied, the following modification can be carried out to tackle some of the problems faced in the siRNA delivery system developed and reported in this thesis.

Based on the observation of IS/C, GIS/C and FGIS/C, the cytotoxicities are still high. Therefore, reduction in toxicity could be beneficial and a shielding domain can be added for reducing toxicity. A higher density of PEG and/or higher molecular weight of PEG can be added to the PEI-SA polymer. Also, a mixture of various molecular weight of PEG can be used. One of the loopholes is that PEG might actually reduce the binding of PEI-SA to CNT, which might be overcome by using a sonicator with a higher power. Another possible loophole is, over-functionalization, which would lead to low siRNA binding and protection. This has to be carried out in a series of different ratios of PEI to PEG as well as low molecular PEG to high molecular weight PEG.

Another approach is the synthesis of degradable PEI or other polycation [30-42]. Synthesis of lipid conjugated crosslinked PEI with disulphide bond for CNT dispersion would be beneficial. The rationale is based on reducing the cytotoxicity of DGI/C. Low molecular weight PEI has minimum cytotoxicity while high molecular weight PEI has high transfection efficiency. Crosslinking low molecular weight PEI combines the low cytotoxicity of low molecular weight PEI and the high transfection efficiency of high molecular weight PEI. Also, crosslinked PEI can release the siRNA better than the original PEI. Crosslinked PEI might have a worse CNT dispersing ability and thus a lipid tail is more preferable for dispersing CNT. Also, the disulphide conjugated PEI will be reduced specifically by the reduced-glutathione in cell [43, 44], therefore, the CNT can

still be dispersed in water after it gained entry into the cell and releases the siRNA. The first introduction of disulphide crosslinkers was done by Gosselin et al [45]. His idea is to synthesize a biodegradable high molecular weight PEI with low molecular weight PEI for pDNA delivery. If the crosslinkers or the bonds formed between the crosslinkers and PEI are degradable, the crosslinked PEI would be suitable for systemic application. Nonetheless, most of the crosslinked PEI have shown reduced cytotoxicity in the *in vitro* experiment but the transfection efficiencies are usually similar to or are lower than that of high molecular weight PEI. It has been reported that the transfection efficiencies of some crosslinked PEI is higher than that of PEI 25 kDa and other commercially available transfection reagents [46]. One of the possible loopholes of this system is that the crosslinked PEI/CNT cannot condense siRNA due to reduced siRNA binding. If the crosslinked PEI/CNT has a lower siRNA binding capacity, more hydrophobic lipid can be attached to the PEI as to enhance the stability of the complexes as demonstrated by the conjugation of an oleate tail to PEI600, PEI1200, or PEI1800 for siRNA delivery [47].

Covalent functionalization is more favorable than non-covalent functionalization of CNT because the CNT will be more stable. Also, the degree of functionalization can be controlled better than non-covalent approach. Various degrees of amination or attachment of cationic oligomer can be carried out for testing the optimal cell penetrating effect or transfection efficiency. However, it is more difficult to carry out and it is also possible that the degree of functionalization can be so low such that it cannot carry siRNA or protect siRNA effectively. Also, it is difficult to find a good solvent for dispersing both the CNT and the groups to graft, thus, the functionalized CNT may suffer from lack of homogeneity.

Finally, for the overall prospect of utilizing CNT as gene delivery vector. First of all, most of the researchers are interested in the toxicity of CNT in the field of biology. Recently, the toxicity of nanomaterials was uncovered and various mechanisms for nanomaterials to induce toxicity [48, 49]. It is because of the increased surface area and activity of the nano-sized surface. Indeed, most of the "effective" non-viral delivery systems are too toxic to be used *in vivo* [50-53]. The toxicity issue of CNT is the core concern of scientists as well as physicians, not to mention the efficacy of non-viral

delivery system is still far behind the gene transfection rate of viruses. Second, the intrinsic property of CNT is hydrophobic and it cannot bind to siRNA effectively. Therefore, in theory, it is more preferable to use CNT for hydrophobic drug delivery than siRNA or DNA delivery although CNT which can deliver siRNA or DNA can be used to deliver hydrophobic drug at the same time. Third, the solubility of CNT is too low for modification without specific equipment. Established experimental platforms for both CNT modifications as well as *in vitro* and/or *in vivo* gene/siRNA delivery are necessary before any discoveries could happen. It is one of the reasons why it hampers the overall progress of research on CNT. Based on the studies covered by this thesis, however, non-covalent functionalization might serve as a way for early research and discovery and the covalently functionalized CNT analogue might be synthesized for late stage research or application.

