Multifunctional nanomaterials for cancer theranostics applications

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NANYANG
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**MULTIFUNCTIONAL NANOMATERIALS FOR
CANCER THERANOSTICS APPLICATIONS**

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SCHOOL OF ELECTRICAL AND ELECTRONIC ENGINEERING

2017

MULTIFUNCTIONAL NANOMATERIALS FOR CANCER
THERANOSTICS APPLICATIONS

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Multifunctional Nanomaterials for Cancer Theranostics Applications

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Abstract

Limited success in early diagnosis of pancreatic cancer, combined with lack of effective treatment options have resulted in low survival rate for pancreatic cancer patients. Current therapy option such as chemotherapy poses toxic side effects and low therapeutic efficacy. In our work, we aim to solve this challenge by utilizing multifunctional nanomaterials for cancer theranostics applications. Organic biodegradable nanomaterials such as cationic polylactides were applied for RNA interference gene therapy for pancreatic cancer and their efficiency and therapeutic efficacy were evaluated. We investigated applications of inorganic materials with interesting properties, such as 1-dimensional carbon nanotubes, 0-dimensional graphene quantum dots, and 2-dimensional transition metal dichalcogenides. Functionalization of these nanomaterials was carried out to improve their biocompatibility and allow conjugation with therapeutic materials to act as efficient transfection agent for gene therapy in pancreatic cancer cells. Intrinsic properties of these nanomaterials are also advantageous for application such as imaging of cancer cells. In these works, we investigated and demonstrated the application of nanomaterials and their potential for improvements in cancer theranostics, especially in gene therapy and imaging of pancreatic cancer.

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List of Abbreviations

AFM	atomic force microscopy
BCPV	biodegradable charged polyester-based vectors
CNT	carbon nanotubes
CPLA	cationic polylactide
DI Water	deionized water
DMSO	dimethylsulfoxide
FA	folic acid
FTIR	Fourier transform infrared microscopy
GS	graphene sheets
GO	graphene oxide
GQD	graphene quantum dots
GS	graphene sheets
IR	infrared
LA	lipoic acid
MWNT	multiwalled nanotubes
NIR	near infrared
PAH	polyallylamine hydrochloride
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEI	polyethylenimine
PCR	polymerase chain reaction
PL	photoluminescence
QY	quantum yield

RNA	ribonucleic acid
RNAi	RNA interference
siRNA	small interfering RNA
SEM	scanning electron microscope
SWNT	single walled nanotubes
TEM	transmission electron microscope
TMDC	transition metal dichalcogenide
UV	ultraviolet

Chapter 1

Introduction

1.1. Background & Motivation

Throughout the years, cancer has remained as one of the most fatal diseases among many. Cancer is responsible for high numbers of incidence and mortality cases around the world, causing estimated deaths close to 0.6 million in United States alone last year.¹ Pancreatic cancer, in particular, has been a significant contributor as 4th highest contributor of cancer mortality in the US and 7th worldwide.¹⁻³ Current state in diagnostics and therapy of cancer also is a factor to this fact. Early diagnosis of pancreatic cancer is difficult, which is why most pancreatic cancer patients have suffered from advanced stage of cancer upon their diagnoses. On top of it, their 5-year-survival rate is very low, standing at a mere 8%, in contrast to that of other types of cancer, which has shown increase over the years.¹ This is partly due to detection of such cancers at advanced stage, at which only 2% of 5-year-survival rate is achieved. Furthermore, the future outlook does not provide an optimistic view, as mortality rates in the last decades has shown increasing trend in cases of pancreatic cancer.^{1, 3} With these in mind, a lot of effort has to be made to urgently improve the current approaches in cancer theranostics to help in early detection and effective therapy options for cancer.

Figure 1.1 Estimated new cases and deaths from cancer worldwide. (Adapted)²

Figure 1.2 Stage distribution of pancreatic cancer and its five-year survival rate in United states. (A) Stage distribution of pancreatic cancers by race, United States, 2005 to 2011, and (B) Five-year relative survival rates for selected cancers by race and stage at diagnosis, United States, 2005 to 2011. (Adapted)¹

Cancer diagnostics and therapy is one of the biggest challenge in current medicine. One of the major challenges in cancer theranostics is the early detection of cancer. While cancer growth is restricted to local tumour environment in its early stage, therefore allows curative treatment to be carried out effectively, once cancer cells enter locally advanced and metastatic stage, effective treatment becomes a great challenge. Presently, several main treatment approaches are available for cancer patients: surgery, chemotherapy, and radiotherapy.⁴⁻⁵ In practice, treatment of pancreatic cancer cases depends on the severity of cancer. Pancreatic cancers are generally classified into stage 0-4, with progressing growth of tumour.⁶⁻⁷ The classification begins with stage 0 denoting carcinoma *in situ* stage, signifying existence of abnormal cells in pancreas. At cancer stage 1A, cancer has grown into small local lesion smaller than 2cm in size, while tumours larger than 2cm is classified as stage 1B. When cancer case reaches stage 2, tumour has spread to surrounding areas and organ. In case of stage 2B, cancer has invaded to lymph node and begin metastasis from the lymph node. Cancers were typically classified as locally advanced when it reached stage 3, when tumour growth has affected pancreatic arteries and considered unresectable. Finally, at stage 4 cancer has reached metastatic stage and spread to organs throughout the patient's body.

Figure 1.3 Trends in Death Rates Overall and for Selected Sites by Sex, United States, 1930 to 2012.
(Adapted)¹

Thus far, surgery remains the first choice of treatment option with curative results, although surgical resection is only applicable for early cases of cancers in stage 1 or even 2, when tumours are still local and resectable.⁷ However, the result of this treatment has not been always satisfactory. In cases of local pancreatic cancer, further treatment by chemotherapy is carried out to improve progression-free and survival rate.⁸⁻¹⁰ For more advanced cancers in stage 3, surgical resection is no longer applicable, and hence treatment by chemotherapy, radiotherapy, or combination of both is used as standard procedure.¹¹ In some locally advanced cancers, chemotherapy can potentially help to downstage patients, therefore allowing potentially curative treatment with surgery.⁷ However, for metastatic cancer, although it can alleviate some symptoms, chemotherapy might not provide curative result and considered as palliative effort.¹² Furthermore, in order to improve therapy efficacy and prolong patient survival, recent studies have even explored potential of chemotherapy with drug combinations,

although significant improvement is rarely seen.¹³⁻¹⁴ Application of these conventional chemotherapy are also limited, as systemic administration of drugs causes nonspecific drug distribution and toxic side effects for patients.¹² In addition, limited solubility of cancer drugs in aqueous media also provides a constraint for their application. Combination of such challenges have therefore put a limitation on the current state of chemotherapy as treatment option for cancer. Hence, novel therapeutic approach to overcome such challenges with potential to improve therapeutic results is an urgent need.

In the last decade or so, nanotechnology has become a field which attracted massive enthusiasm. Capability of engineering materials and structures at nanometer scale is an exciting prospect which can revolutionize applications in multiple fields. Nowadays, numerous nanomaterials, ranging from gold nanorods¹⁵⁻¹⁷ to mesoporous silica¹⁸⁻¹⁹ and carbon nanomaterials including nanotubes²⁰⁻²² and graphene²³⁻²⁵ have been engineered towards various applications. In addition to their extremely small size, these nanomaterials possess unique and interesting properties, compared to their bulky counterpart, such that they attracted huge interest for their potential applications in electronics, photonics, energy, and biomedical engineering. By engineering and employing their extraordinary properties, nanomaterials could be utilized to be a potential solution in cancer therapy. Because nanomaterials can be chemically modified and imparted with chemical functional groups and functionalized with various biochemical substance and polymers, they can be modified to act as novel nanomedicine agent to improve the conventional chemotherapy approach. Nevertheless, application of nanomaterials in biomedical settings also raises concern towards toxicity and biocompatibility of the nanomaterials. Despite their potential as nanomedicine agent, nanomaterials by themselves typically exhibited some degree of toxicity, which becomes a challenge in this area. Therefore, studies of toxicity of nanomaterials have become imperative in order to allow their application especially in biological media. Efforts to minimize the

toxicity of these nanomaterials have to be made in order to realize the application of nanomaterials for nanomedicine applications. All of these reasons have directed development of biocompatible nanomaterials with potential multifunctional capabilities ranging from simultaneous imaging agent²⁶, combinatorial therapy²⁷, or even targeted nanocarrier²⁸ for effective therapy.

1.2. Objectives

Our main direction in these works is to explore and develop multifunctional nanomaterials for applications in nanomedicine. We focused on functionalization and application of nanomaterials and investigated their potential as nanoagents in cancer theranostics as drug or gene delivery system and imaging agents against pancreatic cancer. We speculate that application of these nanomaterials in cancer imaging and therapy can provide great advantages in treatment of cancer as compared to conventional approaches such as chemotherapy. By exploiting these nanomaterials as carriers for therapeutic materials, we were able to form a nanocomplex with these drugs with high loading capacity improved delivery efficiency and therapeutic efficacy of these molecules.

In our works, we are interested in application of novel cancer therapy approach known as gene therapy. In comparison to conventional chemotherapy, gene therapy such as RNA interference (RNAi) involves the delivery of genetic materials such as siRNA into cancerous cells instead of conventional cancer drugs. RNAi leads to inhibition of target gene expression and pathways. We speculate that by targeting oncogenes in pancreatic cancer cells, we can affect their viability and cancerous properties. By applying nanomaterials as transfection agents, we are able to overcome bottlenecks in RNAi therapy and achieve safe and efficient transfection of siRNAs and successfully induce RNAi in pancreatic cancer cells.

We investigated the application of organic nanomaterial such as CPLA, or also referred to as BCPV. This nanomaterial is an excellent candidate for usage in gene therapy as it exhibited positive charges to allow complexation with siRNA molecules. The usage of CPLA were shown to form stable complexes with siRNA and were able to protect siRNA from degradation in biological media. Furthermore, upon application into pancreatic cancer cell lines Panc-1 and Mia PaCa-2, CPLA nanocomplexes were able to enter the cells along with the siRNA molecules, as examined by fluorescence imaging and flow cytometry analysis. Furthermore, cell viability assays have shown that CPLA posed low cytotoxicity effect at high concentrations on its own towards the cells, which notifies that the application and exposure of this nanomaterial in biological media is safe. To further investigate the efficacy of these nanomaterials as gene carriers in RNAi therapy, we carried out several assays to observe the therapeutic efficacy of the nanocomplex. We found that siRNA delivered by this approach can successfully initiate cellular processes such as gene knockdown and inhibit growth of the pancreatic cancer cells. In addition, we were able to confirm the success of the RNAi therapy by observing the wound healing and cell invasion capability of the treated pancreatic cancer cells, which showed the suppressed ability in both areas from the treated cancer cells.

In order to develop nanomaterials with high drug loading capacity, we utilized single walled nanotubes (SWNTs) as nanocarriers due to their high aspect ratio and their extremely high surface area to volume ratio. We applied facile noncovalent functionalization of SWNTs with amphiphilic phospholipid to render water solubility on the otherwise insoluble SWNTs. Upon further functionalized with PAH, the SWNT-PAH nanoparticles were able to act as carrier to siRNA molecules to carry the siRNAs into the cells successfully. Subsequent knockdown of targeted oncogene was also found, suggesting that the nanoparticles were also able to release the siRNA molecules in cancer cells to initiate its therapeutic function.

Application of novel carbon allotrope, graphene quantum dots, allows for cellular imaging due to its intrinsic photoluminescent properties. Using graphene sheets, we synthesized graphene quantum dots following acidic exfoliation and hydrothermal treatment. These GQDs possess water solubility and photostability after long periods of continuous excitation. They were able to enter the cytoplasm of multiple cell lines and used as fluorescent imaging agent. Furthermore, we carried out cellular viability assays of these nanomaterials at extremely high concentrations, in which we observed great biocompatibility of these nanomaterials at relatively high concentration. We believe that upon further functionalization, this nanomaterial can act as gene delivery and imaging agent for simultaneous monitoring and treatment of cancer cells.

A relatively novel 2-dimensional material, transition metal dichalcogenide is considered as graphene analogue with high surface area and drug loading capacity. Despite similarity with graphenes, there were limited studies on their bioapplications. Therefore, we investigated their application as targeted nanocarrier by functionalization with polyethylene glycol and folic acid to act as targeting moiety. Upon functionalization with PAH to regulate their zeta potential, MoS₂ nanosheets were successful to deliver of 2 types of siRNA, targeting different oncogenes simultaneously into pancreatic cancer cells by fluorescence imaging and flow cytometry. By transfection of siRNA combinations, simultaneous gene knockdown was achieved and more pronounced inhibition in cell viability and migration. Throughout these works, we investigated and applied multiple nanomaterials with various advantages and exploit their beneficial properties for improvement in nanomedicine through application in gene therapy for pancreatic cancer cells.

1.3. Thesis Organization

In chapter 1, we describe the motivation and objectives of our research. In motivation section, we present the main reasons toward our focus on this research area. In objectives section, the focus in each of our specific research works was carefully outlined.

In chapter 2, we present literature review on current state in nanomedicine applications focusing on cancer theranostics of organic and inorganic nanomaterials. Beneficial properties of these nanomaterials and their applications in cancer theranostics are presented to justify the potential and capability of their usage for cancer therapy.

In chapter 3, we discuss our work on investigation of pancreatic cancer treatment by gene therapy via RNA interference technique. Organic nanomaterials such as biodegradable cationic polylactide molecules were used as gene delivery agents to aid successful transfection of therapeutic genetic material. We evaluated by multiple assays their biocompatibility and the efficiency of their application as nanocarriers into cancer cell.

In chapter 4, further examination was carried out on the application of these CPLA nanocarriers (also referred to as BCPV) for their effect in cancer therapy. Here, our investigation was conducted beyond transfection capability and into the degree of interference in cellular behaviour upon treatment of the nanocomplex. By targeting K-ras oncogene for pancreatic cancers, alteration in cancerous behaviours such as cell migration and cell invasion capabilities could be observed upon successful treatment. This work can signify that application of nanomaterials not only acts as successful transfection agent, but also induces anticancer effect in some properties of pancreatic cancer.

Chapter 5 describes our effort in utilizing single walled nanotubes (SWNTs) for application as gene nanocarriers, due to their unique morphology with extremely high surface to area ratio. Facile functionalization approach was carried out on pristine SWNTs to enable

water solubility and improve their biocompatibility and subsequent application as siRNA nanocarriers.

In chapter 6, we synthesized graphene quantum dots (GQDs) as a nanomaterial capable for potential application in simultaneous imaging and therapy of cancer cells. Extensive characterizations on morphological and optical properties of these as-prepared GQDs were presented. Fluorescence imaging in multiple cell lines was achieved by using these GQDs. Furthermore, for potential application in nanomedicine, cytotoxicity of GQDs was evaluated in multiple cell lines.

Chapter 7 outlines our work in functionalization and bioapplication of MoS₂ as TMDC nanomaterials for targeted multigene therapy of pancreatic cancer. Upon functionalization with cationic polymer, large surface area of MoS₂ can be applied for loading of multiple siRNA molecules. Furthermore, functionalization with folic acid as targeting moiety can improve specific uptake in the delivery of the nanoagents. Multigene therapy exhibited improved efficacy than single gene therapy. These results were achieved consistently across multiple cell assays. Additional NIR exposure to the treated cells were also observed to improve these results.

Finally, chapter 8 provides a summary and conclusion on our works throughout this thesis.

Chapter 2

Literature Review

In our works, we focus on gene therapy as potential therapeutic option for pancreatic cancer with curative results. In particular, RNA interference (RNAi) is a relatively novel approach with promising potential. For application of RNAi in clinical setting, several obstacles of RNAi must be overcome. In this thesis, we explore the potential of nanomaterials with favourable properties as transfection agent to solve these challenges. These nanomaterials can be classified as organic nanomaterials, such as Cationic polylactides (CPLAs), and inorganic nanomaterials, including single walled nanotubes (SWNTs), graphene quantum dots (GQDs), and transition metal dichalcogenide (TMDCs) such as MoS₂. In this chapter, literature review of RNAi as gene therapy and application of these nanomaterials in cancer theranostics is presented.

2.1. RNA interference

Although gene therapy is a relatively novel approach for cancer therapy, it holds a promising potential for the future. Gene therapy is a therapeutic strategy with the goals of modifying the genetic disease at its source by delivering nucleic acid into cells.²⁹ In gene therapy, genetic materials such as plasmid DNA, small interfering RNA, microRNA, or short nucleotides acted as therapeutic molecules upon their entry to target cells. Since the discovery of recombinant DNA as a revolutionary tool for gene therapy in the 1970s,³⁰⁻³¹ gene therapy has attracted great interest and has been extensively utilized for therapeutic effect in many diseases, such as cardiovascular diseases,³² diabetes,³³ immunodeficiency diseases,³⁴ and cancer.³⁵ RNA

Figure 2.1 The mechanism of RNA interference. Long double-stranded RNA (dsRNA) is introduced into the cytoplasm, where it is cleaved into small interfering RNA (siRNA) by the enzyme Dicer. Alternatively, siRNA can be introduced directly into the cell. The siRNA is then incorporated into the RNA-induced silencing complex (RISC), resulting in the cleavage of the sense strand of RNA by argonaute 2 (AGO2). The activated RISC–siRNA complex seeks out, binds to and degrades complementary mRNA, which leads to the silencing of the target gene. The activated RISC–siRNA complex can then be recycled for the destruction of identical mRNA targets. (Adapted)³⁶

interference (RNAi), first discovered in the late 1990s,³⁷ is a relatively novel approach with great potential as effective method of gene therapy.³⁸ RNAi is a process of post-transcriptional gene silencing where a sequence specific double-stranded RNA is introduced into cells to trigger the degradation of the targeted homologous messenger RNA (mRNA).³⁹⁻⁴⁰ It can be initiated by endogenous microRNAs (miRNAs) or synthetic small interfering RNAs (siRNAs), the latter of which mimic the structure of Dicer products and are incorporated in the pathway downstream of the Dicer enzyme.⁴¹ siRNAs are double-stranded RNA molecules with a typical

length of around 21 base pairs. In the cytoplasm, the siRNAs are incorporated into a nuclease complex, which then identifies and binds to the target mRNA.⁴² Consequently the bound mRNA is rapidly degraded and interrupted from being translated into protein.⁴³⁻⁴⁶ RNAi-based therapeutic strategies have been extensively explored for cancer treatment purpose in the past decade.⁴⁷⁻⁵¹ In recent years, siRNAs have been widely used for targeted gene silencing in pancreatic cancer therapy. For example, siRNAs targeting HIF-1 α suppressed pancreatic cancer cell proliferation and induced cell apoptosis.⁵² Zhi et al. used siRNAs against matrix metalloproteinase-2 (MMP-2) to decrease pancreatic cancer cell adhesion and invasion.⁵³ Another study demonstrated that the silencing of sphingosine kinase-1 can sensitize pancreatic cancer cells to chemotherapy drugs.⁵⁴ These results have shown great promise towards a breakthrough in gene therapy for pancreatic cancer.

Among gene therapy approaches, RNAi displayed several advantages. For example, materials such as siRNA only need to reach the cell cytosol in order to initiate RNAi gene therapy. In contrast, gene therapy based on pDNA requires successful entrance of pDNA into cell nucleus, which provides further obstacle for material transfection.⁵⁵ In addition, RNAi effect can also be observed with lower dose of siRNA.⁵⁶⁻⁵⁷ Inside the cells, functioning siRNA can provide transient therapeutic effect, ranging from 3-7 days in dividing cells to weeks in non-dividing cells.⁵⁷ These reasons show that RNA is quite accessible and controllable gene therapy approach.

Application of RNAi still suffers from several barriers for successful therapy. Firstly, siRNAs are small molecules with labile nature which degrade easily outside cellular environment.³⁶ Secondly, siRNAs are negatively charged due to the existence of phosphate groups in the nucleotides. Combined with their tiny size of ~5nm, naked siRNAs are not able to enter cell membranes efficiently. Whilst this can be solved by utilizing ‘carriers’ to bind with siRNA molecules to form complexes, the nanocomplexes should possess stability to

protect siRNAs outside cellular environment and function as transfection agent. Furthermore, upon successful entrance through cell membrane, siRNA molecules still have to enter cell cytosol in order to initiate mRNA coupling and degradation. In cases of cellular entrance via endosomal pathways, siRNA molecules should be able to escape from endosomes successfully prior to lysosomal degradation. Currently, to achieve high transfection efficiency and therapeutic efficacy, application of RNAi still relies on application of transfection agents such as liposomes and viral vectors.^{36, 55, 58-61} However, these carriers suffer from high toxicity and raises immunogenic concerns which inhibits their use for clinical applications.^{36, 58-62} Therefore, development of new options for application as RNAi therapy agents is a necessity in order to accelerate the realization of RNAi as a potent therapeutic option for cancer patients.