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Appendix ii Animal Protocol

Western 🛡

AUP Number: 2009-006
PI Name: Min, Weiping
AUP Title: Cancer Therapy By Immune Modulation

Approval Date: 05/21/2013

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new
Animal Use Protocol (AUP) entitled "Cancer Therapy By Immune Modulation

" has been APPROVED by the Animal Use Subcommittee of the University
Council on Animal Care. This approval, although valid for four years, and is
subject to annual Protocol Renewal.2009-006::5

1. This AUP number must be indicated when ordering animals for this
   project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared
   through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all
associated safety components (biosafety, radiation safety, general laboratory
safety) comply with institutional safety standards and have received all necessary
approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

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Appendix iii Synthesis and characterization of DSPE-PEG-PLL and DSPE-PEG-PLL/CNT (DGL/C), cellular uptake mechanism of DGL/C

Synthesis of DSPE-PEG-PLL (DGL) was carried out the same way as DSPE-PEG-PEI. Briefly, 200 µL (1.75 µmol) of DSPE-PEG-COOH was added to a round bottom flask and it was dried with compressed air. Then 5 mL DMSO and 10.6 mg (55.3 µmol) of EDC was added. The mixture was stirred for 15 minutes and then desired amount of PLL (Mw 8,000) solution in MES buffer (0.1 M, pH 5.5) was added. The reaction was allowed to proceed overnight and then the reaction mixture was dialyzed (50k Da MWCO) against deionized water for 48 hours. The product was then lyophilized for 2 days.

CNT was dispersed with a similar method with DGI/C. Briefly, 5 mg of DGL was dissolved in water and then 5 mg of CNT was added. The mixture was sonicated for 1 hour and then the mixture was centrifuged at 24000g at 4 degree Celsius. The pellet was discarded and the supernatant was concentrated with ultracentrifugation column. The solution was then lyophilized.
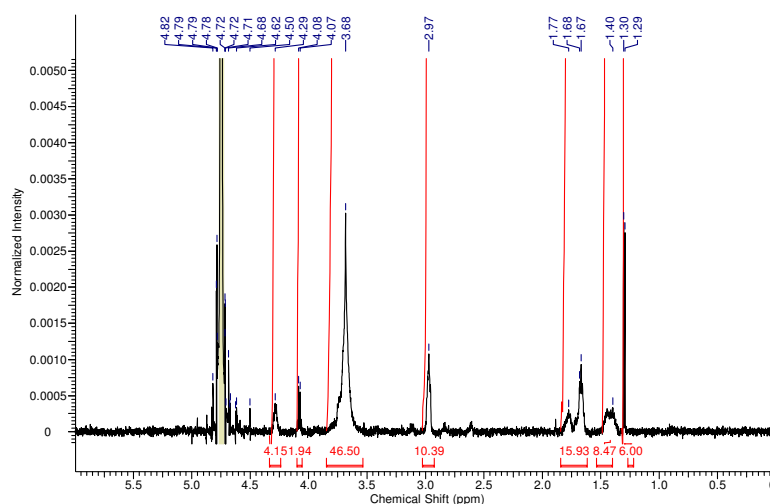
Figure A 1 ^{1}H-NMR of DSPE-PEG-PLL

^{1}H-NMR of DSPE-PEG-PLL, the ratio between PLL and DSPE-PEG was found to be 1:6
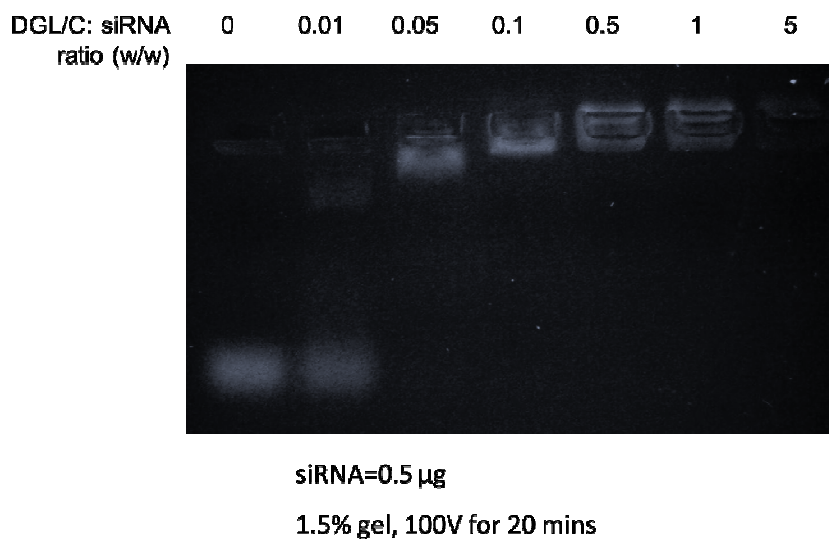
Figure A 2 Gel shift assay of DGL/C and siRNA

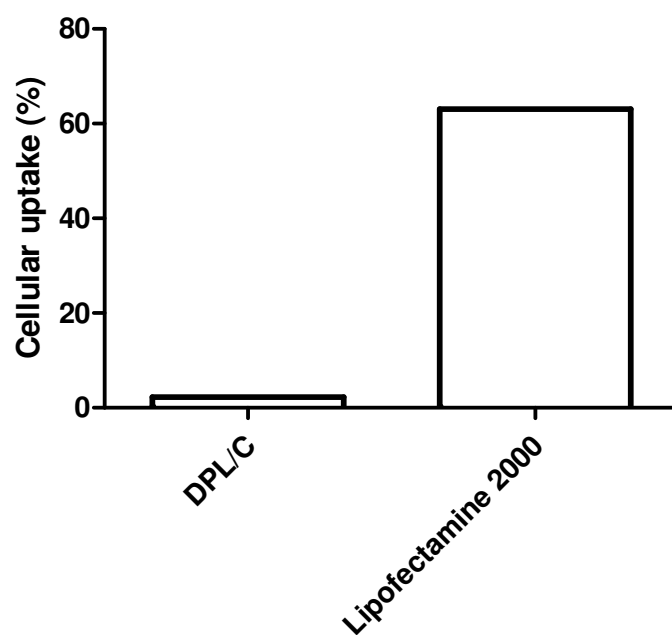Gel shift assay of DGL/C and siRNA. The siRNA condensation ratio is 1:10 (w/w, DGI/C : siRNA)

Figure A 3 Cellular uptake of DGL/C

B16-F10 cells were seeded 1.5×10^5 cells per well in 24 well plate. The cells were transfected with DGL/C (1:1 w/w to siRNA) and lipofectamine 2000 (2μL) and Cy3-labelled siRNA (0.5 μg) for 24 hours in serum containing media. The cells were trypsinized and analysized with flow cytometry. The cellular uptake of DPL/C/siRNA is 2.8%.

Appendix iv Cellular uptake mechanism determination
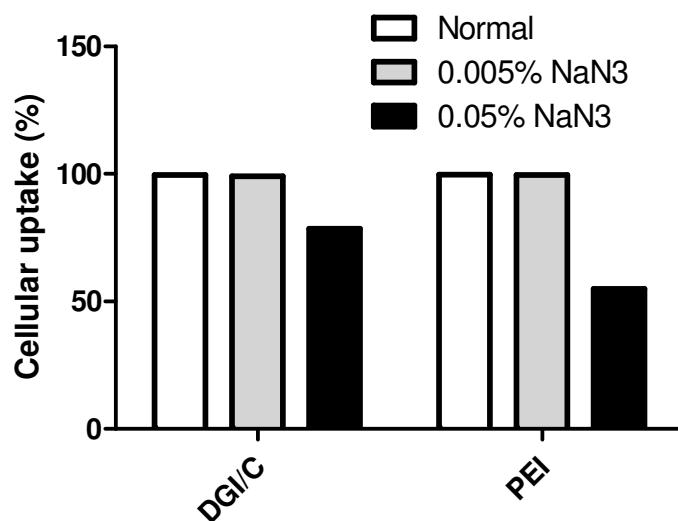
Figure A 4 Cellular uptake of siRNA with energy depleted condition