2.2. Organic nanomaterials for cancer theranostics

Cationic Polylactides (CPLAs) are linear cationic polymers which can be synthesized from allyl functionalized lactide and L-lactide molecules to form their polymeric backbone.⁶³ The formation of the allyl functionalized polymeric structure can be achieved by living ring-opening polymerization (ROP) reaction. Cationic groups can then be added into this backbone by conjugation of 4-dimethylaminopyridine (DMAP) to the allyl sites via thiol-ene reaction. The entire scheme of this synthesis is shown in Figure 2.2. There are several advantages in the design of this nanomaterial, including the ability to control the cationic density of the polymers by altering the ratio of precursors during thiol-ene reaction to create nanomaterials with varying amount of tertiary amine groups. One particular advantage of CPLA among organic nanomaterials is its biodegradable properties. In their work, CPLA synthesized by Chen, et al. were shown to be able to undergo rapid degradation from hydrolytic reaction and that this property can be controlled by content of amine group in the CPLA molecules.

Figure 2.2 Synthesis of CPLA via ROP and thiol-ene functionalization. (Adapted)⁶³

In their experiment, these CPLA nanomaterials were applied for usage in gene delivery for prostate cancer cells and were able to completely degraded into oligomers in 9 hours. It was also observed that biocompatibility of these CPLAs were dependent on the amine density of the nanomaterials, showing higher toxicity for higher amine density of the nanomaterials. Furthermore, CPLAs with different amine densities were also seen to exhibit varying transfection efficiency, and even 1 variety of CPLA was able to generate higher transfection efficiency than positive control used in the experiment. Further studies on this nanomaterial have also observed that they are also suitable for delivery of other genetic material such as plasmid DNA with similar degree of success observed.⁶⁴ Nevertheless, these are early studies on the synthesis and application of the CPLAs for gene therapy, such that further results would be required to confirm their efficacy across multiple targets and explore their full potential.

CPLA molecules exhibited linear structure on its own, but molecules of CPLA can be formed into CPLA nanoparticles by crosslinking reaction between them. In the structure of nanocapsules, they can act as a more robust scaffold for loading of biomolecules as compared to the linear structure and exhibit higher surface area to mass ratio to act as more efficient carrier for gene therapy.⁶⁵ Lin, et al. first utilized the CPLA nanocapsules for transfection of siRNA into pancreatic cancer cells. CPLA nanocapsules with average hydrodynamic size of 32 nm were able to act as transfection agent with successful results as measured by transfection efficiency and gene knockdown with comparative result to commercial transfection agent.

These nanocapsules were also shown to be biocompatible even with exposure of up to 400 μ g ml⁻¹ for 48 hours.

2.3. Inorganic nanomaterials for cancer theranostics

In recent decades, various inorganic nanomaterials have been subject of interest for bioapplications due to their extraordinary properties. Inorganic nanomaterials such as quantum dots, gold nanorods, and carbon nanotubes exhibited properties such as photoluminescence and interesting Raman profiles. Some of these nanomaterials may also exhibit large surface area for loading of therapeutic materials such as gene or drug. The ability to engineer and exploit these properties for applications in imaging, sensing, drug and gene delivery, etc have led nanomaterials to become a leading candidate to solve current challenges in cancer theranostics

A major challenge in the application of inorganic nanomaterials lies in the biocompatibility of the nanomaterials. Due to the composition of nanomaterials, such as in Cd-based quantum dots⁶⁶⁻⁶⁷, or their surface properties, such as in graphenes⁶⁸, they exhibit limited aqueous solubility and toxicity which hinders the application of the pristine nanomaterials. The ability to functionalize nanomaterials have enabled the application of inorganic nanomaterials for biological media. Functionalization typically involves covalent or noncovalent chemical approaches to attach or modify surface of nanomaterials to adjust the properties and function of the nanomaterials. Functionalization becomes an important aspect of the engineering of nanomaterials for bioapplications. Primarily, functionalization on nanomaterials is carried out to impart aqueous solubility onto nanomaterials, which is necessary for their use in biological media, and improve their pharmacological properties. Furthermore, chemical functionalizations can be carried out to engineer a specific function for the nanomaterials, such as modification with tumour targeting molecule, imaging contrast agent, chemotherapeutic

drug, etc.⁶⁸⁻⁶⁹ This has hence opened vast possibilities for design and application of multifunctional nanomaterials for nanomedicine.

Therefore, in our works we are interested to study the functionalization and application of inorganic nanoparticles with extraordinary morphology with high surface area-to-volume ratio to enable high loading capacity of therapeutic molecules. In this thesis, we focused on 1-dimensional carbon nanotubes, 0-dimensional graphene quantum dots, and novel 2-dimensional transition metal dichalcogenide (TMDC) nanosheets with high surface area-to-volume ratio and outstanding intrinsic properties.

2.3.1. Carbon Nanotubes in cancer theranostics

Carbon nanomaterials provide an interesting class of nanomaterials, due to the ability of carbon atoms to form covalent bonds among themselves and grow numerous structures based on carbon frames. Carbon-based nanomaterials exist in various allotropes, including fullerenes, nanotubes, nanohorns, nanodiamonds, and graphene. Although these nanomaterials share the same structures composed of primarily carbon atoms, due to differences in morphology, these nanomaterials exhibit distinct properties.

Figure 2.3 Illustrations of the various carbon-based nanomaterials. (A) Single-walled carbon nanotube. (B) Graphene. (C) Fullerene (C₆₀). (D) Carbon nanohorn. (E) Nano-diamond. (Adapted)⁷⁰

Carbon nanotubes (CNTs) were firstly introduced after Iijima's discovery in 1991.⁷¹ CNTs possess extraordinary mechanical, electrical, thermal, and optical properties which has been exploited for many applications.⁷² Morphologically, carbon nanotubes are made of sp^2 hybridized carbon atoms forming tubular structure with diameters ranging from around 1 to several nanometers. As the length of these CNTs can reach up to several micrometers, they are considered as quasi 1-dimensional nanomaterial due to its extraordinarily high aspect ratio. Carbon nanotubes are usually classified onto Single Walled Nanotubes (SWNTs) and Multi-Walled Nanotubes (MWNTs). SWNTs are formed of single layer of carbon tube, hence possess low diameters at typically 0.8-2 nanometers. MWNTs, on the other hand, are composed of at least 2 layers of concentric carbon tubes, such that the diameter can reach tens of nanometers and hence considered relatively large compared to SWNTs. SWNTs typically exhibited intact sp^2 structure, smaller size, and higher aspect ratio, and higher surface area to volume ratio, due to its small diameter.

Figure 2.3 Structure of SWNTs. SWNT chirality is displayed as chiral indices (n,m). Chiralities affects the structure of carbon atoms. This can be observed clearly at the tube ends for armchair and zigzag tubes. (Adapted)⁷³

In practice, pristine SWNTs tend to appear in bundles, due to hydrophobic and π - π interactions among individual tubes.⁷⁴⁻⁷⁵ As SWNTs surfaces are inert and hydrophobic, they are insoluble and quickly reaggregate in aqueous media, which renders them not readily applicable for applications in biological media.⁷⁶ For applications in biological media, individual SWNTs of smaller size and water solubility would be required instead of insoluble bundles with larger size. Therefore, functionalization of SWNTs is a necessary process to create individual functionalized SWNTs which are soluble in aqueous media and biocompatible in biological environment.

In bioapplication of inorganic nanomaterials, functionalization is usually carried out to modify the surface properties of the utilized nanomaterials. Structure of SWNTs provides rich carbon chemistry and hydrophobic surface for diverse functionalization approach by covalent or noncovalent methods. Covalent functionalizations employ carbon chemistry to modify the carbon atoms on the surface of the SWNTs by forming domains of functional groups.⁷⁷ Various reactions such as oxidation, cycloaddition, amidation, esterification, etc. can be performed towards surface of SWNTs to create sites with oxygenated or amidated functional groups, which usually act as a secondary functionalization sites for biomolecules.^{73, 77-79} These approaches require creation of defect sites and damages the pristine carbon structure, which in turn affect the optoelectronic properties of the SWNTs.^{73, 77-79} On the other hand, noncovalent functionalization of SWNTs typically employs intermolecular forces such as electrostatic interaction, hydrophobic interaction, or π - π stacking between inert surface of SWNTs and biomolecules.⁷⁶ Through this approach, water soluble SWNTs can be constructed by attachment with amphiphilic surfactants through hydrophobic interaction. Hydrophobic interaction is considered as a highly strong noncovalent interaction and provides a stable force for attachment of molecules. Hydrophobic domains of amphiphilic surfactants would adsorb onto hydrophobic surface of pristine SWNTs, leaving hydrophilic ends of the surfactants to

Figure 2.4 Different possibilities of the functionalization of SWCNTs. (a) Noncovalent exohedral functionalization with polymers. (b) Defect-group functionalization. (c) Noncovalent exohedral functionalization with molecules through π -stacking. (d) Sidewall functionalization. (e) Endohedral functionalization, in this case C60@SWCNT. (Adapted)⁷⁷

extend into the aqueous media and impart water solubility. Similar tactics can also be employed in subsequent loading of biomolecules and contrast agents for theranostics applications. Noncovalent functionalization offers the advantage of facile modification method of SWNT while maintaining the integrity of the carbon backbone. This becomes an important factor especially during application which utilizes the electronic or optical applications of SWNTs. One such example is Raman imaging, which requires strong Raman signals generated from the carbon backbone structure.⁸⁰

Functionalized SWNTs soluble in biological media is readily applicable for various bioapplications. Application of SWNTs in cancer theranostics revolved around the utilization of their unique morphology and optical properties. Due to their unique 1 dimensional structure, SWNTs exhibited extremely high aspect ratio and surface area to volume ratio. This latter property is an important property which is highly desirable for application for nanocarrier of herapeutic materials in cancer therapy. In addition, various functionalization options for surface

Figure 2.5 Schematic drawings of doxorubicin π -stacking onto a nanotube. Nanotube was prefunctionalized noncovalently by phospholipid (PL)-PEG (left) and covalently by PEGylation of a sidewall -COOH group (right), respectively. (Adapted)⁸¹

chemistry of SWNTs offers an interesting nanomaterial framework for applications as nanocarriers. By utilizing pristine surface of CNTs, CNTs can be functionalized with molecules of cancer drug doxorubicin (DOX) on their surfaces by hydrophobic interaction and π - π stacking and act as drug nanocarrier. Pi-pi (π - π) stacking is the interaction between π electrons in sp^2 carbon structure and molecules containing benzene ring structure or other saturated rings such as naphthenic rings. This loading of DOX onto surface of PEG functionalized SWNTs was estimated to achieve weight loading capacity as high as 400%.⁸¹ These SWNTs were able to act as carrier for these DOX molecules as observed in successful transfection into cells. Moreover, loading of DOX onto SWNTs were observed to be pH dependent, as drugs were released from CNTs in environment of low pH. This is a beneficial property which can assist in more efficient drug release in cancer cell environment with acidic pH.⁸² They also demonstrated the addition targeting moiety such as RGD peptide to impart tumour targeting function to these materials. These RGD-functionalized SWNTs-DOX were able to minimize non-targeted delivery of these cancer drugs, as shown by the lower toxicity on non-target cells induced by this nanocomplex as compared to non-targeting DOX. Mouse model study of SWNT-DOX nanoconjugate showed that they were able to induce toxicity *in vivo*.⁸³ They also

displayed much higher cancer-killing efficacy than naked DOX due to their longer circulation compared to DOX. Although their efficacy was lower compared to that of liposomal doxorubicin DOXIL used in clinical setting, SWNT-DOX possessed much lower toxic side effects towards the mice compared to DOXIL. Using SWNTs modified with chitosan as surfactant and folic acid as targeting moiety, similar outcome in DOX loading and administration were also observed⁸⁴. Furthermore, these nanocarriers exhibited lower systemic toxicity *in vivo* and higher therapeutic efficacy with the same dose of DOX. Besides DOX, SWNTs can be functionalized for loading of other drugs such as paclitaxel⁸⁵, cisplatin⁸⁶, SN-38⁸⁷, etc. This loading of drugs with SWNTs improved the solubility of these drugs and improved their transfection efficacy.

Functionalization of CNTs for applications in gene delivery commonly involves modification with polycations rich in positively charged amine groups. Several approaches of surface modification have been demonstrated from cationic polymer⁸⁸, cationic dendron⁸⁹⁻⁹⁰, polyethylenimine (PEI)⁹¹⁻⁹², etc. Functionalization of oxidized SWNTs with hexamethylenediamine (HMDA) allows introduction of amine groups, however due to low amount of groups on the surface, they were unable to complex siRNA efficiently.⁸⁸ This properties is improved upon further noncovalent functionalization with polydiallyldimethylammonium chloride (PDPA) to introduce more amine groups to allow siRNA complexation. Battigelli, et al. explored covalent functionalization of multiwalled carbon nanotubes (MWNTs) with diaminotriethylene glycol based dendrons to introduce multiple termini of amine groups, which can act as sites for further modification with guanidium.⁹⁰ Evaluation on siRNA carrying ability of ammonium and guanidium MWNTs showed higher transfection efficiency and siRNA release leading to higher gene silencing capability of ammonium groups. Although these nanocarriers were able to achieve much more efficient transfection of the genetic materials compared to transfection by naked materials

alone, they were still not more efficient than current standard such as liposomes. Functionalized MWNTs have been applied for siRNA based gene silencing *in vivo*.⁹³ Although some gene silencing effect can be observed, the therapeutic efficacy is still limited. Dai's group has functionalized SWNTs with pH-responsive delivery of thiol-modified siRNA materials, which showed higher efficiency than liposomes in *in vitro* and *in vivo* settings.⁹⁴⁻⁹⁵ However, the need of thiol-modified siRNAs for such application might cause the method to be not widely feasible. Hence, development of efficient SWNT-based nanocarriers for gene therapy is still an ongoing progress.

One key advantage of application of inorganic nanomaterials is the utilization of their intrinsic properties. By exploiting intrinsic optical properties of CNTs, they can be employed for other therapeutic applications such as photothermal therapy and photodynamic therapy (PDT). Photothermal therapy utilizes high absorbance of CNTs in NIR region. By irradiation of NIR wavelength to CNTs, absorption of NIR photons by the CNTs induces heat build-up and rise in temperature surrounding the nanomaterials.⁹⁶ By irradiating these nanomaterials following their transfection in cancer cells, heat induced in cancer cells can lead to cell death. By utilizing the intrinsic properties of CNTs, this therapy approach can be applied in combination with imaging application or in combination with chemotherapy. Recently, combination of chemotherapy using DOX and photothermal therapy was able to produce synergistic effects, resulting in much improved cancer killing efficacy in the *in vivo* animal model.⁹⁷

Photoluminescence is another intrinsic property which is advantageous for application in cancer theranostics. Photoluminescent property of SWNTs in near infrared (NIR) spectrum, which is applicable for as imaging agent. To prepare biocompatible SWNTs as NIR imaging agent, noncovalent functionalization with surfactants can be carried out to minimize damage on SWNT structure and their optoelectronic properties.⁹⁸ SWNTs functionalized with cholate

Figure 2.6 Absorption and photoluminescence spectrum of SWNTs. Different chirality of SWNTs possess different absorption and emission peaks (left). After purification from mixture of SWNTs, isolated (6,5) SWNTs possessed pronounced absorption and photoluminescent peaks (right).

(Adapted)⁹⁹

and Phospholipid-PEG were prepared with surfactant exchange process. While direct-SWNT with PL-PEG exhibited broad absorption and weak emission spectrum, cholate-SWNT and exchange-SWNT possess sharper absorption peaks and stronger emission. From this method, SWNT imaging probe with excitation at 800 nm and emission at 1,100-1,300 nm were obtained. Functionalized SWNTs have been shown to be able to be used as imaging contrast for high tumour uptake in 2nd NIR window at 1.1-1.4 μ m in mice.¹⁰⁰ Semiconducting SWNTs were able to act as fluorophores in NIR-IIa range at 1.3-1.4 μ m wavelength for noninvasive imaging of brain and cerebral vasculature with *in vivo* mouse model.¹⁰¹ Functionalized SWNTs *in vivo* can accumulate in primary and metastatic cancer cells.¹⁰² In combination with photothermal therapy, this can achieve noninvasive imaging guided therapy of metastatic cancer cells. As NIR imaging agent, emission of the SWNTs were detectable outside small animal model with resolution up to several micrometers, which showed the ability of deep tissue excitation and emission with high resolution.

As electronic properties of SWNTs are highly dependent on their chirality, their absorption and emission spectra varies among different SWNT chiralities.^{99, 103} In order to obtain highly specific absorption and emission peaks, separation of SWNTs by chirality is necessary. Gel filtration and selective functionalization allowed purification of (12,1) and (11,3) chirality SWNTs.¹⁰³ Compared to unsorted SWNTs, these SWNTs exhibits sharp and

Figure 2.7 NIR imaging of (6,5) SWNTs. (a) Higher purity (from left to right) of (6,5) SWNTs resulted in higher NIR signal. Compared to PBS (b) and unsorted SWNTs (c), isolated (6,5) SWNTs exhibited much stronger NIR fluorescence. (Adapted)⁹⁹

specific absorption and emission peaks and hence stronger emission intensity. Such approach allows application of SWNTs with dose 6 times lower compared to unsorted SWNTs. Recently, using density gradient ultracentrifugation, single SWNT chirality at (6,5) were isolated.⁹⁹ In addition to NIR imaging, these SWNTs are also applicable for photothermal therapy and showed better efficiency than unsorted SWNTs in mouse model. However, absorption peak of these (6,5) SWNTs at 570nm was not optimal for these applications.

Another application of CNTs in imaging is through the utilization of photoacoustic imaging. De La Zerda, et al. demonstrated the application of RGD functionalized SWNTs as targeted photoacoustic imaging agents against U87MG tumour in mice.¹⁰⁴ RGD functionalization improved the uptake and photoacoustic signal of SWNTs in tumour lesion by ~8 times. Optical absorption of these SWNTs was enhanced by modification with indocyanine green by π - π stacking, introducing a new absorption peak at 780nm by 20 times and increased the photoacoustic signal for the nanocomplex.¹⁰⁵ In addition to ICG, similar approach with other dyes, for example, QSY₂₁ enhanced absorption at 710nm by 17 times and improved the sensitivity by over 100 times compared to plain SWNTs.¹⁰⁶ Not only this approach improved

their sensitivity as contrast agents, but by applying both the SWNTs modified with ICG and QSY₂₁ as targeted photoacoustic contrast agent *in vivo*, it also provides a platform for potential applications simultaneous multiplex photoacoustic imaging.

Some studies have also observed that CNTs to be able to enter cells by nonendocytotic pathways, probably contributed by the high aspect ratio of the CNTs.¹⁰⁷⁻¹⁰⁸ However, contradictory results have also been observed,¹⁰⁹ and thus further investigations would be required to confirm this phenomenon. Nevertheless, this is an interesting finding in regards to properties of CNTs as cellular delivery agent.

For applications in nanomedicine, inorganic nanomaterials such as carbon nanotubes possesses advantage such as high surface to volume ratio, which is desirable for an efficient therapeutic nanocarrier. Functionalization of carbon nanotubes is able to not only improve their biocompatibility properties but also introduce new application to the nanoparticles. In recent years, CNTs have displayed the potential to be an efficient nanocarriers for drug and gene delivery. Furthermore, CNTs possess beneficial intrinsic properties which can be exploited for applications such as imaging and photothermal therapy.

2.3.2. Graphene Quantum Dots in cancer theranostics

Introduced in 2004, graphene is a quasi 2-dimensional carbon nanomaterial composed of a single layer of hexagonal sp² hybridized carbon structure.¹¹⁰ Similar to CNTs, graphenes exhibit excellent mechanical and electronic properties, such as mechanical strength, high electronic conductivity and thermal conductivity.¹¹¹ Graphenes share the same surface structure to pristine CNTs. In fact, structure of CNTs can be understood as rolled-up graphene sheets. Hence, similar property and phenomenon in form of inert and hydrophobic surface and the subsequent outcome of aqueous insolubility and stacking of graphene sheets were also observed.

Likewise, graphene sheets have to be functionalized to impart aqueous solubility for application in biological environment. In practice, graphene derivatives such as graphene oxides (GOs) and reduced graphene oxides (rGOs) are commonly used for application in biological media. Graphene oxides are oxidized form of pristine graphene, usually derived from graphite via Hummers' method.¹¹² Graphene oxides typically contain functional groups such as hydroxyl, carboxyl, and carbonyl groups on their surface, which render them to be water dispersible and thus applicable in biological environment. Reduced graphene oxides are formed from reduction of graphene oxide and contain some functional groups in the surface which were not removed in reduction, such that they still exhibit aqueous solubility.

Graphenes are zero bandgap nanomaterials, which indicated that they possess no photoluminescent properties. However, works by Sun, et al. firstly demonstrated the photoluminescence properties of nanographene oxides and their capability to be used as imaging agent.¹¹³ Nanographene oxides at lateral size below 300 nm were observed to possess photoluminescence in NIR region. In recent years, such equivalent of nanographene derivatives in smaller scales were referred as graphene quantum dots. These GQDs were typically defined as nanographene equivalent in sub-100 nm scale, although most synthesized GQDs possess lateral size less than 20 nm. Due to their small lateral size, GQDs exhibits different properties compared to graphenes or GOs of larger size. In particular, the photoluminescent properties of GQDs has sparked massive interest, due to the contrasting properties as compared to zero bandgap graphene.

In the recent years, synthesis of GQDs from various precursors has been the main focus in this area. As such, in general synthesis of GQDs were classified to top-down and bottom-up approaches. Top down approaches typically involve acidic oxidation, hydrothermal and electrochemical processes. In general, graphitic precursor of larger size, including carbon fibers¹¹⁴, carbon black¹¹⁵, coals¹¹⁶, graphite¹¹⁷⁻¹²⁰, graphene¹²¹⁻¹²³, graphene oxides¹²⁴⁻¹²⁵, etc.

were broken down by exfoliation, oxidation, and reduction reactions to produce nanoscale GQDs. Bottom up approach, on the other hand, utilizes various small carbon-rich precursors, ranging from carbohydrates¹²⁶, citric acid,¹²⁷ pyrene¹²⁸, L-glutamic acid¹²⁹, acetylacetone¹³⁰, etc. followed by carbonization, pyrolysis, or hydrothermal treatment to create photoluminescent GQDs. Due to the number of options and parameters available, synthesis of GQDs among the researchers have been varied and GQDs with diverse properties have been produced. In general, GQDs exhibited photoluminescence in visible region and high photostability.¹³¹ GQDs may contain hydrophilic chemical groups such as hydroxyl, carboxyl, or carbonyl upon synthesis,^{114, 122, 124, 132} which imparts aqueous dispersibility to these nanomaterials. In addition, they generally have excellent biocompatibility and low toxicity which are beneficial for bioapplications. GQDs are relatively novel carbon nanomaterial and a lot of efforts so far have been put towards the understanding of its optical properties, such as the source of its photoluminescence and controls of the photoluminescence properties.¹³³

Figure 2.8 Synthesis of GQDs from carbon fiber. (A) Representation scheme of oxidation cutting of CF into GQDs. (B) Proposed mechanism for the chemical oxidation of CF into GQDs. (Adapted)¹¹⁴

Primary application of GQDs in cancer theranostics is by utilizing its intrinsic photoluminescence in bioimaging purposes. Early results have displayed various synthesized GQDs as imaging agent in multiple cell lines. Pan, et al. introduces hydrothermal approach for GQD synthesis, which resulted in GQDs with green emission and shown their preliminary study for imaging of HeLa cervical cancer cells.¹²² Upon transfection for 2 hours, these GQDs were brightly fluorescent inside cell nucleus and shown to be photostable as their fluorescence was stable even after 10 minutes of continuous excitation. In addition to applying GQDs for imaging, Qian, et al. also assessed toxicity of the GQDs on HeLa cells by MTT assay, showing relatively low toxicity for pristine GQDs and 1,2-ethylenediamine functionalized GQDs.¹¹⁷ However, in contrast to Pan's work, their GQDs were seen to accumulate in cytoplasm despite transfection for 24 hours. This might be due to different size of GQDs, their GQDs were 10 nm in lateral size, compared to 5nm in Pan's work. In general, most as-synthesized GQDs have been shown to readily enter various cell lines, such as T47D breast cancer cells¹¹⁴, MCF-7 breast cancer cells¹¹⁵, A549 cells¹²³, 4T1 cells¹²⁸, . In these studies, most GQDs were seen to accumulate in cytoplasm, although some accumulation in nucleus were also observed¹¹⁵.

A lot of synthesized GQDs exhibit excitation-dependent emission¹²⁵, a phenomenon where the emission wavelength of GQDs were shifted as excitation wavelength were changed. Zhu, et al. utilizes this excitation-dependent emission of GQDs to demonstrate fluorescence imaging with flexibility in multiple excitation wavelengths. They transfected GQDs on MG-63 cells, and shows that emission from the GQDs can be detected with excitations at both 405nm to 488nm.¹²⁴ Moreover, these GQDs possess low toxicity towards the MG-63 cells *in vitro*. Similar approach was also attempted by Wu, et al. to demonstrate the capability of GQDs to be applied for imaging with excitations at 458 and 514nm.¹²⁹ Another potential application for GQDs also includes imaging of cancer stem cells. While efficient imaging of cancer stem

Figure 2.9 Cytotoxicity of solvothermal GQDs and excitation-dependent imaging. Intrinsically soluble GQDs exhibited relatively low toxicity. The excitation-dependent photoluminescence of GQDs can be applied for cancer imaging with different excitation lengths (b-d). (Adapted)¹²⁴

cells with semiconductor QDs and fluorophores are still challenging, GQDs were able to transfect cancer stem cells and emitted bright fluorescence after transfection.¹²⁰

Therapeutic application of GQDs is still in early stages and has been revolving around its use as drug delivery agents. Wang, et al. carried out early investigation on GQDs as drug delivery agent for DOX. They found increased delivery of DOX into nucleus by GQD-DOX and increased toxicity with increasing GQD ratio. They speculated that GQDs contributed to the enhanced DNA cleavage by DOX. However, further studies are necessary to confirm this phenomenon. GQDs were functionalized with hyaluronic acid (HA) as targeting moiety against CD44 receptors.¹³⁴ Targeted delivery of GQD-HA was displayed with higher uptake in target A549 cells as compared to non-target MDCK cells *in vitro*. This is further shown by increased uptake of GQD-HA compared to GQD in A549-tumour-bearing mouse model. Finally, they loaded DOX onto GQDs for drug delivery application *in vitro*. Wang, et al. demonstrated application of GQDs as simultaneous imaging and drug delivery agent.¹²⁸ Synthesized GQDs of 1-4 nm were loaded with DOX by using sp^2 sites of GQDs as modification surface for π - π stacking and hydrophobic interaction. Conjugation of folic acid embeds targeting function onto these GQDs to improve their uptake on target cells such as HeLa cervical cancer cells. Huang,

et al. further demonstrate the application of GQDs as multimodal imaging and drug delivery agent.¹³⁵ In addition to fluorescence imaging, MRI imaging was achieved by functionalizing these GQDs with Gd^{3+} and folic acid as targeting moiety. These GQDs were also used as DOX delivery agent and exhibited improved efficacy than DOX alone.

2.3.3. Transition Metal Dichalcogenide (TMDC) in cancer theranostics

TMDC is a novel class of nanomaterials which attracted interest recently due to its similarity to 2-D graphene, especially in the electronics and optical field.¹³⁶⁻¹³⁷ TMDCs are considered as 2-D nanomaterials composed of a transition metal atom covalently bonded and sandwiched between 2 chalcogen atoms, forming a layer of 3 atoms thick nanosheets. Examples of such TMDCs include MoS_2 , WS_2 , $MoSe_2$, and WSe_2 nanosheets. As an analogue to 2-D graphene, which have been widely studied for bioapplications,¹³⁸ there are increasing interest towards investigations of the TMDCs application in biomedical field ranging from biosensors¹³⁹⁻¹⁴⁰ to nanomedicine.¹⁴¹⁻¹⁴⁴

Pristine TMDC nanosheets such as MoS_2 exhibited poor solubility in aqueous medium and biological buffer. In contrast to graphene, which contains carbon atoms on the surface applicable for covalent functionalization, MoS_2 nanosheets possess covalently bonded S and Mo atoms, such that these atoms cannot be readily functionalized in covalent chemistry. An option of functionalization of any ligands to MoS_2 nanosheets was developed by exploiting defect sites due to vacated Sulphur atoms. With the existence of defect sites on MoS_2 surface, ligands containing thiol group can be introduced into the MoS_2 solution, where the Sulphur atoms in the thiol groups can attach themselves into the vacated defect sites on the MoS_2 .¹⁴⁵⁻¹⁴⁶ Further approach has included the use of ligands containing disulphide bonds instead of thiol group, suggesting that the existence of 2 Sulphur atoms in disulphide bonds should provide a stronger bond between the ligand as compared to 1 Sulphur atom in thiol group.¹⁴² By applying

this approach, the MoS₂ were able to form stable solution in PBS after functionalization with lipoic acid-PEG conjugate.

Similar to graphenes, the 2-D morphology of TMDCs means that these nanoparticles exhibit high surface area to volume ratio, which allows high loading efficiency of therapeutic materials such as drugs and genes. Liu, et al. speculated that TMDC such as MoS₂ can be used as drug delivery agents by utilizing this analogous property of graphenes and TMDCs.¹⁴² PEGylated MoS₂ were loaded with multiple drugs, including doxorubicin, SN38, and Ce6. The results displayed that these MoS₂ nanosheets were able to act as drug nanocarriers with higher loading efficiency as compared to graphene. Moreover, the MoS₂ nanosheets also exhibited high absorbance in NIR region, allowing them to act as simultaneous photothermal and chemotherapeutic agent. Similar photothermal properties were also observed in other TMDCs, such as WS₂ nanosheets. This enables the applications of TMDCs as multi-modal nanomedicine and theranostic applications, such as photothermal therapy in combination with photodynamic therapy¹⁴⁷ and CT imaging.¹⁴¹

Figure 2.10 Synthesis and characterization of MoS₂ and MoS₂-PEG. A scheme showing the fabrication process of MoS₂-PEG and the subsequent drug loading. (Adapted)¹⁴²

To date, application of TMDCs in as nanocarrier for gene therapy is still limited. Kim, et al. modified MoS₂ nanosheets with PEG-SH and LA-PEI to simultaneously impart biocompatible and cationic functional groups for plasmid DNA delivery into cells.¹⁴⁴ Moreover, application of the MoS₂ is accompanied with NIR irradiation upon transfection of the nanosheets. This approach was speculated to be able to promote the rupture of cellular endosomes, as the higher release of the nanoparticles from cellular endosomes and release of plasmid DNA from the nanocomplex was observed, allowing the genes to undergo cellular processes.

Despite advantageous properties including high surface area and drug loading efficiency and photothermal properties, investigation on TMDC application in nanomedicine is still in early stages and inadequate to fully explore their capabilities. This might be limited by the lack of options in functionalization approaches available thus far. Further development in this area might open more possibilities for engineering these nanomaterials as multifunctional nanomedicine agents.

Chapter 3

Interleukin-8 gene silencing on pancreatic cancer cells using biodegradable polymer nanoplexes

3.1. Introduction

Despite efforts towards utilization of polymeric nanomaterials for gene delivery, development of efficient polymeric gene delivery system is still an ongoing case. As polymeric nanomaterials can exist in many forms and structures, various formulation of polymers ranging from polyethyleneimine, chitosan, cyclodextrin, poly-L-lysine, etc. have been investigated.¹⁴⁸⁻¹⁵⁰ However, many of them suffers from setbacks including toxicity, low gene loading efficacy, or low transfection efficiency into cells. Hence, proper design of polymeric nanomaterials to achieve nontoxic property and high efficacy becomes an important aspect. Cationic Polylactic acid (CPLA) is a polymeric nanomaterial synthesized by polymerization of allyl functionalized lactide and L-lactide.⁶³ Cationic property of CPLA was introduced by addition of 4-dimethylaminopyridine (DMAP) to the allyl sites via thiol-ene reaction. The existence of the tertiary Nitrogen in the DMAP provides cationic functional group in the polymeric structure and positive charge. This positive surface charge enables loading of siRNA onto CPLA by electrostatic interaction. Main advantage of CPLA over other polymeric nanomaterials typically used for siRNA delivery, such as polyethyleneimine (PEI) or Poly-L-Lysine, is primarily its low cytotoxicity and biodegradability.⁶³ CPLAs are able to undergo complete degradation rapidly by hydrolytic reaction within 9 hours. Furthermore, due to its controllable synthesis, structures of CPLAs can be designed to yield CPLAs with different amine group densities.

Interleukin-8 (IL-8), also known as CXCL8, was originally identified as an 8 kDa proinflammatory CXC chemokine which is secreted by multiple cell types, including neutrophils, monocytes, and endothelial cells.¹⁵¹ IL-8 is produced in response to multiple stimuli and is associated with recruiting neutrophils, basophils, and T cells during immune system activation. It exerts its biological effects by binding to two cell surface G protein-coupled receptors, CXCR1 and CXCR2, which are produced by macrophages and other cell types such as epithelial and endothelial cells.¹⁵²⁻¹⁵³ However, IL-8 was also found to be overexpressed in many tumours,¹⁵⁴ such as ovarian epithelial tumours¹⁵⁵ and lung cancer,¹⁵⁶ and exert profound effects on the tumour microenvironment as a proangiogenic cytokine.^{154, 157} Tumour-derived IL-8 activates endothelial cells in the tumour vasculature to accelerate angiogenesis and promote the proliferation, invasion, migration and survival of cancer cells through autocrine signalling pathways.¹⁵⁴ As such, the extensive effects of IL-8 activity on tumour progression and development make it an ideal therapeutic target in pancreatic cancer.^{154, 158} Targeting of IL-8 signalling may have great implications in the halting of tumour progression and assist in enhancing the sensitivity of tumours to chemotherapy or radiotherapy.

In this study, we demonstrated the use of highly biocompatible and degradable cationic polylactic acid nanoparticles (CPLAs) as delivery agent of siRNAs targeting the IL-8 gene into pancreatic cancer cells for gene therapy. Two different pancreatic cancer cell lines, Panc-1 and MiaPaCa-2, were used in the experiments as comparison. Firstly, we examine the role of IL-8 in both pancreatic cancer lines. The IL-8 siRNAs were then loaded to CPLAs for transfection into both cells. We measured the transfection efficiency of the nanocomplexes and their capability to affect the IL-8 gene expression. In addition, we evaluated the effect of IL-8 gene silencing on the proliferation of both cell lines. We found that the CPLAs were able to deliver siRNAs into the cells in efficient manner and induced the suppression of gene expression of IL-8. Successful downregulation of the IL-8 gene was shown to attenuate the proliferation of

the pancreatic cancer cells. This work indicates the potential of CPLAs with RNAi as a novel effective and safe way towards pancreatic cancer therapy.

3.2. Materials and methods

3.2.1 Synthesis of CPLAs with 26 mol% amine group relative to PLA backbone repeat units

Materials: 4-dimethylaminopyridine (DMAP; 99+%) and L-lactide (L-LA) (98%) were purchased from Sigma-Aldrich, and 2,2'-dimethoxy-2-phenylacetophenone (DMPA; 98%) was purchased from Acros Organics. Dichloromethane (DCM; HPLC grade), acetone (HPLC grade), and benzyl alcohol (BnOH; HPLC grade) were purchased from Fisher Chemical. 2-(Diethylamino)ethanethiol hydrochloride (DEAET, 98+%) was purchased from Amfinecom Inc. All other chemicals were used without further purification.

CPLA was prepared according to the method reported previously.⁶⁵ In brief, in a 10 mL flask, allyl-functionalized PLA, DEAET, and photoinitiator DMPA were dissolved in CDCl₃ (5 mL), resulting in a particular molar ratio. Then, the thiolene reaction was induced by UV irradiation ($\lambda_{\text{max}} = 365 \text{ nm}$) for 30 min to yield CPLA with 26 mol% tertiary amine cationic groups relative to backbone repeat units.

3.2.2 Cell culture

Human pancreatic cancer cells, Panc-1 (CRL-1469, American Type Culture Collection) and MiaPaCa-2 (CRL-1420), were maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 $\mu\text{g mL}^{-1}$ penicillin (Gibco) and 100 $\mu\text{g mL}^{-1}$ streptomycin (Gibco). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂.

3.2.3 RNA extraction and real-time quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted from Panc-1 cells using TRIzol reagent (Invitrogen) and the amount was quantified by a spectrophotometer (Nano-Drop ND-1000). Total RNA (2 µg) was reverse transcribed to cDNA using the reverse transcriptase kit from Promega according to the manufacturer's instructions. Relative expression of IL-8 mRNA was assessed using the SYBR green master mix from Promega to perform Q-PCR using a real-time PCR instrument (Applied Biosystems 7500) that detects and plots the increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR amplification. To provide a precise quantification of initial target in each PCR reaction, the amplification plot was examined at a point during the early log phase of product accumulation. This was accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification plot reached the threshold (defined as the threshold cycle number or CT). Differences in the threshold cycle number were used to quantify the relative amount of PCR target contained within each tube. Relative mRNA expression was quantified and expressed as transcript accumulation index ($TAI = 2^{-\Delta\Delta CT}$), calculated using the comparative CT method. All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.2.4 ELISA assay

The level of IL-8 in cell culture supernatants was determined using standard sandwich ELISA Kits (EH2IL8, Thermo Scientific) following the manufacturer's instructions.

3.2.5 Transfection

The day before transfection, cells were seeded onto 6-well plates in DMEM medium without antibiotics to give 30–50% confluence at the time of transfection. A 20 µL CPLA (1

mg ml⁻¹) dispersion was mixed with 20 µL of 10 µM IL-8 siRNA^{FAM} (sense: 5'-FAM-GGAUUUCCUAGAUAUUGCdTdT-3; antisense: 5-GCAAUAUCUAGGAAAAUCCdTdT-3) with a gentle vortex and left undisturbed for 20 minutes. Before transfection, the culture medium was replaced with 960 µL of OPTI-MEM (Invitrogen), the above mentioned CPLAs-siRNA^{FAM} mixture was then added to the medium and the cells were continuously cultured. Four hours later, 500 µL DMEM medium with 30% FBS was added to the medium. Free siRNA^{FAM} was also used in another parallel experiment at the same dosage level. A commercial transfection reagent Oligofectamine (Invitrogen) coupled siRNA^{FAM} was used as a positive control. Gene expression was monitored at 48 hours post-transfection. For transfection efficiency examination, fluorescence imaging and flow cytometry analysis were performed at 4 hours post-treatment.

3.2.6 Fluorescence imaging

In vitro fluorescence microscopy images were obtained using a fluorescence microscope (Eclipse-Ti, Nikon). The cells were washed and fixed with 4% formaldehyde before imaging, and the nuclei were stained with DAPI (Sigma). To image the cells, filter sets for DAPI (excitation at 405 nm and emission was collected with a band pass filter 450/50 nm) and FITC (excited with 488 nm laser and emission was collected with a band pass filter 525/50 nm) were applied for DAPI and FAM (with excitation/ emission maximum at 492/518 nm) signals, respectively.

3.2.7 Flow cytometry

For the flow cytometry experiments, the cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The FAM served as the luminescent marker (filter set for FITC was applied) to determine the transfection efficiency quantitatively.

The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mississauga, CA, USA).

3.2.8 Gene expression analysis

48 hours after transfection, total RNA was extracted from Panc-1 cells using TRIzol reagent (Invitrogen) and the amount was quantified by a micro-spectrophotometer (Nano-Drop ND-1000). Total RNA (2 µg) was reverse transcribed to cDNA using the reverse transcriptase kit from Promega according to the manufacturer's instructions. Semi-quantitative PCR was used to determine the IL-8 relative mRNA expression level normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the most commonly used housekeeping genes adopted in comparisons of gene expression. The PCR products were electrophoresed on 2% ethidium bromide-stained agarose gel and observed under a UV transilluminator (Bio-Rad). Primers used were as before (Table 3.1).

3.2.9 Cell viability

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay. Cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated with different concentrations of CPLAs for 24 or 48 hours. 20 µL of 5 mg ml⁻¹ MTS in PBS was added and the cells were incubated for 4 hours at 37 °C with 5% CO₂. 150 µL of 100% dimethylsulfoxide (DMSO, Sigma) was then added to solubilize the precipitate with 5 minutes gentle shaking. Absorbance was measured with a microplate reader (Bio-Rad) at a wavelength of 490 nm. The cell viability was obtained by normalizing the absorbance of the sample well against that from the control well and expressed as a percentage, assigning the viability of non-treated cells as 100%.

3.2.10 Statistical analysis

All data were presented as means $\pm$ SD. The results were compared by analysis of variance (ANOVA). All statistical calculations were performed with the SPSS 11.0 software package. A p value less than 0.05 was regarded as a statistically significant difference.