Before the transfection, the cell media was replaced with fresh media, media with 0.005% sodium azide or 0.05% sodium azide. Cells were then transfected with DGI/C 9 or PEI. 0.5 µg of Cy3-labeled siRNA and 2:1 (w/w) of PEI and 5:1 (w/w) of DGI/C 9 were used for transfection. 4 hours after transfection, the cells were washed with cold PBS and were trypsinized for flow cytometry analysis. PEI is well-known to gain into the cell by energy depended mechanism. Also, it can escape from endosome effectively. The mechanism of both transfection are energy depended because the siRNA uptake was reduced significantly with the sodium azide concentration.

Appendix v ¹H-NMR spectrum of the compounds 1b-1e, 2b,2c in Chapter 4

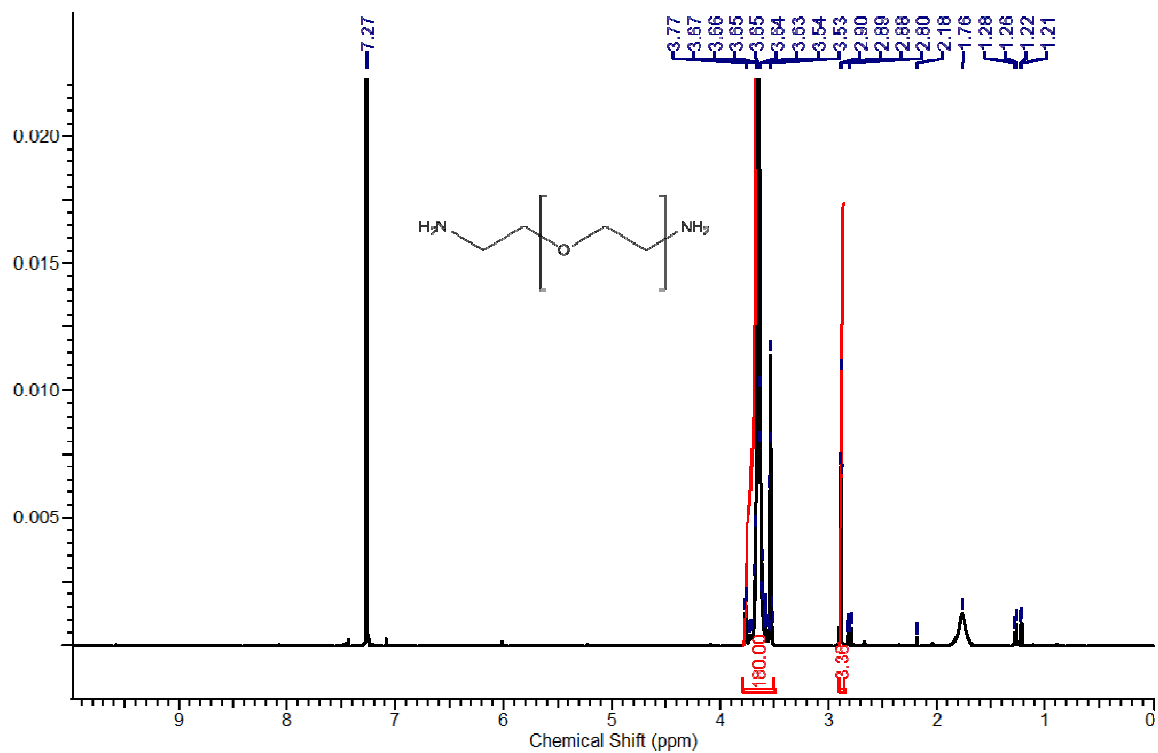**Figure A 5 ¹H-NMR of 1b**

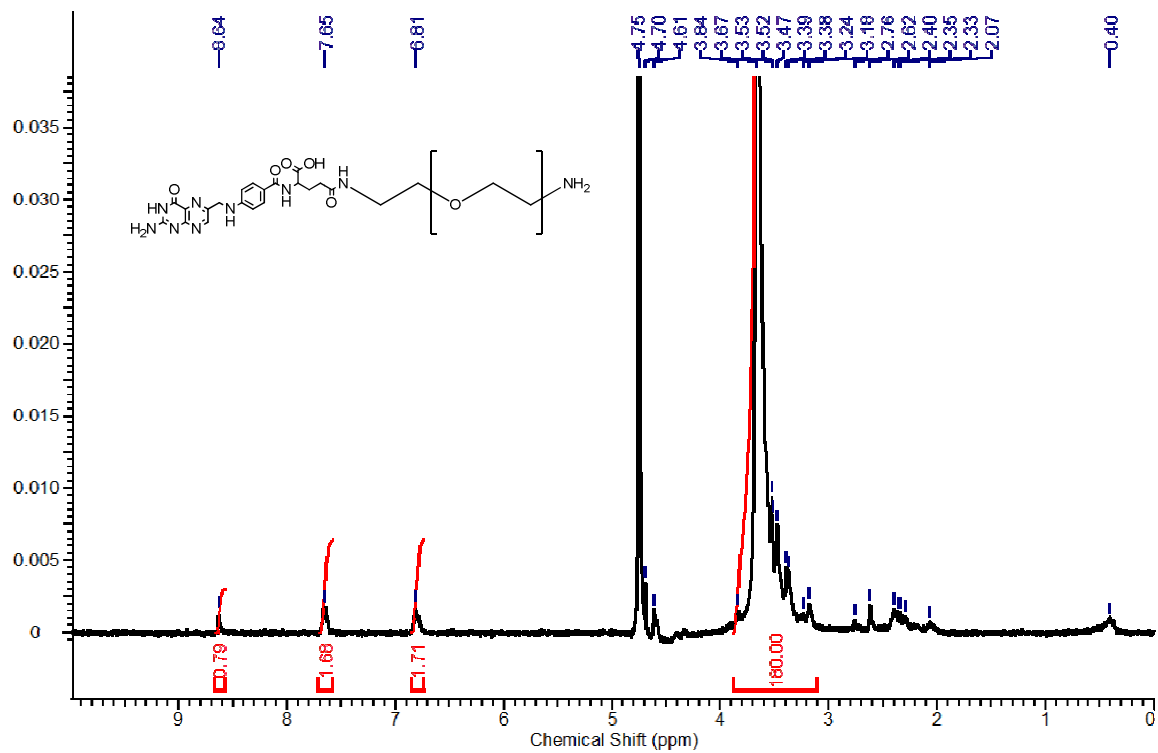**Figure A 6 ¹H-NMR of 1c**