3.3. Results and discussion

Increased expression of IL-8 and/or its receptors has been identified in cancer cells.¹⁵⁴ The tumour-derived IL-8 will then increase the NF κ B transcription and the anti-apoptotic protein expression. As a result, profound effects on the tumour microenvironment will be initiated to maintain the viability of tumour cells.¹⁵⁷ We conducted experiments by using CPLA nanoparticles as nanocarriers of IL-8 siRNA for *in vitro* gene therapy on two pancreatic cancer cell lines, Panc-1 and MiaPaCa-2. Furthermore, the roles of IL-8 and its receptors in the pancreatic cancer cells were also examined.

Table 3.1 Primer sequences for real-time PCR

Gene name	Primers
IL-8	5'-CTTCTAGGACAAGAGCCAGGAAGAAACCAC-3' 5'-GTCCAGACAGAGCTGTCTTCCATCAGAAAG-3'
CXCR1	5'-GAGCCCCGAATCTGACATTA-3' 5'-GCAGACACTGCAACACACCT-3
CXCR2	5'-ATTCTGGGCATCCTTCACAG-3' 5'-TGCACTTAGGCAGGAGGTCT-3
GAPDH	5'-ACCACAGTCCATGCCATCAC-3' 5'-TCCACCACCCTGTTGCTGTA-3'

Figure 3.1 Expression of IL-8 and its receptors in pancreatic cancer cell lines (Panc-1 and MiaPaCa-2) of different aggressive potential. (A) The gene expression of IL-8 receptors, CXCR1 and CXCR2 detected by RT-PCR. (B) The relative mRNA level of IL-8 detected by Q-PCR. Values are means $\pm$ SD, $n = 3$; *, $P < 0.001$ vs. Panc-1 cells. (C) Protein levels of IL-8 in Panc-1 and MiaPaCa-2 cells detected by ELISA assay. Values are means $\pm$ SD, $n = 4$; *, $P < 0.001$ vs. Panc-1 cells.

Gene expression of the IL-8 gene and its receptors, CXCR1 and CXCR2 from the pancreatic cancer cells were obtained by RT-PCR. It was observed that IL-8 expression is significantly higher in MiaPaCa-2 than in Panc-1 cells, as characterized in both mRNA and protein levels (Fig. 3.1B and C). It is worth mentioning that the doubling time of MiaPaCa-2 cells in culture is about 40 hours, which is much less than that of Panc-1 cells (~ 52 hours). This result might indicate a possible relation between IL-8 level and the aggressiveness of the cancer cells. This might hint towards silencing of the IL-8 gene as an effective way to halt the proliferation of pancreatic cancer cells.

Figure 3.2 demonstrates the RNAi effect towards the IL-8 gene in Panc-1 and MiaPaCa-2. In Figure 3.2A, commercially available transfection agent, Oligofectamine was used to demonstrate the RNA interference on IL-8. Panc-1 and MiaPaCa-2 were treated with Oligofectamine-IL-8 siRNA nanocomplex (Oligo-IL-8) and the transfection results were shown as gene expression levels. Figure 3.2A shows the gene expression of IL-8 gene in Panc-1 and MiaPaCa-2 of different treatments at 48 hours post-treatment. Blank cells were non-treated cells, while Oligo-SC treated cells were transfected with Oligofectamine-scramble siRNA, which does not target any specific gene. From Figure 3.2B, it was observed that Oligo-

Figure 3.2 Silencing of IL-8 gene expression by specific siRNAs attenuates the proliferation of pancreatic cancer cells. (A) The gene expression of IL-8 in Panc-1 and MiaPaCa-2 cells after treatment with Oligo-IL-8. (B) Protein levels of IL-8 in Panc-1 and MiaPaCa-2 cells after IL-8 silencing detected by ELISA assay. Values are means $\pm$ SD, $n = 4$; *, $P < 0.001$ vs. mock and scrambled. (C) Cell proliferation assays of Panc-1 and MiaPaCa-2 treated with Oligo-SC and Oligo-IL-8 for 72 hours. Values are means $\pm$ SD, $n = 5$.

IL-8 treated cells exhibited significantly low IL-8 gene expression, as compared to the blank and Oligo-SC treated cells. Furthermore, cell proliferation assay by MTT assay (Figure 3.2C) also displayed the lower cell proliferation ($p < 0.01$) in cells treated with the IL-8 siRNA, indicating the anti-cancer effect of the RNAi on IL-8 gene.

Oligofectamine has been widely used as a carrier system for siRNAs delivery in vitro and in vivo.¹⁵⁹ Although it has shown promising results, a high dose of Oligofectamine is known to be cytotoxic.¹⁶⁰ Furthermore, it may initiate an immune response and change the expression of non-target genes that are involved in critical cellular processes.^{159, 161} For

example, reports have shown that Oligofectamine can induce the overexpression of apoptosis related genes, resulting in an increased tendency for early cell apoptosis.¹⁵⁹⁻¹⁶⁰ Due to these reasons, application of Oligofectamine for clinical application is severely limited. In contrast, degradable materials with superior biocompatibility and suitable for biological clearance could be used in its place.

CPLAs are cationic polymer nanomaterials which are soluble in aqueous media and possesses positive charge. This positive charge allows the formation of nanocomplex of CPLAs with nucleic acids by electrostatic interaction between them. In addition, CPLAs can undergo degradation into oligomers (fragments of CPLAs) after 9 hours under physiochemical conditions, which is advantageous in biological applications.⁶⁴⁻⁶⁵ In this work, we utilized CPLAs to form nanocomplex with IL-8 siRNAs at 1:128 ratio. Upon the formation of the CPLA-IL-8 nanocomplex, characterization with dynamic light scattering were carried out to measure the hydrodynamic size. As seen in Figure 3.3, the hydrodynamic size of the nanocomplex were typically 60-80 nm. Zeta potential measurement on this nanocomplex determined the zeta potential of +13.1 mV.

Figure 3.3 Hydrodynamic size distribution of CPLA-siRNA nanoplexes.

Figure 3.4 Fluorescent images of MiaPaCa-2 cells with various treatments. Displayed images are of cells treated with (A) blank, (B) CPLA, (C) siRNA^{FAM}, (D) CPLA-siRNA^{FAM} and (E) Oligo-siRNA^{FAM}. The cell nucleus was stained with DAPI (pseudo-colored in blue) and the signals from FAM are assigned in green. Oligo-siRNA^{FAM}: a commercial transfection reagent Oligofectamine conjugated siRNA^{FAM}.

Figure 3.4 shows the fluorescent images of MiaPaCa-2 cells transfected with CPLA and siRNA combinations for 4 hours. In this Figure, siRNAs were tagged with FAM (siRNA^{FAM}) as fluorescent label for imaging purpose. Observation of FAM in the fluorescent images indicated that the siRNA^{FAM} were successfully delivered into cells. In Figure 3.4A-C, no observable FAM signals were seen, even in siRNA^{FAM} treated cells. This is probably due to the degradation of naked siRNA^{FAM} in biological media before they can enter the cells. In

addition, the negative charge of siRNA^{FAM} might also contribute towards difficulty in entering the cells. In Figure 3.4D and 3.4E, strong FAM signals were observed in cells treated with CPLA-siRNA^{FAM} and the positive control Oligo-siRNA^{FAM}, demonstrating that the CPLAs can protect the siRNA^{FAM} from rapid degradation and act as nanocarrier for siRNA^{FAM} to enter the cells successfully.

Figure 3.5 Transfection efficiency of MiaPaCa-2 cells determined by flow cytometry analysis. (A) Representative pictures, where cells were treated with (i) blank, (ii) CPLAs, (iii) siRNA^{FAM}, (iv) CPLA-siRNA^{FAM} and (v) Oligo-siRNA^{FAM}. (B) Transfection efficiency from experiments shown in (A). Values are means $\pm$ SD, n = 4; *, P < 0.001 vs. blank, CPLA and siRNA.

Transfection efficiency of the CPLA nanocomplex were quantified by flow cytometry analysis as shown in Figure 3.5. Representative plots of the fluorescence intensity in MiaPaCa-2 cells treated with the different formulations for 4 hours were shown. In agreement to results in Figure 3.4, almost no FAM signal was observed in non-treated cells and cells treated with CPLA only ($2.45 \pm 0.84\%$) and siRNA^{FAM} only ($3.55 \pm 1.20\%$). In contrast, much higher FAM signal was observed in cells treated with CPLA-siRNA^{FAM} ($94.72 \pm 1.25\%$) ($P < 0.001$), and is comparable to signal from Oligo-siRNA^{FAM} treated cells ($93.94 \pm 1.38\%$). This result was proportional to the delivery efficiency of siRNAs by CPLAs, and therefore strongly demonstrates that the CPLAs can be used as efficient transfection reagents for siRNAs.

Following the transfection of CPLA-siRNA nanocomplex, the gene silencing efficiency of this nanocomplex was evaluated. Figure 3.6 displays the RT-PCR results of the IL-8 gene mRNA expression in Panc-1 and MiaPaCa-2 cells. It shows that CPLA only and naked siRNA treated cells displayed minimal effect towards gene expression, similar to non-treated negative control cells. In comparison, cells treated with CPLA-siRNA showed significant decrease in the gene expression, with knockdown efficiencies of 50.81% and 53.27% for Panc-1 and MiaPaCa-2 cells, respectively (Figure 3.6B). This result is similar to the knockdown efficiency Oligo-siRNA treated cells, at 51.19% and 48.82% for Panc-1 and MiaPaCa-2 cells, respectively. Moreover, the protein levels of the IL-8 gene were also quantified and showed similar fashion, where CPLA-siRNA treated cells were able to generate a significant knockdown in the protein expression successfully. This result indicates that the siRNAs were in fact released successfully from the nanocomplex upon its transfection and hence initiated RNAi processes.

Figure 3.6 Expression of IL-8 in Panc-1 and MiaPaCa-2 cells. (A) The gene expression of IL-8 detected by RT-PCR, where a–e represent blank, CPLA, siRNA, CPLA–siRNA and Oligo–siRNA treatment groups, respectively. (B) The relative mRNA level of IL-8 from experiments shown in (A). Values are means $\pm$ SD, n = 4; *, P < 0.001 vs. blank, CPLA and siRNA. (C) Protein levels of IL-8 in Panc-1 and MiaPaCa-2 cells detected by ELISA assay. Values are means $\pm$ SD, n = 4; *, P < 0.001 vs. blank, CPLA and siRNA.

Therapeutic effect of the RNAi therapy was evaluated by cell viability assay. MTT assay was conducted on both Panc-1 and MiaPaCa-2 cells after they were treated with different formulations for 72 hours. As seen in Figure 3.7, no significant difference cell viability was observed in negative control and cells with CPLA only and siRNA only treatment. Cells treated with CPLA-siRNA and Oligo-siRNA were observed to exhibit lower viability with no

Figure 3.7 Growth inhibition of IL-8 siRNA delivered by CPLA. Panc-1 and MiaPaCa-2 cells were treated with different formulations for 72 hours. Cell viabilities were measured by MTS assay. Values are means $\pm$ SD, n = 5. *, P < 0.01 vs. blank, CPLA and siRNA.

significant difference between them. This indicates that targeting and knockdown of the IL-8 gene could be used as a potential therapeutic strategy for pancreatic cancer.

The biocompatibility of the gene nanocarriers is of great importance for their bioapplications. For this CPLA nanomaterial, we conducted cell viability studies on Panc-1 and MiaPaCa-2 cells, as displayed in Figure 3.8. At concentration as high as $160 \mu\text{g mL}^{-1}$, cells treated with the CPLA nanomaterials exhibited viability of over 80%, even 24 and 48 hours after exposure. This result demonstrated the low cytotoxicity of the CPLA nanomaterials for *in vitro* applications.

The aggressiveness of pancreatic cancer has made it one of the most deadly cancers around the world.¹⁶² RNAi-based gene therapy has brought great potential for a curative therapy. However, despite lots of efforts in identification of target genes for pancreatic cancer therapy, effective transfection of the siRNA into diseased cells still poses a challenge, such that biocompatible and high transfection agent for gene delivery is required. In this work, CPLA was used as nanocarrier for siRNA into pancreatic cancer cells and has shown potential for their use for novel cancer therapeutic strategies.

Figure 3.8 Cytotoxicity study of CPLAs detected by MTS assay. Panc-1 and MiaPaCa-2 cells were treated with respective concentrations (0, 2.5, 5, 10, 20, 40, 80, 160 $\mu\text{g mL}^{-1}$) of CPLA for 24 or 48 hours. Values are means $\pm$ SD, n = 5.

3.4. Conclusion

In conclusion, we have conducted a biodegradable nanoformulation based on Cationic Polylactic acid (CPLA) for RNAi-based gene therapy of pancreatic cancer. IL-8 gene overexpressed in tumour cells was chosen as the target gene. Upon our examination, IL-8 gene expression was varied in different pancreatic cancer cell lines and may be correlated with aggressiveness of the cancer. Cationic Polylactic acid (CPLA) was used as biodegradable cationic polymer for complexation and transfection of siRNAs. CPLA-siRNA nanocomplex were shown to be able to effectively enter 2 lines of pancreatic cancer cells with high transfection efficiency of over 90%. This formulation was able to suppress the IL-8 gene expression successfully and inhibit the proliferation of Panc-1 and MiaPaCa-2 cancer cells with comparable efficacy to commercial transfection agent Oligofectamine. Cell viability assay have also shown that the CPLA nanomaterials exhibited high biocompatibility. Overall, these results show that CPLA exhibited great potential for application as gene delivery agent for RNAi cancer therapy.

Chapter 4

Biodegradable nanoparticle-mediated K-ras down regulation for pancreatic cancer gene therapy

4.1. Introduction

In previous chapter, cationic polylactide (CPLA), which can be referred to as biodegradable charged polyester-based vectors (BCPVs), was applied as nanomedicine delivery agent for application in gene therapy. The CPLA and siRNA nanocomplex were able to act as successful gene delivery system in targeting IL-8 gene to lower cell proliferation and viability of pancreatic cancer cells. More importantly, these BCPVs also exhibited very low toxicity, high biocompatibility and considerable hydrolytic degradability. In this chapter, we performed further evaluation on therapeutic efficacy of BCPVs as transfection agent for RNAi gene therapy. In this chapter, we utilized BCPVs for delivery of siRNA targeting K-Ras gene in pancreatic cancer cells. Following studies on gene transfection and gene silencing effect, we also carried out further evaluation on effects of gene therapy by examining cell proliferation and metastasis indicators such as cell migration and invasion in MiaPaCa-2 pancreatic cancer cells.

K-ras gene has recently been found to be a common genetic mutation among pancreatic cancer cell lines which occur mostly in early stages of pancreatic cancer development.¹⁶³ Activation of K-ras oncoproteins are thought to cause alteration on the cellular signal transduction pathways and enhance the neoplastic growth of pancreatic cancers.¹⁶⁴⁻¹⁶⁵ Downregulation of K-ras gene expression has been shown to cause reduced proliferation and migration, and increased apoptosis in MiaPaCa-2 cells.¹⁶⁶ Hence, in this work, we are applying

the formulation of BCPV for gene therapy applications by performing RNAi with K-ras target gene.

4.2. Materials and methods

4.2.1. Synthesis of the BCPVs and characterization of the polyplexes

The BCPVs were synthesized in accordance with the method reported previously.⁶⁴ The resulting vectors were characterized by ¹H-NMR and GPC, and the following structural properties were obtained: MNMR $n \approx 19.3$ kDa; charge density: 28%; PDIGPC ≈ 1.38 . The hydrodynamic size and zeta potential of the BCPV/ siRNA polyplexes with different weight ratios were measured using a Brookhaven 90Plus Particle Size Analyzer. Agarose gel retardation assays were utilized to determine the optimized dosage of the BCPVs required to interact completely with the siRNA. The siRNA was mixed by gentle vortexing with the BCPVs at various ratios. After 20 minutes of incubation at room temperature, the complexes were loaded on a 1.2% agarose gel and electrophoresed at 100 V for 15 minutes. The gel was observed under a UV transilluminator (Bio-Rad).

4.2.2. Cell culture

The human pancreatic cancer cells, MiaPaCa-2 (CRL-1420) obtained from American Type Culture Collection, which were used for this study, were maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units per mL penicillin (Gibco) and 100 mg mL⁻¹ streptomycin (Gibco). The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂, and maintained as described previously.⁶⁵

4.2.3. Cytotoxicity

The MiaPaCa-2 cells were seeded at 5×10^3 per well into 96-well plates and allowed to adhere for 24 h. The cells were treated with different concentrations of the BCPVs for 24 or 48 hours. The cell viability was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay kit as previously described.⁶⁵ The cell viability was obtained by normalizing the absorbance of the sample well against that from the control well and was expressed as a percentage, assigning the viability of non-treated cells as 100%.

4.2.4. Transfection

The cells (1×10^4) were seeded onto 6-well plates in DMEM without antibiotics to give 30–50% confluence at the time of transfection. The BCPV (1 mg mL^{-1}) dispersion was mixed with 20 mL of 10 mM K-ras siRNA^{FAM} (sense: 50-FAM-GUUGGAG CUUGUGGCGUAGUU-3; antisense: 5-CUACGCCACAAGCUCCACUU-3) with a gentle vortex and incubated for 20 minutes. Before transfection, the culture medium was replaced with OPTI-MEM (Invitrogen), the above mentioned BCPV–siRNA^{FAM} mixture was then added to the wells of 6-well plates and the cells were continuously cultured for four hours. After that, some of the cells were prepared for imaging under an inverted fluorescence microscope and the transfection efficiency was obtained using flow cytometry. The culture was continued for the remaining cells in the dish with the addition of 500 mL DMEM with 30% FBS. Free siRNA^{FAM} was also used in another parallel experiment at the same dosage level. A commercial transfection reagent OligofectamineTM (Invitrogen) coupled with siRNA^{FAM} was used as the positive control. The gene expression was monitored at 48 hours post-transfection. For the transfection efficiency examination, fluorescence images were obtained at 4 hours post-treatment.

4.2.5. Fluorescence imaging

In vitro fluorescence microscopy images were obtained using a fluorescence microscope (Eclipse-Ti, Nikon). After 4 hours of incubation, the transfected cells were washed twice with PBS, and fixed with 4% formaldehyde. The cells were incubated with DAPI (Sigma) for nuclear counterstaining before the imaging experiments were performed. To image the cells, filter sets for DAPI (excitation at 405 nm and the emission was collected with a 450/50 nm band pass filter) and FITC (excited with a 488 nm laser and the emission was collected with a 525/50 nm band pass filter) were applied for the DAPI and FAM (with excitation/emission maxima at 492/518 nm) signals, respectively.

4.2.6. Flow cytometry

For the flow cytometry experiments, the cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The FAM served as the luminescent marker (the filter set for FITC was applied) to determine the transfection efficiency quantitatively. The samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson, Mississauga, CA).

4.2.7. Gene expression analysis

Forty-eight hours after transfection, the total RNA was extracted from the MiaPaCa-2 cells using an E.Z.N.AtM Total RNA Kit (OMEGA) and the amount was quantitated using a spectrophotometer (Nano-Drop ND-1000). Reverse transcription was conducted in a 20 mL reaction mixture and cDNA was synthesized from 2 mg of the total RNA using a reverse transcriptase kit (Promega) according to the previous experience.¹⁶⁷ The K-ras relative mRNA expression level was determined using semiquantitative PCR normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the most commonly used housekeeping genes adopted in comparisons of gene expression. According to the previous

report⁶⁵, the primer sequences for different genes were as follows: K-ras, forwards 5'-AGAGTGCCTTGACGATACAGC-3' and reverse 5'-ACAAAGAAAGCCCTCCCCAGT-3'; GAPDH, forwards 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCT-GTTGCTGTA-3'. The PCR products were electrophoresed on a 1.2% ethidium bromide stained agarose gel and observed under a UV transilluminator (Bio-Rad).