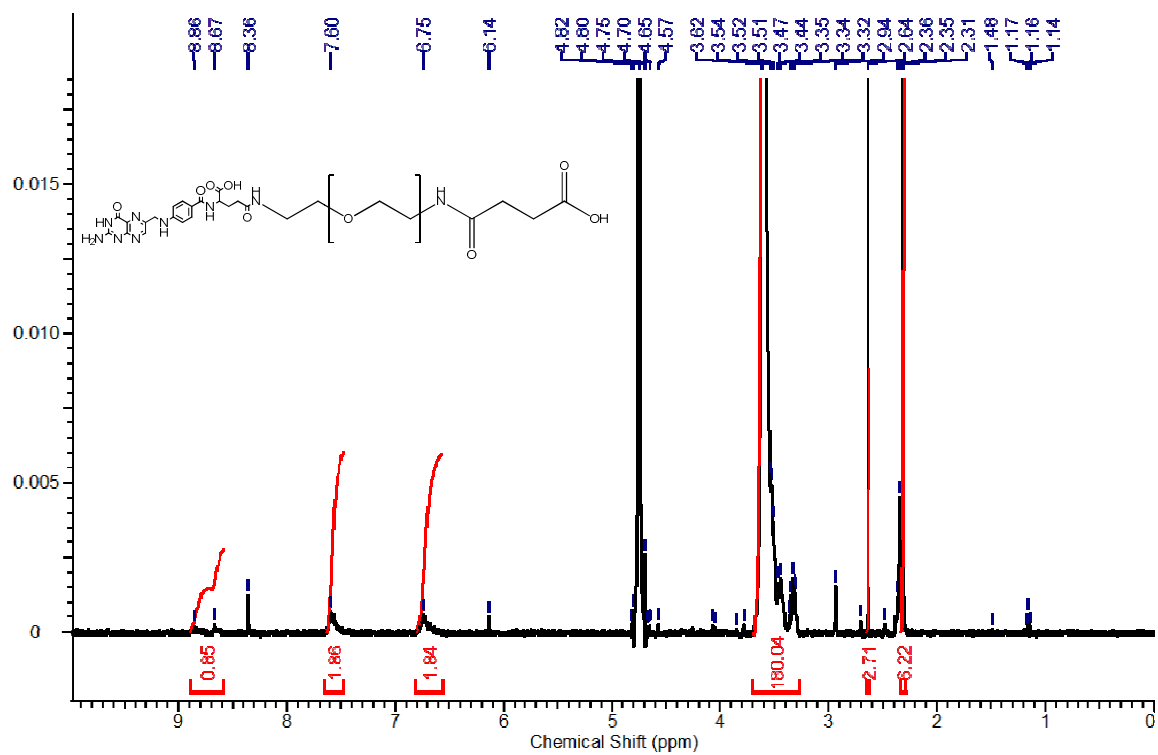
Figure A 7 ¹H-NMR of 1d
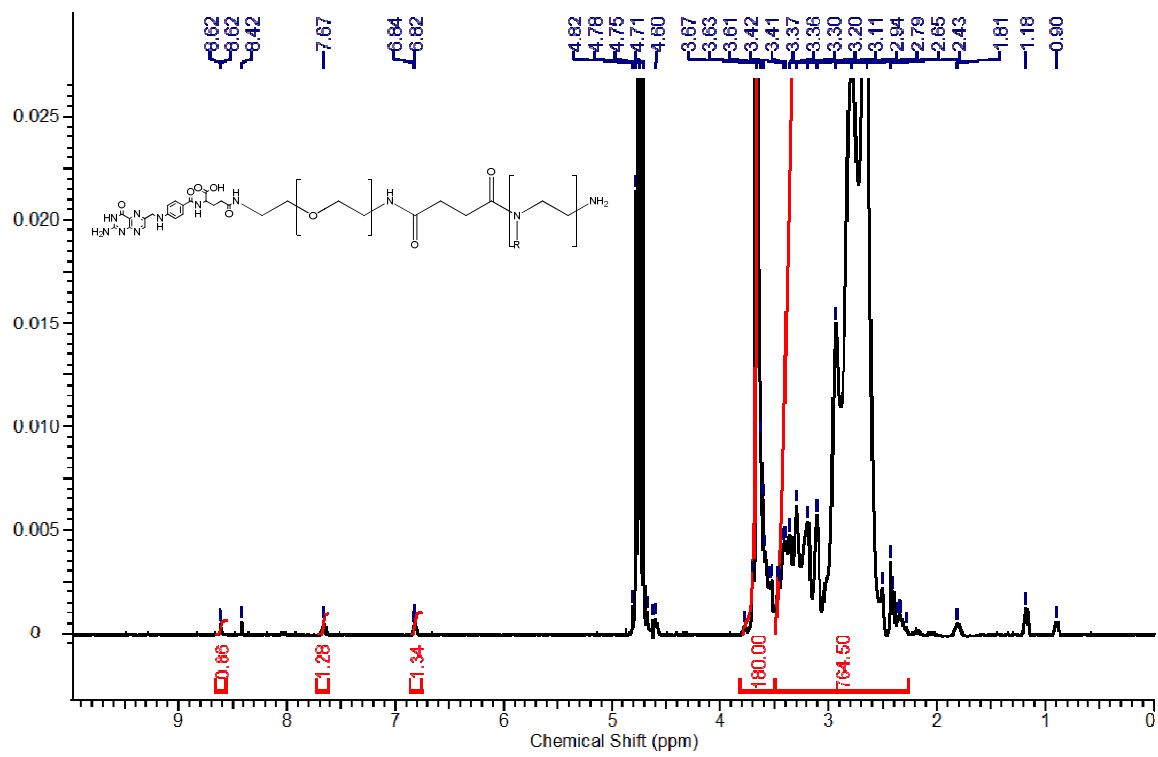
Figure A 8 ^{1}H-NMR of 1e
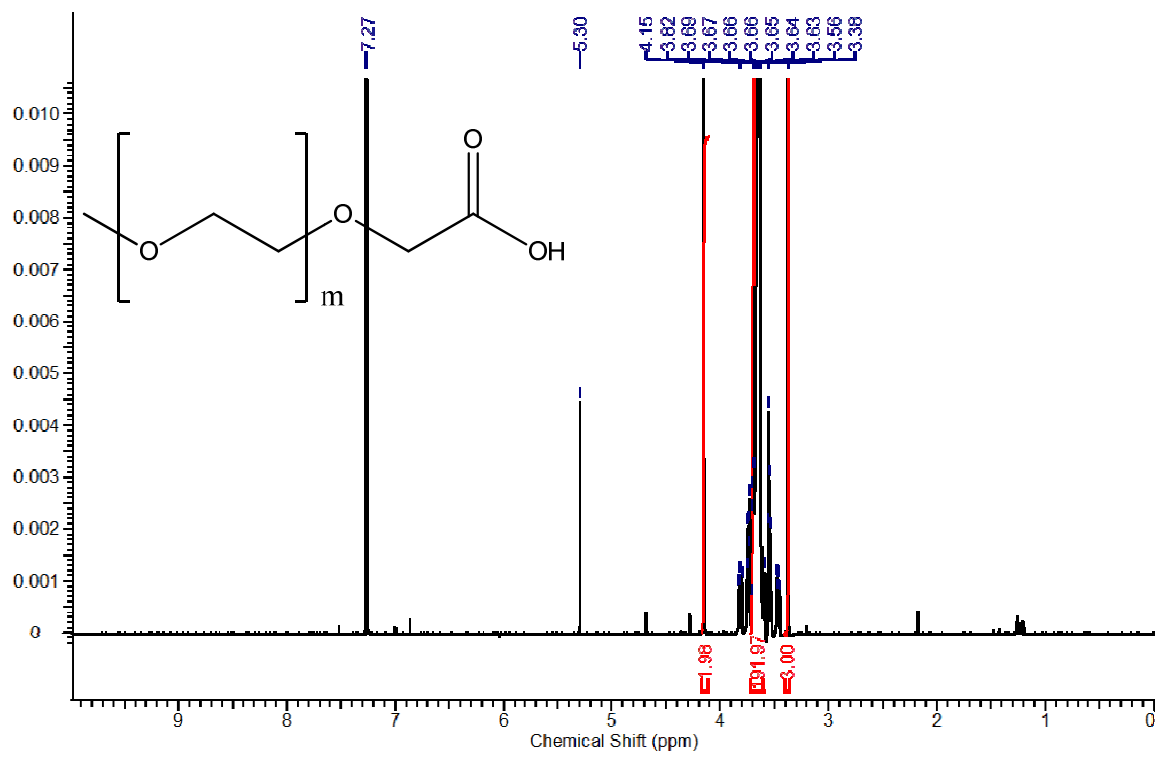
Figure A 9 ^{1}H-NMR of 2b
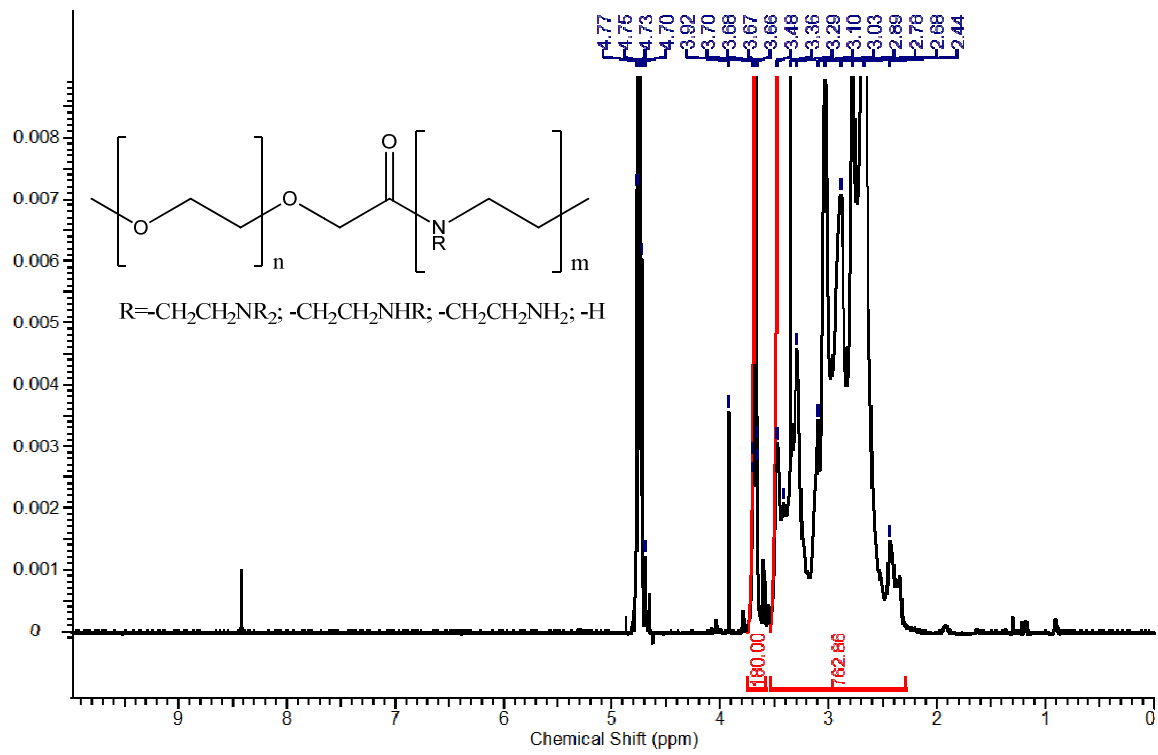
Figure A 10 ^{1}H-NMR of 2c