4.2.8. Cell proliferation, apoptosis, migration and invasion assays

The effect of BCPV-siRNA on the MiaPaCa-2 cell proliferation was detected by MTT assay as described in section 4.2.3 above. A wound-healing assay was conducted to examine the capacity of cell migration. The wound-healing assay was conducted as described previously.¹⁶⁸⁻¹⁶⁹ Briefly, MiaPaCa-2 cells (1×10^4) were seeded into 24-well plates, then transfected with BCPV-siRNA (the weight ratio was 8:1) for 48 hours, in which the cells almost reached confluence. The wound was generated by scratching the surface of the plates with a sterile pipette tip. Then, the cells were incubated in complete culture medium and photographed with a Nikon Eclipse-Ti microscope. Cellular migration towards the scratched area was assessed at 0, 24, 48, and 72 hours (0 hours is the time of scratching). The distances of migration from the cellular monolayer to the wounded area during this time period were measured by NIS Elements Br Analysis 4.0 software. The invasive capacity of the MiaPaCa-2 cells transfected with siRNA was tested using an 8.0 mm BD BioCoat Matrigel invasion chamber assay system (BD Biosciences) according to the manufacturer's protocol with minor modification. Briefly, the MiaPaCa-2 cells were transfected with BCPV-siRNA for 48 hours as described above. Furthermore, the transfected MiaPaCa-2 cells (5×10^4) with a FBS-free medium were seeded into the upper chamber of the system. The bottom chamber of the system was filled with 10% FBS medium as a chemoattractant. After 48 hours of incubation, the cells in the upper chamber were removed with swabs, and the cells that invaded through the Matrigel matrix membrane were stained with 1% crystal violet in PBS for 30 minutes. Then, the invaded

cells were viewed under the microscope, and counted in 5 random fields of vision. The apoptosis assay was conducted using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™) according to the manufacturer. Briefly, approximately 1×10^6 MiaPaCa-2 cells were seeded in a six-well plate. Then, the cells were treated with BCPV–siRNA (the weight ratio was 8:1) for 72 hours. The cells were then collected and washed twice with cold PBS and suspended in binding buffer. Subsequently, the externalization of phosphatidylserine was measured as FITC labelled Annexin V. The cell nuclei were stained with PI. The stained cells were analysed by flow cytometry to determine the apoptotic cells.

4.2.9. Statistical analysis

The data are presented as means $\pm$ SD. Statistical analysis was performed using analysis of variance (ANOVA). All statistical calculations were performed with the SPSS 11.0 software package. When two comparisons were obtained, Student's unpaired two-tailed *t* test was used. A *P* value less than 0.01 is regarded as statistically significant.

4.3. Results and discussion

Previous studies on BCPVs have previously shown promising results as highly efficient carriers for gene delivery,^{64-65, 170} especially as carriers for intracellular siRNA delivery. Chemical structure of BCPVs used in this experiment is shown in Figure 4.1A. The BCPV is a degradable vector with a high surface charge $+62.68 \pm 9.46$ mV suitable for application as siRNA nanocarrier. Characterization on the hydrodynamic size of the BCPV resulted in 13.64 ± 4.96 nm hydrodynamic diameter of the nanoparticles. Prior to bioapplications, cytotoxicity of the BCPVs were also evaluated by MTT assay on MiaPaCa-2 cells. As shown in Figure 4.1B, as 90% cells were viable even at dosage as high as 160 mg mL^{-1} at 24 and 48 hours of treatment, which demonstrates that these BCPVs were highly biocompatible.

Figure 4.1 Structure of BCPV and its cytotoxicity evaluation. (A) The structure of the BCPV. (B) The cell viability of the MiaPaCa-2 cells treated with the BCPVs at different concentrations for 24 and 48 hours. The results are presented as the means $\pm$ SD, n = 6.

In order to ensure the proper loading of siRNA to BCPV, conjugation of BCPV and siRNA was examined at various weight ratios. Figure 4.2A displays the result of gel retardation assay of BCPV and siRNA complex at various weight ratio (1:1, 2:1, 4:1, 8:1, 16:1) with fixed siRNA weight at 1.25mg. In this process, nanocomplexes of BCPV:siRNA were mixed thoroughly to ensure proper complexation and was loaded to the electrophoresis gel. Bright colour observed in Figure 4.2A is attributed to ethidium bromide staining of unbound siRNA, which were able to migrate within the agarose. As the mass ratio of BCPV:siRNA increased, less signal can be observed due to higher amount of siRNA being complexed with BCPV polymers. This complexation between siRNA and BCPV hinders the contact between siRNA and ethidium bromide, such that weaker signal was seen. From Figure 4.2A, nanocomplex at BCPV:siRNA mass ratio of 8:1 were seen to be able to complex with most of the siRNA. Calculation of gene loading content of BCPV at this mass ratio gives a result of 11.1 wt%.

Figure 4.2 Characterization of siRNA loading onto BCPV.(A) Agarose gel electrophoresis of the BCPV/siRNA nanoplexes, Oligo-siRNA as a positive control group. (B) Monitoring of the change in particle hydrodynamic size and zeta potential with different weight ratios of BCPV:siRNA

Characterization of the hydrodynamic size and zeta potential of the nanocomplex were shown in Figure 4.2B. Loading of siRNA onto BCPVs formed nanocomplex with hydrodynamic sizes 56.73 ± 20.62 nm, 93.69 ± 24.06 nm, 84.02 ± 23.59 nm, 74.14 ± 25.48 nm and 73.95 ± 27.38 nm at weight ratios (BCPV : siRNA) of 1 : 1, 2 : 1, 4 : 1, 8 : 1, and 16 : 1, respectively. The change in zeta potential was also clearly seen, as zeta potential drops rapidly as ratio of BCPV:siRNA decreases.

After complexation of BCPV-siRNA, the nanocomplexes were applied for transfection in cancer cells. In Figure 4.3, fluorescence imaging of cells with different ratio of BCPV:siRNA 4 hours post-transfection were displayed. Throughout these figures, amount of siRNA was fixed at 2.5 mg. For this purpose, siRNAs were labelled with FAM to produce green fluorescence during imaging. DAPI was used as stain for cell nuclei with blue fluorescence. Positive control was obtained by using commercial transfection agent Oligofectamine to

Figure 4.3 Fluorescence microscopy images of the MiaPaCa-2 cells treated with different materials. The cell nuclei were stained with DAPI and rendered in blue, and the fluorescence signal from the FAM labeled siRNA is rendered in green.

deliver FAM labelled siRNA into the cell. As observed in Figure 4.3a-c, no fluorescence signal was observed in cells transfected with BCPV alone and FAM labelled siRNA alone, which is similar to negative control. This result confirmed that the siRNA molecules were not able to enter the cells and probably suffer from degradation in the biological media. In contrast, bright FAM fluorescence were seen in cells treated with the various ratio of BCPV:siRNA, which confirms that the BCPV at these ratios were able to deliver the siRNA molecules into the cell safely. Upon closer examination, slight differences can be observed in the fluorescence images of the BCPV:siRNA at different ratio. As the ratio of BCPV was increased, stronger FAM signal was observed, signifying that higher transfection efficiency was achieved. In particular,

at ratio of 8:1, FAM fluorescence signal was observably comparable to that in positive control Oligofectamine group.

Quantification of the transfection efficiency was carried out by flow cytometry analysis. In this measurement, population of fluorescence signals were divided into two populations of M1 and M2 (Figure 4.4b–h), where cells in M1 have low fluorescence signals from autofluorescence while cells in M2 express strong fluorescence from the FAM label. Transfection efficiency is defined as fraction of M2 normalized by total population. The summary of this flow cytometry analysis is provided in Figure 4.4i. Measurement in cells treated with BCPV ($1.05 \pm 0.73\%$) only and FAM labelled siRNA only ($2.14 \pm 0.63\%$) showed that they exhibited negligible level of fluorescence, similar to levels in negative control ($0.35 \pm 0.01\%$). In contrast, cells transfected with BCPV-siRNA have shown strong signals and high transfection efficiencies at $96.69 \pm 1.16\%$, $96.5 \pm 1.40\%$, and $96.15 \pm 2.19\%$ for BCPV:siRNA ratio of 4:1, 8:1, and 16:1, respectively. Moreover, these results are comparable to transfection efficiency of positive control at $84.95 \pm 3.90\%$. Although this result shows a similar level of transfection efficiency of the BCPV:siRNA even at the varying weight ratio, the averaged siRNA delivery efficiency varies for different BCPV ratios. As observed in Figure 4.2A, at ratio of 4:1, there were excess amount of free siRNA in the mixture which were left unconjugated. By increasing the ratio to 8:1, higher concentration of siRNA can be transfected, which translates to transfection of more siRNA in the cells. As compared to 16:1 group, due to the insufficient siRNA for the higher BCPV ratio, this causes no significant difference observed in the fluorescence intensity of the 8:1 and 16: 1 ratio.

Figure 4.4 Transfection efficiency of the MiaPaCa-2 cells evaluated by flow cytometry. (a) Dot plot of the cells, the cells in the R1 region were selected for evaluating the transfection efficiency, (b)–(h) are the statistical counts of the cells treated with PBS, BCPV, siRNA^{FAM}, Oligo-siRNA^{FAM}, and BCPV-siRNA^{FAM} at the different weight ratios of 4 : 1, 8 : 1 and 16 : 1, respectively. Accordingly, the concentrations of the BCPVs were estimated to be 10 mg mL⁻¹, 20 mg mL⁻¹ and 40 mg mL⁻¹ where the weight ratios of the BCPV-siRNA complexes were determined to be 4 : 1, 8 : 1 and 16 : 1. (i) Quantitative evaluation of the results from (b)–(h) showing the average fluorescence intensity and transfection efficiency of each group. The average fluorescence intensity presents the FAM intensity in the cells. The transfection efficiency is defined as the ratio between the cell counts of M2 (transfected cell counts) to M1 + M2 (total cell counts). The relative values shown are the means $\pm$ SD, n $\geq$ 3 (*, P < 0.01 compared to the blank group, #, P < 0.01 compared to the Oligo-siRNA group).

After we were able to confirm the transfection of the nanocomplexes, we evaluate their capability to induce gene therapy in the cells. To measure the knockdown efficiency of the BCPV-siRNA, mutated K-ras gene was selected as target cells. The gene expression was evaluated at mRNA level by agarose gel electrophoresis. mRNA of mutant K-ras gene were

extracted and normalized to control groups, with gene expression of GAPDH gene used as reference. In Figure 4.5, relative gene expressions of mutant K-ras of groups treated with BCPV and siRNA only group did not show any obvious difference as compared to the negative control. Several groups of BCPV-siRNA treated cells have shown significant level of knockdown efficiencies, at $29.56 \pm 6.16\%$, $44.24 \pm 2.01\%$, $47.85 \pm 6.11\%$, for weight ratio from 4:1, 8:1 and 16:1, respectively. Among them, knockdown efficiency at 4:1 ratio was slightly lower possibly due to the existence of unbound excess siRNA during the complexation. Nevertheless, the groups at ratio of 8:1 and 16:1 were able to generate knockdown at level comparative to Oligo-siRNA treated group ($46.67 \pm 6.23\%$). Hence, from these experiments, it can be seen that BCPV:siRNA at ratio of 8:1 were able to sufficiently act as efficient transfection agent. Thus, further experiments using the BCPV:siRNA were conducted at this ratio.

Figure 4.5 Gene expression of K-ras in MiaPaCa-2 cells of different treatment groups. (A) Agarose gel electrophoresis of K-ras relative expression. (B) Quantitative relative gene expression of K-ras after siRNA transfection of different treatment groups. The relative values shown are the means $\pm$ SD, n $\geq$ 3 (*, $P < 0.01$ compared to the blank group. #, $P < 0.01$ compared to the Oligo-siRNA group).

Figure 4.6 Proliferation of the MiaPaCa-2 cells after treatment with different formulations. Cells were first treated with BCPV, siRNA, BCPV-siRNA and Oligo-siRNA, then continued to culture for 24 h, 48 h, and 72 h. The cell viability was determined using MTT cell viability assay. The results are represented as means $\pm$ SD, n = 3.

Figure 4.6 displays the cell proliferation as evaluated by MTT assay of MiaPaCa-2 cells after treatment with the nanocomplex. Cells treated with BCPV only and siRNA only have shown cell growth in similar fashion compared to untreated cells. The cell population were growing up in a steady rate at double after 24 hours and 4 times in 72 hours. However, in cells treated with BCPV-siRNA and Oligo-siRNA, an obviously lower proliferation was observed. This change in the proliferation rate was induced by the successful delivery of the siRNAs.

Figure 4.7A demonstrates the wound healing assay for MiaPaCa-2 cells treated with the different formulations. In negative control group, upon the creation of the wound, the cells would slowly grow to reduce the width of the wound window. At 24 hours post treatment the cells were able to close significant amount of window, and at 48 hours the cells have grown to close and clutter the wound window. After 72 hours the wound were almost completely covered and was barely recognizable. The cells treated with BCPV and siRNA only were also seen with similar fashion. Significant difference was shown by cells treated with BCPV-siRNA and Oligo-siRNA. The cells treated in the 2 groups were growing in much slower rate and were only able to cover almost half the wound after 72 hours, when it took 24 hours for the negative

Figure 4.7 Wound healing assay in MiaPaCa-2 cell culture treated with different formulations. (A) Phase contrast microscope images of the wound healing process monitored for 72 hours post-treatment. (B) Quantitative evaluation on the percentage of the wound window closed after treatment, values were normalized by the initial wound window width.

control. It has been reported that the wound healing process in cell culture is caused by a combination of proliferation and migration of cells in response to the disruption of the cell–cell contacts and an increased concentration of growth factors.¹⁷¹⁻¹⁷² It is a commonly used method to study the cell migration and the underlying biology.¹⁷³⁻¹⁷⁴

In Figure 4.7B, the result is displayed in the normalized percentage. In this Figure, the difference of the wound closing rate between the BCPV-siRNA treated cells and negative controls can be seen. The wound closing rate of BCPV-siRNA and Oligo-siRNA were also showing comparable results. We believe that these results indicate that by applying the BCPV-siRNA complex to target the mutant K-ras gene in MiaPaCa-2 cells, we were able to downregulate the expression of the mutant K-ras gene and the altered the expression of the downstream gene associated with cell proliferation and migration. This results in the slower proliferation and cell migration rate of the MiaPaCa-2 cells treated with the targeting siRNA. Cell migration is a complex process which are regulated by matrix-degrading proteinases, integrins and other cell adhesion molecules.¹⁷⁵ It is a critical event in cancer progression and especially cancer metastasis.¹⁷⁶ By successful inhibition of the cell migration by gene therapy towards mutant K-ras gene in MiaPaCa-2 cells, we can provide a potentially therapeutic approach for treatment of pancreatic cancer.

Metastasis is a complex and multi-step process which starts with the migration and invasion of primary tumour cells to the adjacent tissue.¹⁷⁷⁻¹⁷⁸ Metastatic tumour are able to move around to different location in cancer patients, which causes great challenge for curative treatment. As a result, the ability to block the migratory and invasive capacity of cancer cells is an important approach to treat patients with malignant cancer.¹⁷⁹⁻¹⁸⁰ Hence, we performed assessment of the invasion capacity of the MiaPaCa-2 cells treated by the BCPV-siRNA by an in vitro Matrigel invasion chamber assay.¹⁸¹⁻¹⁸² Laminin and type IV collagen are the main components of Matrigel and give it the structural and biochemical properties of a basement

Figure 4.8 Cell invasion assay based on a transwell chamber model of the MiaPaCa-2 cells after treatment with different formulations. (A) Bright field microscope images of the cells that penetrated through the Matrigel-coated membrane in the transwell chamber. The cells were stained with trypan blue. (B) Quantitative cell counts of the cells that penetrated through the Matrigel-coated membrane. Each bar represents five random microscopic fields under 20x magnification. Error bars indicate the SD, *P < 0.01 compared with the blank group, n = 5.

membrane.¹⁸³ The capability of cells to penetrate the membrane is a good measurement of their invasiveness and metastatic potential.¹⁸⁴ Figure 4.8. shows the result of the Matrigel invasion chamber assay in terms of the population of cell that penetrated through the Matrigel. As observed in Figure 4.8A, population of penetrated cells in groups with BCPV-siRNA and Oligo-siRNA treatment were observed at a much lower level than the untreated cells and cells treated with BCPV only and siRNA only. Quantification of the normalized data shows that the penetrated cells were 60% lower than that in negative control group. These results could

Figure 4.9 FITC Annexin V and Propidium Iodide (PI) co-stained apoptosis assay on the MiaPaCa-2 cells treated with different formulations (blank, BCPV only, siRNA only and BCPV–siRNA) at 72 h post-treatment. (A) Apoptotic cells stained with Annexin V/PI were measured using flow cytometry. (B) Late apoptotic cell counts in the upper right quadrant (Q2) of different treatment groups. Error bars indicate the SD, * $P < 0.01$ compared with the blank group, $n = 5$.

suggest that gene therapy treatment towards mutant K-ras gene by using BCPV nanocomplex could lead to impede the invasion and metastasis of pancreatic cancer cells.

Finally, we carried out apoptosis assay on to the MiaPaCa-2 cells by FITC labelled Annexin V and Propidium Iodide (PI) staining. Annexin V is widely used as early apoptosis indicator. It has a high affinity for the membrane phospholipid phosphatidylserine (PS) which is exposed to the external cellular environment in apoptotic cells. PI is a vital dye which will

be excluded by viable cells with intact membranes. It is commonly used to label dead and damaged cells. Figure 4.9. displays the result after 72 hours of treatment. Among the group of cells with negative control, BCPV only and siRNA only treatment, no clear differences can be observed. However, noticeable difference can be observed between the 3 groups of cells with cells of BCPV-siRNA and Oligo-siRNA treatment, especially in the Q2 zone of the apoptotic cell counts (Figure 4.9A). This is further clarified by quantification of the data, as no significant difference was seen for blank ($17.19 \pm 2.72\%$), BCPV only ($16.11 \pm 2.79\%$), and siRNA only ($17.47 \pm 2.44\%$) group. In the BCPV-siRNA treated group, the number of apoptotic cells was doubled ($37.52 \pm 3.15\%$), and there were no statistically significant differences to the Oligo-siRNA group ($32.67 \pm 4.11\%$).

Cancer therapy has entered a stage of personalized treatment where targeted gene therapy is regarded as the most promising choice. As the K-ras mutation mainly occurs in the early phase of pancreatic ductal carcinogenesis, knockdown of the mutated K-ras gene at the early stage of pancreatic cancer is of vital importance. However, the efficiency of knockdown largely depends on the selection of proper carriers. Our findings demonstrate that the inactivation of mutated K-ras gene by BCPV-siRNA leads to a significant inhibition of cellular proliferation, migration, invasion and anti-apoptosis in MiaPaCa-2 cells. According to such results, the BCPV-siRNA delivery system is far-reaching in gene therapy for pancreatic cancer, and successfully overcomes some challenges in RNA interference and serves as a promising candidate for gene therapy in pancreatic cancer treatment. Yet it is also worth noting that before entering clinics, the stability in the bloodstream, immunotoxicity, and metabolic pathways of BCPV-siRNA in vivo still need to be carefully evaluated and its therapeutic effect in animal tumour models also needs to be assessed.

4.4. Conclusion

In conclusion, we have demonstrated the use of BCPVs as biocompatible and biodegradable cationic polymer based nanoparticle for application in gene therapy to target the K-ras oncogene in pancreatic cancer cells. We performed cytotoxicity assessment of the BCPV nanomaterials and characterization of the BCPV-siRNA nanocomplex at varying weight ratio. We evaluate their efficiency as transfection agent by fluorescence imaging and flow cytometry assessment. Moreover, we evaluated the knockdown of the target gene expression after the treatment with the nanocomplex. In addition, we also investigated the consequences of knockdown of mutant K-ras gene in MiaPaCa-2 cells. The results suggest that this RNA interference process induced changes in the expression pattern of the downstream gene that regulates cell proliferation, migration and apoptosis. By applying BCPV-siRNA with an optimized weight ratio, we significantly reduced the growth, migration, and invasion of MiaPaCa-2 cells, and promote the apoptosis of pancreatic cancer cells. In summary, the BCPV nanomaterials demonstrate great potential for applications as gene nanocarrier for RNAi therapy *in vitro*.

Chapter 5

Pancreatic cancer gene therapy using an siRNA- functionalized single walled carbon nanotubes (SWNTs) nanoplex

5.1. Introduction

Various nanomaterials have been developed for application as nanocarriers of therapeutic biomolecules into cells. Especially, for fragile biomolecules such as siRNA, the use of nanocarriers presents a highly beneficial means for their efficient delivery into target cells. Until now, organic and inorganic nanomaterials ranging from chitosan nanoparticles¹⁸⁵, polymer nanoparticles^{65, 186}, gold nanoparticles¹⁸⁷, silica nanoparticles¹⁸⁸, etc. has been utilized as nanocarriers for cellular transfection of siRNA molecules. Among numerous nanomaterials, carbon nanotubes (CNTs) stands out due to their unique morphology and properties. Their intrinsic carbon structure and 1-dimensional structure provides several advantageous properties for CNTs. Firstly, CNTs were composed of entirely carbon atoms which are intrinsically nontoxic. Due to their 1-dimensional morphology, CNTs possess extremely large surface area compared to their volume. This means that CNTs exhibit higher surface area available for functionalization and loading of biomolecules, as compared to spherical nanomaterials with the same volume. This allows CNTs to act as efficient nanocarriers, as their surface can be attached with high amount of therapeutic materials. Combined with rich chemistry of carbon, CNTs offers a nanomaterial platform with various surface functionalization approaches.⁷⁶⁻⁷⁷ In addition, intrinsic optical properties of CNTs may allow potential application in Raman or NIR photoluminescence imaging.^{98-100, 103}

Similar to many inorganic nanomaterials, naked CNTs suffer from insolubility in aqueous media. In biological applications, which require nanomaterials to be dispersible in biological media, aqueous solubility is imperative. Surface of pristine CNTs were composed of sp^2 carbon structure, which renders them hydrophobic and insoluble in water. Functionalization of the CNTs has to be carried out to impart water soluble moiety on the surface of CNTs and improve its solubility in biological media. This functionalization also lowers the toxicity and improve the biocompatibility of these nanomaterials. In addition, further functionalization may add functionalities onto the surface of the CNT to enable specific applications.