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Publications:

1. <u>King Sun Siu</u>, Di Chen, Xiufen Zheng, Xusheng Zhang, Nathan Johnston, Yanling Liu, Ken Yuan, James Koropatnick, Elizabeth R. Gillies and Wei-Ping Min (2014) Non-covalently functionalized single-walled carbon nanotube for topical siRNA delivery into melanoma. **Biomaterials** (2014 Mar;35(10):3435-42)

2. Jiang N, Zhang X, Zheng X, Chen D, Zhang Y, <u>Siu LK</u>, Xin HB, Li R, Zhao H, Riordan N, Ichim TE, Quan D, Jevnikar AM, Chen G, Min W (2012) Induction of alloimmune tolerance in heart transplantation through gene silencing of TLR adaptors. **Am J Transplant** (12:2675-2688)

3. Rong Li, Xiufen Zheng, Igor Popov, Xusheng Zhang, Hongmei Wang, Motohiko Suzuki, Rosalia De Necochea-Campion, Peter W French, Di Chen, Leo Siu, David Koos, Robert D Inman and Wei-Ping Min (2012) Gene silencing of IL-12 in dendritic cells inhibits autoimmune arthritis. **Journal of Translational Medicine**

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5. Siu, KS, Lin MCM (2008) Development of novel crosslinked poly(ethylenimine) as potential gene transfer agent ESGCT 2008 Poster. **Human Gene Therapy** (10:1098-1202)

6. Sau M. Lai, Mai Y. Yuen, Leo K. S. Siu, and Ka M. Ng (2007) Experimental Determination of Solid-Liquid-Liquid Equilibrium Phase Diagrams. **Journal of AIChE** (53: 1608–1619, 2007)