In this chapter, we demonstrated the use of facile noncovalent functionalization of SWNTs for application as transfection agent for mutant K-Ras siRNAs for gene silencing towards pancreatic cancer cells. SWNTs were functionalized with amphiphilic surfactant and cationic polymer to render aqueous dispersion and modify surface charge, respectively. These functionalized SWNTs were further complexed with siRNAs to form an SWNTs/siRNA nanoplex for gene therapy applications. Our results demonstrated that by using the synthesized nanoplex formulation, efficient delivery of siRNAs into the pancreatic cancer cells can be achieved. Furthermore, transfected siRNAs were able to induce gene silencing in the Panc-1 cancer cells. Cytotoxicity study showed that the functionalized SWNTs are biocompatible over a wide range of concentrations. More importantly, the modification was performed using cationic polyallylamine hydrochloride, which has a lower cytotoxicity than mostly used polymers such as polydiallyldiamine hydrochloride or polyethylenimine.¹⁷⁰ As these SWNTs were functionalized noncovalently, minimal defects and breakage on these SWNT surfaces were caused. This preserves the pristine structure and electronic properties of the SWNTs and would be useful for potential applications such as siRNA delivery with simultaneous Raman imaging or photothermal therapy.

5.2. Materials and methods

5.2.1. Functionalization of carbon nanotubes

Facile functionalized SWNTs were prepared based on a modified version of the procedure of Kam et al.⁹⁴ Briefly, 160 mg of raw HiPCo Carbon Nanotube powder (containing 12.3 wt% of pure SWNT, Nanointegris) was mixed with phospholipid mPEG-DSPE-5000 (Laysan Bio Inc.) at a weight ratio of 1 : 2.5 (SWNT : mPEG-DSPE-5000). Onto the mixture, 20mL of mini-Q DI water was added and solution was sonicated for 4 hours in a water bath. After sonication, black solution containing individual dispersed SWNTs was obtained and followed by several rounds of centrifugation. Centrifugation was first carried out at 6,000 $\times g$ for 30 minutes. Collected supernatant from this round was further centrifuged at 13,500 $\times g$ for another 30 minutes and the procedure was performed for final centrifugation at 21,200 $\times g$ for 1 hour. Upon removal of insoluble and bundled materials by centrifugation, the final supernatant was repeatedly washed by filtration through a 100kDa filter (Corning) to remove any excess mPEG-DSPE. SWNTs were further functionalized by addition of 200 μ L of 1mg mL⁻¹ polyallylamine hydrochloride (PAH, Sigma-Aldrich) into 800 μ L of SWNT solution (Abs = 0.461, at 808 nm) under vigorous stirring. This solution was then centrifuged at 9,400 $\times g$ for 10 minutes to remove any aggregates.

5.2.2. Characterization of SWNT solution

Shimadzu UC-2450 UV-VIS Spectrophotometer was used for measurement of absorption spectra of SWNTs. Samples were loaded into a standard 10mm quartz cuvette and measured against water as the reference. Brookhaven 90Plus Particle Size Analyzer was used for measurement of hydrodynamic size based on dynamic light scattering (DLS) and zeta potential measurement by phase analysis light scattering (PALS). Raman spectra measurement and AFM imaging was carried out with SWNTs samples prepared on a Si wafer. Raman

spectrum was obtained with measurement using a WITec Raman system with an excitation laser at 532 nm. AFM images were obtained with a Shimadzu SPM-9500J2 instrument in the tapping mode.

5.2.3. Cell culture and transfection

The human pancreatic cancer cell Panc-1 (CRL-1469, American Type Culture Collection) was maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 $\mu\text{g mL}^{-1}$ penicillin (Gibco) and 100 $\mu\text{g mL}^{-1}$ streptomycin (Gibco). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂. The day prior to transfection, cells were seeded onto 6-well plates to give 30%–50% confluence at the time of transfection. Before transfection, the cell medium was replaced with 1.1mL of OPTI-MEM (Invitrogen) Serum Free medium. Mutant K-Ras siRNA-FAM (K-Ras siRNA^{FAM}, sense strand: 5'-AM-GUUGGAGCUGAUGGCGUAGUU-3'; Antisense: 5'-CUACGCCAUCAGCUCCAACUU-3', from Shanghai GenePharma, China) was solubilized in DEPC treated water at a density of 10 μM . Functionalized SWNT-PAH solution (10 μL) was mixed with siRNA^{FAM} (20 μL , 10 μM) with gentle vortex mixing and left undisturbed for 20 minutes. The SWNT/PAH/siRNA^{FAM} conjugates were then added to the Panc-1 cell culture and the cells were continuously cultured. For comparison, commercial transfection reagent OligofectamineTM (Invitrogen)-coupled siRNA^{FAM} was used according to the vendor's instructions and used in Panc-1 cells treatment as positive control. In addition, free siRNA^{FAM} was also used in parallel experiment. All siRNA^{FAM} in this experiment were used at the same concentration. For transfection efficiency examination, flow cytometry and fluorescence imaging were performed at 4 hours post-treatment.

5.2.4. Fluorescence microscopy

In vitro fluorescence microscopy images were obtained using a fluorescence microscope (Eclipse-Ti, Nikon). The cells were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde and mounted onto glass slides with DAPI loaded mounting media (Fluoroshield™, Sigma-Aldrich) before imaging. Filter sets for DAPI (two band pass filters 350/50 nm and 460/50 nm, and a 400 nm long pass filter as the dichroic mirror) and FITC (two band pass filters 480/30 nm and 540/50 nm, and a 505 nm long pass filter as the dichroic mirror) were applied for imaging of DAPI and FAM fluorescence signals, respectively.

5.2.5. Flow cytometry

Transfected Panc-1 cells were washed with PBS before collection with trypsin, and then washed again afterwards. The FAM served as the luminescent marker (filter set for FITC was applied) to determine the quantitative transfection efficiency. Measurements were performed using a FACSCalibur flow cytometer (Becton Dickinson, Mississauga, CA).

5.2.6. Gene expression analysis

RNA was extracted from Panc-1 cells using TRIzol reagent (Invitrogen) 48 hours post transfection. The amount was quantified using a spectrophotometer (Nano- Drop ND-1000). A reverse transcriptase kit from Promega was used to reverse transcribe this total RNA (2µg) to cDNA following the vendor's instructions. The K-Ras mRNA expression level was determined by semi-quantitative PCR, and compared to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is a housekeeping gene used for the normalization of gene expression.¹⁸⁹ Electrophoresis of the PCR result was carried out on 1.2% ethidium bromide stained agarose gel and observed under a UV transilluminator (Bio-Rad). Primers: K-Ras 5'-AGAGTGCCTTGACGATACAGC-3' (sense), 5'-ACAAAGAAAGCCCTCCCCAGT-3'

(antisense); GAPDH 5'-ACCACAGTCCATGCCATCAC-3' (sense), GAPDH 5'-TCCACCACCCTGTTGCTGTA-3' (antisense).

5.2.7 Cell viability study

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay. Panc-1 cells were seeded in a 96-well plate at a density of 5×10^3 cells per well and incubated with a range of concentrations of SWNT and SWNT-PAH for 24 hours. After 24 hours, MTT (5 mg mL^{-1} , $20\mu\text{L}$) in PBS was added and the cells were incubated for 4 hours at 37°C with 5% CO_2 . Dimethylsulfoxide (DMSO, $150\mu\text{L}$, Sigma) was added to solubilize the precipitate with 5 minutes of gentle shaking. Absorbance was measured with a microplate reader (Bio-Rad) at a wavelength of 490 nm. The cell viability was determined by normalizing the absorbance of the sample well against absorbance of the control well and was expressed as a percentage, assigning the viability of the control non-treated cells as 100%.

5.3. Results & Discussion

For application in siRNA delivery, we functionalized SWNTs by noncovalent method with PEGylated phospholipids and polycationic polymer. Functionalization of SWNTs with PEGylated phospholipid resulted in dispersion containing water-dispersible SWNTs. Upon centrifugation and filtration to remove large bundles, insoluble materials, and impurities, homogenous SWNTs solution were obtained. In order to enable loading of siRNA via electrostatic interaction, further functionalization of these negatively charged SWNTs were carried out with polycationic polymer polyallylamine hydrochloride (PAH) by electrostatic interaction. PAH is composed of repeating amine groups connected along the carbon chain backbone and is generally less toxic in comparison to other cationic polymers such as

Figure 5.1 Scheme for noncovalent functionalization of SWNTs. (a) SWNTs adsorbed with mPEG-DSPE, (b) Electrostatic loading of PAH on mPEG-DSPE functionalized SWNTs, (c) SWNT/PAH conjugated with siRNA^{FAM} by electrostatic interaction.

polydimethyl diallyl ammonium chloride (PDDAC) and polyethylenimine (PEI).¹⁷⁰ Functionalization of the dispersed SWNTs with PAH creates a layer of PAH surrounding SWNTs and hence renders positive surface charge on the nanoconjugates. Thereafter, loading of siRNAs onto the positively charged surface SWNTs/PAH by electrostatic interaction can be completed successfully.

Firstly, mPEG-DSPE functionalized SWNTs were characterized by Raman spectrometry, UV-Vis spectrophotometry, and dynamic light scattering. Characterization by Raman spectroscopy of SWNTs revealed several unique features which can be used for identification of SWNTs, namely G-Band, D-Band, and radial breathing mode (RBM) peaks.¹⁹⁰ From the Raman spectrum of functionalized SWNTs, graphitic G-Band at 1587 cm⁻¹ is the most noticeable peak due to its high intensity. In this sample, D-Band at 1300cm⁻¹ can be observed, which is possibly due to defects in SWNTs and residual impurities. Even though these SWNTs were functionalized noncovalently, sonication for long duration might cause breaks in surface of SWNTs.⁹⁸ Finally, peaks of RBM of SWNTs, which Raman shift are associated to radial mode and diameter, were also observed at the lower region of 150–250 cm⁻¹. Thus, this result confirmed the existence of SWNTs in the solution. Concentration of SWNTs in our sample was determined based on absorption and molar extinction coefficient.

Figure 5.2 Characterization of SWNTs. (a) Raman spectrum of functionalized SWNTs displayed characteristic G-Band, D-Band, and RBM peaks; (b) absorbance spectrum of SWNT dispersion. Measured absorbance was diluted dispersion of SWNT (28 times dilution); (c) size distribution of functionalized SWNTs as determined by DLS technique; (inset: AFM image of SWNT with a scale bar of 2 μm); (d) Colloidal stability of SWNTs and SWNTs/PAH as assessed by effective hydrodynamic diameter size over time.

Absorption of SWNTs was measured by UV-Vis spectrometry, as shown in Figure 5.2b. From this spectrum, concentration was estimated from molar extinction coefficient of SWNTs as obtained by Kam et al.⁹⁶

Size of mPEG-DSPE functionalized SWNTs can be measured by several modalities. In our experiment, size of SWNTs was represented in their hydrodynamic size as measured by dynamic light scattering (DLS) technique. As compared to measurement of dehydrated SWNTs samples by SEM or TEM, hydrodynamic size can more accurately represent conformation of functionalized SWNTs in aqueous media. This is because hydrodynamic diameter includes the

effect of the surfactant conformation in solution, aqueous solvation layer and Brownian motion as well as diffusion of the nanoparticles.¹⁹¹ Therefore hydrodynamic diameter is a useful and considerably important parameter in application of nanoparticles.¹⁹²⁻¹⁹⁴ Measured effective hydrodynamic diameter of functionalized SWNTs was 110nm, that they were applicable as nanocarriers for cellular transfection application. From further measurement by AFM, the strand-like shape of these dispersed SWNTs was observed.

SWNTs functionalized by mPEG-DSPE exhibited negative surface charge, such that the measured zeta potential was -25 mV. Functionalization with PAH modified the surface charge, yielding a new zeta potential at +40 mV. This was also accompanied with increase in hydrodynamic size to 150nm. Furthermore, colloidal stability of SWNTs and SWNTs/PAH were assessed based on change in hydrodynamic size, where good colloidal stability for over 2 weeks were observed on SWNTs and SWNTs/PAH.

For applications in biological environment, functionalized nanomaterials have to be biocompatible such that the nanomaterials alone are not toxic towards cells. Although previous study has shown that generally, functionalized CNTs possess low toxicity, toxicity of each CNTs was dependent on type and degree of functionalization.¹⁹⁵ Therefore, prior to application of our functionalized SWNTs, cytotoxicity of these nanomaterials was firstly evaluated by MTT assays upon treatment on Panc-1 cells. Treatment of Panc-1 cells with SWNTs and SWNTs/PAH for 24 hours revealed that viability of treated cells was maintained above 80% at concentration as high as 600 pM. In addition, as no signs of morphological damage to the treated cells were observed, these nanomaterials were highly biocompatible and suitable for applications within the concentration range.

Figure 5.3 Cytotoxicity assessment of SWNT and SWNT/PAH by MTT assay. Panc-1 cells were treated with several concentrations of SWNT and SWNT/PAH for 24 hours. Values shown are means $\pm$ SD, n=5.

Therapeutic potential of SWNTs/PAH nanoformulation was explored by application for gene therapy. These SWNTs/PAH can be complexed with siRNAs by electrostatic interaction, forming SWNTs/PAH/siRNA nanoplexes which were stable in aqueous solution for up to 72 hours. For application in gene therapy of pancreatic cancers, SWNTs/PAH were complexed with mutant K-Ras gene for Panc-1 pancreatic cancer cells. K-Ras is an oncogene which encodes important proteins which are responsible for regular cellular function, such as proliferation, differentiation and survival of the cell.¹⁹⁶⁻¹⁹⁷ Mutation on this gene causes abnormal cell function which is observed in most of pancreatic cancers.¹⁹⁸ For visualization of siRNA transfection, fluorescence FAM labeled K-Ras siRNA molecules (siRNA^{FAM}) were used for transfection into Panc-1 cells. To observe the efficiency of transfection, several formulations were used. Firstly, commercial transfection reagent Oligofectamine™ loaded with siRNA^{FAM} (Oligo/siRNA^{FAM}) were used as positive control. For comparison, results from non-transfected (blank) cells and cells transfected with siRNA^{FAM}, SWNTs/PAH, and SWNTs/PAH/siRNA^{FAM} were also presented. From fluorescence microscopy images, high FAM fluorescent signals can be observed from groups of cells transfected with SWNTs/PAH/siRNA^{FAM} and Oligo/siRNA^{FAM}. In contrary, cells transfected with siRNA^{FAM}

Figure 5.4 Fluorescence microscopy of different groups of Panc-1 cells upon treatment by several formulation. Cells were treated with blank (untreated), siRNA^{FAM}, SWNT/PAH, Oligo-siRNA^{FAM}, and SWNT/PAH/siRNA^{FAM}. Fluorescence signal were obtained from cell nuclei staining with DAPI (blue fluorescence) and FAM signal from siRNA^{FAM} (green fluorescence). Scale bar represents 20μm. alone does not emit observable FAM signal. This shows that SWNTs/PAH can be applied as nanocarrier for delivery of siRNAs into cancer cells.

Quantitatively, transfection efficiency upon delivery of SWNTs/PAH/siRNA^{FAM} was evaluated by flow cytometry analysis. In this experiment, FAM fluorescent signal from different transfection groups were recorded and comparison was made with threshold value based on negative control group (SWNT/PAH only group, Fig. 3.5a). Transfection efficiency was determined by fraction of the cell count with fluorescent signals above the threshold (Fig. 3.5b). From the various groups, blank cells and cells treated with SWNTs and SWNTs/PAH alone did not produce fluorescent signal due to the lack of FAM. In the case of cells transfected with siRNA^{FAM} alone, negligible signal were also seen, due to ineffective transfection of

Figure 5.5 Transfection efficiency of Panc-1 cells as measured by flow cytometry. (a) Flow cytometry analysis of Panc-1 cells treated by: (i) blank (untreated), (ii) siRNA^{FAM}, (iii) SWNT, (iv) SWNT/PAH, (v) Oligo-siRNA^{FAM}, and (vi) SWNT/PAH/siRNA^{FAM}. (b) Transfection efficiency from different siRNA^{FAM} delivery formulation as determined from flow cytometry analysis in (a). Values shown are means $\pm$ SD, n=3 (*, $P < 0.001$ compared to negative control).

siRNA^{FAM} alone. In contrast, significantly stronger FAM signal were observed in cells treated with the Oligo/siRNA^{FAM} ($92.6 \pm 2.1\%$) and SWNTs/PAH/siRNA^{FAM} nanoplex ($95.3 \pm 4\%$). This hence quantitatively demonstrates the potential functionalized SWNTs as efficient nanocarriers of siRNA for cell transfection.

Upon successful delivery of siRNAs into cells, siRNAs need to be released from nanocarrier to subsequently initiate RNAi process. To ensure that transfected siRNAs were released from cells and initiated RNAi, analysis of mRNA expression of corresponding target gene by PCR was carried out. In this case, expression levels of mutant K-Ras mRNA in Panc-1 cells after treatment for 48 hours was assessed. Based on the expression levels, whilst no

knockdown was seen in untreated cells ($100 \pm 12.2\%$), cells treated with SWNT ($109.7 \pm 5.73\%$) or SWNT/PAH ($113.1 \pm 4.11\%$), and cells treated with siRNAs ($106 \pm 8.8\%$), a notable knockdown was observed in the group of cells treated with SWNTs/ PAH/siRNA nanoplex, where the expression level of the targeted mutant K-Ras mRNA was suppressed to ($66.88 \pm 5.14\%$). Although the gene expression is not as low as that of the positive group Oligo/siRNA formulation ($56.92 \pm 1.27\%$), the knockdown by SWNTs/ PAH/siRNA is still comparable. Interestingly, SWNTs functionalized by covalent methods for electrostatic complexation and delivery of siRNAs also exhibited similar knockdown efficiency to our functionalized SWNTs, relative to positive controls.⁹⁰⁻⁹¹ Nevertheless, SWNTs functionalized to siRNA via disulphide bonds exhibited higher knockdown efficiency, although it requires thiol-modified siRNA.⁹⁴ Moreover, duration of treatment could be a factor in the assessment of knockdown efficiency, especially for siRNA electrostatically loaded to nanocarriers.^{13, 185, 187} Conjugates of siRNA electrostatically loaded onto nanocarrier might be more stable than the conjugates with transfection agents in positive control group, such that they release siRNAs at slower rate.⁹⁰ Regardless, the result showed that upon transfection with these SWNTs/PAH/siRNA nanoplex, siRNA molecules were released within the cell and subsequently initiated the RNAi process for specific gene knockdown.

Figure 5.6 Relative gene expression of mutant K-Ras mRNA of treated Panc-1 cells. Inset: gel electrophoresis of mutant K-Ras and GAPDH mRNA from Panc-1 cells. Values shown are means $\pm$ SD, n=3. (*, $P < 0.001$ compared to negative control).

5.4. Conclusion

In this work, we demonstrated a facile approach of SWNTs functionalization for pancreatic cancer gene therapy. SWNTs were noncovalently functionalized with PEGylated phospholipids to improve its solubility in aqueous media and its biocompatibility. For application as siRNA transfection agent, further modification with cationic polyallylamine hydrochloride (PAH) was performed on the SWNTs to alter their surface potential and allow effective complexation with siRNAs by electrostatic interaction. These SWNTs/PAH/siRNA nanoplexes displayed high gene transfection efficiency as assessed by flow cytometry. They were also able to release siRNAs upon cellular internalization and induced effective knockdown of target gene expression in pancreatic cancers. Furthermore, the formulated SWNTs exhibited relatively low toxicity over a wide range of concentrations. Based on these results, we envision that further developments on these functionalized SWNTs, by utilizing their large surface area and intrinsic optical properties, can potentially provide improved formulation of these nanomaterials as multifunctional platform for targeted cancer theranostics with high efficiency.

Chapter 6

Synthesis and characterization of graphene quantum dots (GQDs) for pancreatic cancer imaging

6.1. Introduction

In recent decades, there have been a lot of emphasis towards development of nanomaterials with multifunctional capability upon their transfection into cells. Nanomaterials with application as nanotherapeutic and imaging agents can provide effective mode for simultaneous monitoring and treatment of cancer cells. Previously, semiconductor quantum dots,^{67, 199} gold nanorods,¹⁶ carbon nanotubes,¹⁰²⁻¹⁰³ etc. have been investigated due to their optical properties for these applications. However, several bottlenecks have led to difficulties for widespread application of these nanomaterials. For example, photoluminescence of carbon nanotubes was heavily dependent on the bandgap tuned by chirality of individual nanotubes, which limits their effective usage in large capacity. Major concerns over intrinsic cytotoxicity and biocompatibility of nanomaterials have also been a raised, especially in materials like Cd based quantum dots.⁶⁶⁻⁶⁷ This has hence led efforts towards strategies for biocompatible functionalization of Cd-based quantum dots⁶⁷ and development of Cd-free quantum dots¹⁹⁹ to provide lower the toxicity properties of these nanomaterials.

Along with the single atom thickness, graphene possesses extraordinary mechanical, electrical, thermal properties which allows them to be potentially advantageous for applications in various field.²⁴⁻²⁵ Graphene has also been used for biological applications in recent years for applications such as carrier of therapeutic and imaging agents. However, due to their infinite

Bohr radius and zero bandgap, graphene possesses no intrinsic photoluminescence, which limits their application as intrinsic imaging agent. Graphene Quantum Dots (GQDs) are considered as a novel allotrope of carbon nanomaterials due to its development in recent years. GQDs are usually defined as mono or few-layer sp^2 allotrope of carbon nanomaterials, typically at sub-100nm scale. Although GQDs share similarity with the well-known graphene such as their monolayer sp^2 lattice structure, GQDs possess distinctive properties, including photoluminescence, photocatalytic and electronic properties²⁰⁰ which were unseen before in commonly larger graphene sheets. In particular, due to their photoluminescence properties in visible region, GQDs have attracted large interest for potential applications in bioimaging and biosensing.²⁰⁰⁻²⁰¹

In this chapter, we described our work on graphene quantum dots (GQDs) synthesized from graphene sheets as precursor. Graphene sheets were oxidized and exfoliated with mixture of concentrated sulfuric and nitric acid. Further hydrothermal treatment on the oxidized graphene resulted in photoluminescent GQDs. We performed cytotoxicity evaluation of the as-prepared GQDs with multiple cell lines. The GQDs were shown to possess low cytotoxicity at relatively high concentration and were suitable for application in cellular imaging. Furthermore, upon non-covalent functionalization with cationic polymer such as polyallylamine hydrochloride (PAH), the nanocomplex were able to bind with siRNA to act as theranostic agent for simultaneous imaging and therapeutic agent.

6.2. Materials and methods

6.2.1. Synthesis and functionalization of graphene quantum dots from graphene flakes

Graphene quantum dots were synthesized by acidic exfoliation¹¹⁴ followed with hydrothermal treatment¹²² by using graphene sheets as precursor. In this work, 30mg graphene flakes 8nm (graphene Supermarket) were oxidized with 8ml of sulfuric acid 98% (J.T. Baker) and nitric acid 65% (Sigma Aldrich) at 3:1 ratio and sonicated at 80°C for 24 hours. Acid solution containing oxidized graphene were centrifuged and filtered to remove precipitates. Sodium hydroxide (Merck) were added to neutralize the pH of the solution. Upon dialysis with 1kDa dialysis bag (Spectrum Labs) for 5 days to remove ions, the solution was lyophilized to obtain graphene oxide.

5mg of graphene oxide in 25ml solution was added with 2.5ml sodium hydroxide (6M) to adjust to pH=13, and were treated in hydrothermal autoclave at 200°C for 12 hours. Following hydrothermal process, the obtained graphene quantum dots (GQDs) solution were neutralized with sulfuric acid and dialyzed in 1kDa dialysis bag for 5 days to remove any ions. GQDs were obtained in powder form following lyophilization of the solution.

As-prepared GQD were functionalized with polyallylamine hydrochloride (PAH) (Sigma) by mixing and vortexing various mass ratio of 1mg ml⁻¹ GQD solution and 1mg ml⁻¹ PAH for 15 minutes.

6.2.2. Characterization

The absorption spectra were measured using a Shimadzu UV-2450 UV-VIS Spectrophotometer. Samples were loaded into a standard 10mm quartz cuvette and measured against water as the reference. Photoluminescence spectrum were measured with Fluorolog-3 Fluorometer (HORIBA Jobin Yvon, Edison, NJ USA). Zeta potential measurement by phase

analysis light scattering (PALS) were performed using a Brookhaven 90Plus Particle Size Analyzer. Fourier Transform Infrared (FT-IR) measurement was performed by using Shimadzu FT-IR spectrometer. Raman spectroscopy was carried out using a WITec Raman system with an excitation laser at 532nm. Transmission electron microscopy (TEM) images were captured using a FEI Tecnai microscope. AFM images were obtained with Asylum Research ARC2™ SPM instrument. The photostability study was performed by continuous excitation using UVGL-58 UV lamp (watts: 6W, spectral output: 365nm) for irradiation. The fluorescence lifetime was measured using a DCS-120 confocal scanning fluorescence lifetime imaging system (excitation wavelength: 375nm, repetition rates: 20MHz). Measurement of quantum yield was performed against Quinine Sulfate (QY=54%) in 0.1M sulfuric acid. Measurement was performed based on absorbance band of GQD at 360nm.

6.2.3. Cellular Imaging

Cell lines such as human pancreatic cancer cell Panc-1 (CRL-1469, American Type Culture Collection) and MiaPaCa-2 (CRL-1420), liver cancer cell Hep G2 (HB-8065), and Raw 264.7 macrophage cells were maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 $\mu\text{g mL}^{-1}$ penicillin (Gibco) and 100 $\mu\text{g mL}^{-1}$ streptomycin (Gibco). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂. Cells were seeded onto petri dishes to give 30%–50% confluence at the time of transfection. GQDs were added into the cells at concentration of 200 $\mu\text{g mL}^{-1}$ and were incubated for 4 hours. After 4 hours, the cells were washed with PBS and fixed with 4% formaldehyde prior to imaging.

6.2.4. Cell Viability Study

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay. For this, cells were seeded in a 96-well plate at a

density of 5×10^3 cells per well and incubated with a range of concentrations of GQDs for 24 hours. The cells were transfected with GQDs at varying concentration and incubated for 24 hours at 37 °C with 5% CO₂. After 24 hours, MTT (5 mg mL⁻¹, 20 µL) in PBS was added and the cells were incubated for another 4 hours. Dimethylsulfoxide (DMSO, 150 µL, Sigma) was then added to solubilize the precipitate with 5 minutes of gentle shaking. Absorbance was measured with a microplate reader (Bio-Rad) at a wavelength of 490 nm. The cell viability was determined by normalizing the absorbance of the sample well against that from the control well and was expressed as a percentage, assigning the viability of the non-treated cells as 100%.

6.2.5. Gel Retardation Assay

Gel retardation assay was carried out to evaluate the net charge of GQD-PAH nanocomplex at various mass ratio. Electrophoresis was carried out on a 1% agarose gel with a current of 80 V for 15 min in a TAE buffer solution (40 mM Tris-HCl, 1 v/v % acetic acid, and 1 mM EDTA). UV light was illuminated onto the agarose to show the position of the GQD bands.

6.3. Results & Discussion

The as-prepared graphene quantum dots were synthesized by hydrothermal method by using graphene sheets as precursor. Oxidative exfoliation of graphene sheets was carried out by sonication of graphene in 3:1 ratio of concentrated sulfuric and nitric acid at 80°C for 24 hours. The resulted oxidized graphene in solution exhibited weak fluorescence, in contrast to similar process with carbon fiber, which produced brightly fluorescent graphene quantum dots. We believe that this is caused by the pristine and uniform sp² structures across graphene nanosheets which presents a harder structure to oxidize and cut, as compared to sp² clusters in precursor like carbon fiber¹¹⁴. This hence has produced graphene oxide of larger scale.

Subsequent treatment of these graphene oxides through hydrothermal process resulted in fluorescent GQDs. Hydrothermal treatment was speculated to act as reduction step of the graphene oxides. Addition of sodium hydroxides can act as reducing agent in combination with hydrothermal process.²⁰² The combination of hydrothermal reductions were expected to simultaneously remove oxygenated groups such as epoxy on the surface and lead to cutting of the nanosheets into smaller sizes.¹²¹ Figure 6.1 represents the synthesis scheme for these GQDs from graphene precursor and the overview of this chapter.

Figure 6.1 Synthesis scheme of GQDs from graphene nanosheets. Graphene quantum dots were characterized to study their morphology and optical properties. They were applied as imaging agents and their biocompatibility was evaluated. Inset shows the graphene oxide and graphene quantum dots solution in visible light and under excitation with 365nm UV light.

In Figure 6.2A, TEM characterization of the as-prepared graphene oxide (GO) and graphene quantum dots (GQDs) is shown. The GO produced were measured around 30-50nm in lateral size, and were observed to form multi-layered clusters. Measurement of lateral size of GQDs resulted in size ranging from 6-12nm, showing crystalline structure. Further characterization by AFM on GO showed the height of the sample ranging from 5-12nm, showing that the particles were composed of few layers of graphene oxide. Considering atomic thickness and interlayer spacing of graphene at 0.34nm, the GO nanoparticles obtained in this process contained approximately 8-20 layers, although fewer layer might exist due to increased thickness from oxygenated functional groups on their surface. The as-prepared GQDs possess height of 0.56-1.3 nm, suggesting monolayer or 2-3 layer atomic thickness.

FTIR spectroscopy was carried out to examine the chemical composition of the GS, GO, and GQDs. From the IR spectrum shown in Figure 6.2E, no peaks from oxygenated functional groups were seen in FTIR spectrum of GS as expected. Oxygenated functional groups such as -OH ($>3000\text{ cm}^{-1}$), C=O (1730 cm^{-1}) and C-O ($1251, 1416\text{ cm}^{-1}$) were observed in both GO and GQD. Whilst these peaks in GO were attributed by multiple sites of oxygenated groups due to the oxidation process, the finding of these peaks in GQD signifies that incomplete reduction was achieved, leaving oxygenated groups such as carboxyl, aldehyde, and ketone groups in GQD. These remaining functional groups most likely contribute to water solubility of the GQDs. Moreover, the existence of these functional groups explained the negatively charged surface of these GQDs, which zeta potential were measured at -10.51 mV (table 6.1). Finally, Raman spectrum of the GQDs were measured, where G peak and D peaks of the graphitic can be observed. Measurement of the I_D/I_G peaks provides result of 0.92, signifying that the GQDs exhibit relatively small sp^2 cluster and relatively high functional groups on the surface.

Figure 6.2 Characterization of GO and GQD nanoparticles. TEM characterization of (A) GO and (B) GQD (inset: HRTEM of single crystalline GQD). AFM characterization of (C) GO and (D) GQD. (E) FTIR characterization of graphene sheets (GS), GO, and GQD. (F) Raman spectroscopy of GQD.

Optical properties of the as prepared GQDs were used in this process were shown in Figure 6.3. In Figure 6.3A, the absorbance spectra of graphene sheets (GS), graphene oxide (GO), and graphene quantum dots (GQDs) are shown. Graphene oxide exhibited relatively broad absorbance spectrum with no distinct absorbance peak. In contrast, the absorbance spectrum of the as-prepared graphene quantum dots displayed 2 noticeable features, the

absorbance shoulder at 283 nm, contributed to π - π^* transition of the sp^2 configuration, and an absorbance band at 360 nm.

Similar to other GQDs, the as prepared GQD exhibited excitation dependent photoluminescent properties. Upon excitation at 360 nm, the GQDs exhibited emission at 498 nm. However, despite their absorbance shoulder at 360 nm, maximum emission intensity was observed when the GQDs were excited with 420 nm wavelength. Subsequently, longer wavelengths were also seen to be able to excite the GQDs, although they resulted in weaker emission intensity and red shifted emission. Quantum yield of this GQDs were measured against quinine sulfate based on absorbance and excitation at 360 nm, resulting QY of 2.03%. Nevertheless, in practice the GQDs were typically used with excitation at 420nm due to the maximum emission intensity at this excitation wavelength.

Fluorescence lifetime measurement of the GQDs were displayed in Figure 6.3C. Exponential fitting of the fluorescence decay of the GQDs showed 2 decay components. The parameters resulted were shown in inset, where the lifetime components were $\tau_1=3.3271$ ns and $\tau_2=0.4830$ ns. Figure 6.3D displayed the photostability of the GQDs, where emission intensity was measured at 505 nm peak with 420 nm excitation. After 10 and 24 hours of continuous excitation, the GQD emitted at 97.88% and 95.24% of the emission intensity, respectively, as compared to emission intensity at beginning of the measurement. This result clearly demonstrated the photostability of the as prepared GQDs.

We further examined the photoluminescent properties of the GQDs by examining the photoluminescence in varying pH and temperature conditions. Absorbance and photoluminescence of the GQD at 420 nm excitation in various pH were shown in Figure 6.4A and 6.4B. It was observed that in acidic pH that absorbance shoulder at 360 nm was more pronounced than at neutral, while conversely in alkali pH, absorbance at 283 nm was more

Figure 6.3 Characterization of optical properties of GQDs. (A) Absorbance of GS, GO, and GQD. (B) Photoluminescence spectrum of GQD. (C) Fluorescent lifetime measurement of GQD. (D) Photostability of GQD.

noticeable in a similar manner. Examination of the GQD emission at various pH displayed that while no significant shift of emission peak was observed, the emission intensity was heavily dependent on the pH of the environment the GQDs were dissolved in. Maximum emission intensity of the GQDs was achieved when GQDs were dispersed in neutral as compared to alkali or acidic solution. However, the emission was much subdued in acidic than alkali environment, as observed in Figure 6.4C. Figure 6.4C presented the relative emission intensity of GQDs at 505 nm with 420 nm excitation in varying pH. From this Figure, it was observed that at pH 1 and 3, respectively, the peak emission intensity was only at 23.86% and 42.51% as compared to 82.79% at pH 11.

Figure 6.4 Optical properties of GQDs in varying pH and temperature environment. Measurement of optical properties of GQDs in varying pH: (A) absorbance and (B) photoluminescence. (C) Comparison of peak emission intensity in varying pH, relative to neutral pH. (D) Measurement of photoluminescence and (E) the comparison of photoluminescence peak intensity relative to intensity at 20°C of GQDs in varying solution temperature. The photoluminescence spectra were measured were 420 nm excitation wavelength.

Furthermore, effects of the solution temperature towards emission intensity was also examined. Figure 6.4D displayed the emission of the GQD at 420 nm excitation. It was observed that the emission was the highest when the temperature of the GQD solution was at 10°C. Following at higher temperatures, the peak emission intensity drops off to a relatively stable level. Similarly, no peak shift across the different temperatures was observed in this experiment. From Figure 6.4E, relative towards peak emission intensity at 20°C, no major changes were observed other than slopes from 10°C to 20°C and 20°C to 30°C. Furthermore, at higher temperatures the emission intensity was relatively stable, as no major changes were seen. At temperature as high as 80°C, the peak intensity was still 81.89% of the intensity at 20°C. This shows that the GQDs were relatively stable towards temperature and could be used for applications in high temperature environment.

Figure 6.5 Fluorescence imaging of multiple cell lines without (blank) and with GQD transfection (GQD). (A) Raw 264.7 macrophage cells, (B) Hep G2 liver cancer cells, (C) Panc-1 and (D) Mia PaCa-2 pancreatic cancer cells.

We utilized the photoluminescent properties of GQDs as bioimaging agent for fluorescence cellular imaging. The GQDs were transfected into several cell lines for 4 hours

prior to imaging. Figure 6.5 shows the cell imaging results of multiple cell lines with and without GQDs. Green fluorescence can be seen in the various cell lines transfected with GQDs. This result shows the ability of the as-synthesized GQDs to be used as imaging agent for multiple cell lines for *in vitro* applications.

Thus far, we have evaluated the cytotoxicity of the GQDs on Raw 264.7 macrophage and liver cancer cell Hep G2 at high concentration of nanoparticles. Figure 6.6 shows the cytotoxicity of the GQDs starting from $100\mu\text{g ml}^{-1}$ as the lowest concentration as evaluated by MTT assay. To our knowledge, most studies have set such concentration as the highest concentration used for cytotoxicity assay. As observed in Figure 6.6, the GQDs possess relatively low cytotoxicity at concentration up to $200\mu\text{g ml}^{-1}$. Moreover, at concentration as high as 1mg ml^{-1} and 2mg ml^{-1} , Hep G2 cells still displayed cell viability at $74.3\pm 7.86\%$ and $66.42\pm 6.42\%$, respectively. Although GQDs were typically not utilized at such high concentration in biological media, this provides a relatively useful indication of the biocompatibility of GQDs in biological media. Furthermore, these GQDs did not undergo further modification to improve its biocompatibility. As a result, we believe that the by functionalizing these GQDs, a further increase in biocompatibility might be achieved.

Figure 6.6 Cytotoxicity assay of GQD as evaluated by MTT assay. Multiple cell lines: (A) Raw 264.7 macrophage cells and (B) liver cancer cells Hep G2 were incubated with GQDs at various concentration for 24 hours. Values shown are means $\pm$ SD, n=5.

Figure 6.7 Gel retardation assay of GQD to PAH at increasing ratio. Unmodified GQD with negative charge moving towards positive terminal of agarose gel. From left to right: GQD only; GQD and PAH at mass ratio: 1:0.125; 1:0.25; 1:0.5; 1:0.75; 1:1; 1:1.5; 1:2; 1:3; 1:5)

Table 6.1 Zeta potential of GQD-PAH at various mass ratio

GQD-PAH ratio	1:0	1:0.25	1:0.5	1:0.75	1:1	1:1.5	1:2
Zeta Potential (mV)	-10.51	-13.42	9.74	12.97	21.55	30.63	35.09

We speculated that functionalization of the as-prepared GQDs can potentially allow the GQDs to act as multifunctional nanoparticles for application in simultaneous gene delivery and bioimaging. Hence, we performed noncovalent functionalization of the GQDs with cationic polymer polyallylamine hydrochloride (PAH). PAH was chosen due to the relatively lower cytotoxicity in contrast to polyethylenimine (PEI) which consists of high density of tertiary nitrogen groups.¹⁷⁰ Upon mixing the GQDs with the PAH at various concentration ratio, gel retardation assay was carried out to evaluate the potential of the nanocomplex. As shown in Figure 6.7, GQDs themselves were negatively charged, hence moving towards positive terminal of agarose gel. As ratio of PAH to GQDs was increased and more GQDs were functionalized, and the nanocomplex become more positively charged. Some negatively charged GQDs were still observed until GQD-PAH ratio at 1:1, where no movement of negatively charged GQDs were observed. The result is further confirmed by zeta potential measurement of the GQD and the GQD-PAH nanoparticles, shown in table 6.1. Zeta potential

of the GQDs were measured to be -10.51mV, and the zeta potential of the complex increases at higher GQD-PAH ratio. With the current properties of the GQD-PAH complex, we believe the nanoparticles can be further complexed with siRNA for application as gene delivery agent for RNA interference therapy.

6.4. Conclusion

Graphene quantum dots (GQDs) were synthesized by hydrothermal process with graphene as precursor material. The GQDs were exhibited oxygenated functional groups which renders them soluble in aqueous media and contribute to their negative charge. These GQDs exhibited excitation dependent emission and great photostability. We also investigated the photoluminescent properties were investigated in varying pH and temperature conditions. These GQDs readily transfected into multiple of cancer cell lines and macrophage cells and used as fluorescence imaging agent. Cytotoxicity assay of the as-prepared GQDs on macrophage and liver cancer cells revealed that they be highly biocompatible without any further surface functionalization. By noncovalent functionalization of GQDs with cationic polymer, the zeta potential of the GQDs can be turned into positive, as shown by gel retardation assay and zeta potential measurement. The positively charged nanoparticles can be advantageous for applications in drug and gene delivery system and act as simultaneous imaging and therapeutic agent.

From current perspective, cytotoxicity assay of GQDs were still necessary to evaluate their biocompatibility across multiple cell lines. While current observation has shown that these GQDs possess relatively low cytotoxicity, functionalization on the surface of these GQDs might also be designed to improve their biocompatibility. Furthermore, application of the functionalized GQDs as drug or gene delivery agent will also be investigated to observe their efficacy as theranostics agent.

Chapter 7

Functionalization and Application of MoS₂ Nanosheets as Gene Delivery System for Multi-Gene Therapy of Pancreatic Cancer

7.1. Introduction

Since its discovery,¹¹⁰ graphene has become a well-known 2D nanomaterial extensively used in various fields, including solar cells,²⁰³ sensors,²⁰⁴ electronics,²⁰⁵ and energy storage.²⁰⁶ Subsequently, functionalized graphene nanosheets with biocompatible polyethylene glycol have been applied in cancer therapy successfully,²⁰⁷ and were used for generate tumour ablation by utilizing the photothermal effect of graphene.²⁰⁸ Furthermore, the large surface area endows graphene as the ideal drug delivery system for various therapeutic agents. In the recent years, another class of 2D nanomaterials, transition metal dichalcogenides (TMDCs), have become subject of great interest for applications in electronics and optics.²⁰⁹ Similar to graphenes, TMDCs possess a quasi 2-Dimensional layered structure, typically composed of MX₂, in which M represents a transition metal atom (Mo, W, V, etc.) and X represents a chalcogen atom (S, Se or Te), forming the sandwich structure of M atoms in between two layer of X atoms (Figure 7.1). As a typical layered transition-metal, Molybdenum disulphide (MoS₂) is a promising nanomaterial and considered to potentially surpass graphene in sensors and electronics.²¹⁰⁻²¹¹ Due to their structural similarity to graphene, MoS₂ nanosheets have also been attracting likewise interest for their potential application in biomedical field. Recently, MoS₂ based DNA sensor was used to detect DNA and small molecules,¹³⁹ and functionalized MoS₂ nanosheets have been recently reported to be applied as drug delivery systems for cancer treatment.¹⁴²

Although these reports have shown early promise of utilizing MoS₂ as nanotherapeutic agent, only few reports have been made so far and further investigations for their biomedical application, including gene therapy, is necessary.

Previously, co-delivery of siRNA and anticancer drug doxorubicin was achieved by using gold nanorods (AuNRs) as nanocarriers for combinatorial therapy of pancreatic cancer *in vitro* and *in vivo*.²¹² Synergistic combination of chemotherapy, RNA silencing and photothermal therapy displayed superior anticancer efficacy.²¹² In this work, we fabricate PEGylated MoS₂ nanosheets as a gene delivery system to perform co-delivery of HDAC1 siRNA and K-Ras siRNA (against a G12D mutant K-Ras gene, special mutant siRNA for Panc-1 cells) for pancreatic cancer therapy *in vitro*. Histone deacetylase 1 (HDAC1) is the first discovered histone deacetylase, which specifically removes the acetyl group from acetylated histone H3 at lysine 56 (H3K56).²¹³⁻²¹⁴ The unique role of HDAC1 in maintaining the pluripotency of embryonic stem cells and cancer stem cells makes it one of the most interesting genes in epigenetics. Furthermore, HDAC1 is highly expressed in pancreatic adenocarcinoma and other malignant tumours,²¹⁵ which can regulate cell transformation, survival, invasion and metastasis.²¹⁶⁻²¹⁸ Thus, HDAC1 has been considered as a promising target for cancer therapy. The mutational K-Ras gene occurs in over 90% of pancreatic cancers and is a considerable target for pancreatic cancer therapy.²¹⁹ In this chapter, we described our work on utilizing functionalized MoS₂ nanosheets as gene delivery agents for the co-delivery of HDAC1 siRNA and K-Ras siRNA into pancreatic cancer cells. The MoS₂ nanosheets were functionalized with folic acid-Polyethyleneglycol (PEG) polymers and polyallylamine hydrochloride (PAH) for complexation with siRNAs. The nanosheets showed a high rate of internalization in Panc-1 cells and exhibited a 55% inhibition of cell proliferation. Moreover, the synergistic effect of gene therapy and photothermal effect contributed by the MoS₂ nanosheets under NIR light was able to inhibit the pancreatic cancer cell growth rate by 70%. Therefore, our work demonstrates

the promising potential of MoS₂ nanosheets as a gene delivery system and novel and low-toxic nanoplatform with targeted gene therapy and photothermal effect for pancreatic cancer treatment.

7.2. Materials and methods

7.2.1. Chemicals and reagents

Monolayer MoS₂ nanosheets (62 µg mL⁻¹, dissolved in ethanol) were purchased from 2D-Semiconductors Inc (USA). mPEG-NH₂ (5K) and NH₂-PEG-Boc (5K) were obtained from Laysan Bio, Inc (USA). Lipoic acid (LA), dichloromethane, N,N'-dicyclohexylcarbodiimide (DCC), triethylamine (TEA), sodium bicarbonate, trifluoroacetic acid (TFA), Folic acid (FA), N-Hydroxysuccinimide (NHS), dimethyl sulfoxide (DMSO), diethyl ether, polyallylamine hydrochloride (PAH, molecular weight 15,000) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromides (MTT) were purchased from Sigma Aldrich. Ultrapure water produced with a Milli-Q Integral 5 system was used in all experiments. Antibodies against HDAC1 (ab53091), K-Ras (ab55391) and Actin (ab8227) were obtained from Abcam.

7.2.2. Synthesis of LA-PEG and LA-PEG-FA polymers

Synthesis of LA-PEG:

The synthesis of mPEG-LA polymer followed a previous protocol with subtle changes.²²⁰ Briefly, 500 mg of 5K mPEG-NH₂ was mixed with 50 mg LA, 10 mg DCC and 6 µL TEA in 3 mL dichloromethane and stirred for 24 hours at room temperature. Then the reaction solution was evaporated, yielding a solid in the bottle to which 10 mL water was added. The insoluble solid was removed by filtration. After adjusting the solution pH to 8 with sodium bicarbonate (0.1M), the filtrate solution was extracted by dichloromethane for three times.

After evaporating the organic solvent, the product was dissolved in water and then lyophilized. The final product was LA-PEG.

Synthesis of LA-PEG-FA:

To synthesize LA-PEG-FA, a Boc protected bi-functional NH_2 -PEG-Boc polymer was used to replace mPEG- NH_2 . Briefly, 500 mg NH_2 -mPEG-Boc was mixed with 50 mg LA, 10 mg DDC and 6 μL TEA in 3 mL dichloromethane under nitrogen under magnetic stirring for 24 hours at room temperature. Subsequently, the dichloromethane solvent was evaporated and 2 mL TFA was added under magnetic stirring for 3 hours at room temperature to de-protect the Boc group. After evaporating the TFA solvent, the leftover solid was dissolved in 10 mL water. The same purification procedure was carried out to obtain the LA-PEG- NH_2 mid-product in solid.

FA conjugated LA-PEG (LA-PEG-FA) was prepared by conjugating the amine-functionalized LA-PEG- NH_2 with activated FA. Briefly, 100 mg of FA was mixed with 50 mg DCC and 35 mg NHS in 3 mL anhydrous DMSO for 15 minutes at room temperature. 500 mg LA-PEG- NH_2 in 5 mL DMSO was added afterwards. After reaction at room temperature under stirring for 24 hours, cold diethyl ether (50 mL) was added to precipitate PEG from the mixture solution. The product was washed with 5 mL diethyl ether 3 times and dried under vacuum. The yellowish product was then dissolved in water and filtered to remove any insoluble solid. The final product, LA-PEG-FA, was lyophilized and stored at $-20\text{ }^\circ\text{C}$ until use.

7.2.3. Preparation of PEG or FA-PEG-coated MoS_2 nanosheets

For the PEG functionalization, modified protocol was adopted from literature. In brief, 1 mg of the MoS_2 solution ($62\text{ }\mu\text{g mL}^{-1}$, ethanol) was evaporated to remove the ethanol solvent, yielding a solid in the bottle, and 10 mg of LA-PEG or LA-PEG-FA was added into the bottle redispersed in 2 mL of ultrapure DI water. Subsequently, the resulting PEGylated MoS_2

nanosheets mixture was sonicated for 20 minutes and stirred overnight vigorously. The excess unbound PEG or FA-PEG polymers was removed with two cycles of centrifugation (10000 rpm, 25-30 minutes). precipitate was resuspended and sonicated again in 1 mL of ultrapure water. A final PEGylated MoS₂ nanosheets concentration of 1mg/mL was obtained.

For siRNA loading, PAH was predissolved in ultrapure DI water. PEGylated MoS₂ nanosheets (1mg mL⁻¹) were mixed with PAH (10 mg mL⁻¹) in 2 mL ultrapure DI water at volume ratio of 1:1. The mixture was stirred 36 hours under room temperature. Free PAH was removed by centrifugation.

7.2.4. MoS₂ characterizations

The UV-visible absorption spectra were obtained from a spectrophotometer (Shimadzu UV-2450). Samples were loaded into a standard 10 mm quartz cuvette and measured against water as the reference. The hydrodynamic size distribution profile and the zeta potential of the PEGylated MoS₂ nanocomplex were measured by a particle size analyser system (90 Plus, Brookhaven Instruments). AFM images were obtained with Asylum Research ARC2™ SPM instrument. Fourier Transform Infrared (FT-IR) spectrum was measured by Shimadzu FT-IR spectrometer.

7.2.5. Gel retardation assay

The siRNA binding ability of the PEGylated MoS₂-based nanocarriers was studied by agarose gel electrophoresis. MoS₂/PEG/PAH (1 mg mL⁻¹) was mixed with siRNA (130 µg mL⁻¹) at five different mass ratios (0.5µg: 1.3µg, 1µg: 1.3µg, 2µg: 1.3µg, 4µg: 1.3µg and 6µg: 1.3µg). Electrophoresis was carried out on a 1% agarose gel with a current of 100 V for 15 min in a TAE buffer solution (40 mM Tris-HCl, 1 v/v % acetic acid, and 1 mM EDTA). The retardation of the complexes was visualized by staining with ethidium bromide and then

analysed on a UV illuminator to show the position of the complex siRNA band relative to that of naked siRNA.

7.2.6. Tumour cell line and culture

Human pancreatic cancer cell line, Panc-1 (CRL-1469, American Type Culture Collection), were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone) and penicillin/streptomycin (100 $\mu\text{g mL}^{-1}$, Gibco). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 .

7.2.7. siRNA transfection

The day before transfection, Panc-1 cells were seeded onto 6-well plates in DMEM medium with 10% FBS to give 30%-50% confluence at the time of transfection. $\text{MoS}_2/\text{PEG}/\text{PAH}$ or $\text{MoS}_2/\text{PEG}/\text{FA}/\text{PAH}$ nanosheets dispersion (1 mg mL^{-1} , 6 μL) was mixed with a G12D mutant K-Ras siRNA^{FAM} (130 $\mu\text{g mL}^{-1}$, 10 μL) or HDAC1 siRNA^{Cyc3} with gentle vortex and left undisturbed for 30 min. Before transfection, the culture medium was replaced with OPTI-MEM (950 μL , Invitrogen), the above mentioned PEGylated $\text{MoS}_2/\text{siRNA}$ mixture was then added to the medium and the cells were continuously cultured. Four hours later, DMEM medium (500 μL) with 30% FBS was added to the medium. Free siRNA^{FAM} or siRNA^{Cyc3} was also used in another parallel experiment at the same dosage level. The gene expression was monitored at 72 hours post-transfection. For transfection efficiency examination, fluorescent imaging and flow cytometry assays were performed at 4 hours post-treatment.

7.2.8. Flow Cytometry

For the flow cytometry of transfection efficiency experiments, Panc-1 cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization. The FAM (filter set for FITC was applied), and Cyc3 (filter set for PE was applied), served as luminescent

markers for K-ras siRNA and HDAC1 siRNA respectively, to determine the transfection efficiency quantitatively. The samples were analysed using a FACScalibur flow cytometer (Becton Dickinson, Mississauga, CA).

7.2.9. RNA isolation and quantitative RT-PCR

48 hours after transfection, total RNA was extracted from Panc-1 cells using TRIzol reagent (Invitrogen) and the amount of RNA was quantitated by a spectrophotometer (Nano-Drop ND-2000). Total RNA (2 µg) was reverse transcribed to cDNA using the reverse transcriptase kit from Promega according to the manufacturer's instructions. The mRNA levels of the target genes were quantified by real time PCR using SYBR green (Promega) in an ABI Prism 7500 real-time PCR system (Applied Biosystems).

7.2.10. Western Blotting

For Western blot analysis, cells were seeded in 6-well plates and treated for 24 hours with various treatment as described for the RT-PCR assay. To isolate protein, cells were washed with PBS and harvested using the lysis buffer (50 mM Tris·Cl PH=6.8, 2% SDS, 6% Glycerol, 1% β-mercapitoethanol, 0.004% bromophenol blue). Total cellular protein concentrations were determined by a spectrophotometer (Nano-Drop ND-2000). 20 µg of denatured cellular extracts were resolved by 10% SDS-PAGE gels. Protein bands in the gel were then transferred to Nitrocellulose Blotting membranes and incubated with the appropriate primary antibody. The antibody dilutions were as follows: 1:1000 for K-Ras, 1:1000 for HDAC1 and 1:1000 for actin. Membranes were incubated overnight at 4 °C and washed the next day with buffer (1xPBS, 0.05% Tween 20). Goat anti-rabbit or anti-mouse secondary antibodies were used for secondary incubation for 1 hour at room temperature. Proteins were then visualized with chemiluminescent substrates.

7.2.11. Cell viability test

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay. Cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and incubated with different PEGylated MoS₂ nanoformulations for indicated time points. For combination with NIR light, Panc-1 cells were incubated with FA/MoS₂ nanosheets or FA/MoS₂/si(HDAC1+Kras)RNA nanoformulations for 4 hours, washed with PBS twice, and then irradiated by a 808 nm NIR light at power densities of 1W/cm² for 5 minutes. MTT (5 mg mL⁻¹, 20 μ L) in PBS was added and the cells were incubated for 4 hours at 37 °C with 5% CO₂. DMSO (Dimethylsulfoxide, 150 μ L, Sigma) was then added to solubilize the precipitate with 5 min gentle shaking. Absorbance was measured with a microplate reader (Bio-Rad) at a wavelength of 490 nm. The cell viability was obtained by normalizing the absorbance of the sample well against that from the control well and expressed as a percentage, assigning the viability of non-treated cells as 100%.

7.3. Results & Discussion

As a graphene analogue, the layered structure of MoS₂ is composed of one layer of Mo atoms and two layers of S atoms to form 3-atomic layer quasi 2D S-Mo-S structure (Figure 7.1). The most common method to prepare MoS₂ nanosheets is chemical exfoliation method with different lithium intercalating agents as reported.^{142, 211} Figure 7.1 displayed the illustration of the formation of MoS₂-PEG-FA from MoS₂ nanosheets. Whilst naked MoS₂ exhibits solubility in water, they are unstable in buffer solution and functionalization is necessary to improve their solubility to form stable solution.¹⁴² To improve its biocompatibility for their application in biological media, the MoS₂ nanosheets have to be functionalized with polyethylene glycol (PEG). Functionalization of MoS₂ nanosheets followed defect site

Figure 7.1 Schematic illustration of the formation of MoS₂/PEG/FA and the gene loading by the engineered MoS₂-based nanocarriers.

conjugation approach, where Sulphur atom such as in thiol groups were introduced to bind to defects sites generated in MoS₂ surface.¹⁴⁵ In this work, polyethylene glycol (PEG) and folic acid modified PEG were both conjugated with lipoic acid, to form lipoic acid-PEG (LA-PEG) or folic acid modified LA-PEG (LA-PEG-FA). Lipoic acid possesses dithiol group on its end, which provide the Sulphur atoms for stable binding of the polymers to MoS₂.^{142, 145} Upon attachment of LA-PEG or LA-PEG-FA onto MoS₂, PAH polymer was introduced onto the surface of the nanosheets to render positively charged surface. This positively charged MoS₂ nanosheets were then suitable as siRNA delivery system.

In our work, there were 6 types of MoS₂-based nanoformulations as follows: (i) naked MoS₂ nanosheets functionalized with lipoic acid modified PEG (MoS₂/LA/PEG); (ii) naked MoS₂ nanosheets functionalized with folic acid and lipoic acid modified PEG (MoS₂/LA/PEG/FA); (iii) MoS₂/LA/PEG formulation modified with PAH polymer (MoS₂/LA/PEG/PAH, MoS₂); (iv) MoS₂/LA/PEG/FA formulation modified with PAH polymer (MoS₂/LA/PEG/FA/PAH, FA/MoS₂); (v) siRNA molecules delivered by

MoS₂/LA/PEG/PAH (MoS₂/siRNA); (vi) siRNA molecules delivered by MoS₂/LA/PEG/FA/PAH (FA/MoS₂/siRNA).

In our experiments, we have obtained the monolayer 2D MoS₂ nanosheets from 2D-Semiconductors Inc, which exhibited thickness from 1-2.6 nm and lateral size ranging from 58-88 nm, as shown in Figure 7.2A. Upon functionalization with LA-PEG-FA, the dimensions of the MoS₂/PEG/FA was examined. As shown in Figure 7.2B, the functionalized MoS₂ exhibited thickness from 1.8-4.3 nm and lateral size from 42-100nm. This increased thickness and lateral dimension most likely originated from addition of the PEG/FA groups on the surface of MoS₂.

To enable conjugation and delivery of siRNA molecules, PEGylated MoS₂ were functionalized with PAH polymer to form positively charged MoS₂/PEG/PAH or MoS₂/PEG/FA/PAH. As shown in Figure 7.2C, the absorbance spectra of MoS₂ was not changed by PEG or PAH. The hydrodynamic diameters of MoS₂ nanoformulation were determined by dynamic light scattering (DLS) technique (Figure 7.2E), resulted in estimated hydrodynamic diameter of naked MoS₂ nanosheets of 90.84 ± 3.76 nm. The zeta potential of these naked MoS₂ nanosheets was measured to be -23.86 ± 1.94 mV (Figure 7.2F). With different materials functionalized on the surface of MoS₂, the values of hydrodynamic diameter and zeta potential were changed. Hydrodynamic size of MoS₂ nanosheets were increased after functionalization with LA-PEG and LA-PEG-FA, in agreement with the AFM characterization results. Further functionalization with PAH was successful in modifying the surface charge of the nanosheets, as observed in the change of zeta potential from negative to positive (Figure 7.2F). Complexation with siRNA molecules by electrostatic interaction introduced negatively charged molecules onto the surface of the nanocomplexes, resulting in negatively charged surface. When the incubation time between MoS₂/PEG/FA and PAH was prolonged, the values

Figure 7.2 Characterization of the engineered MoS₂-based nanocarriers. AFM characterization of (A) MoS₂, (B) MoS₂/PEG/FA (C) Absorption spectra of different MoS₂-based nanocarriers. (D) The quantitative analysis of gene loading ability of MoS₂/PEG/FA/PAH nanocarriers. Gel retardation quantitative analysis of siRNA loading by MoS₂/PEG/FA/PAH, using free siRNA as reference. The mass ratio between MoS₂/PEG/FA/PAH (1 mg/mL) and siRNA (130 μg/mL) were set to be 0.5:1.3, 1:1.3, 2:1.3, 4:1.3 and 6:1.3, respectively. (E) Hydrodynamic size and (F) surface zeta potential of the different complex of MoS₂. All experiments were performed in duplicates with consistent results.

Values are means±SEM, n=3.

of zeta potential and hydrodynamic diameter of the complex were increased (Figure 7.3). Based on the results, we had chosen 36 hours as the optimized time to mix FA/MoS₂ and PAH. In Figure 7.2D and 7.4, gel retardation study was used to confirm the total amount of positively

charged FA/MoS₂ nanosheets needed to completely bind with a given amount of siRNA molecules. Figure 7.4 displayed the agarose gel electrophoresis of siRNAs with varying siRNA:FA/MoS₂ ratio. A sharp decrease in the total amount of free siRNA molecules has been found with the increasing addition of FA/MoS₂ nanosheets. Based on this gel electrophoresis, quantification of this siRNA loading ability was calculated (Figure 7.2D), by measuring the amount of unbound siRNA left relative to total siRNA. Based on the gel retardation results, the optimized mass ratio between FA/MoS₂ and siRNA molecules was found to be 6 μ g: 1.3 μ g. At this ratio, all siRNA molecules were bound with no free siRNA observed and has been used for all the *in vitro* experiments.

Figure 7.3 Characterization of complexation of MoS₂/PEG/FA with PAH. (A) Surface zeta potential and (B) hydrodynamic of the complex of MoS₂/PEG/FA with PAH at different mixing time. All experiments were performed in duplicates with consistent results. Values are means $\pm$ SEM, n = 3.

Figure 7.4 Gel retardation analysis of siRNA loading by MoS₂/PEG/FA /PAH, using free siRNA as reference (lane 1). The mass ratio between MoS₂/PEG/FA/PAH (1 mg/mL) and siRNA (130 μ g/mL) were set to be 0.5:1.3 (lane 2), 1:1.3 (lane 3), 2:1.3 (lane 4), 4:1.3 (lane 5) and 6:1.3 (lane 6), respectively.

Figure 7.5 Fluorescent images of Panc-1 cells treated with different MoS₂/siRNA nanocomplex formulations four hours after treatment. (A) PBS as blank control, (B) MoS₂/PEG/PAH/FA (FA/MoS₂) and (C) free Kras-siRNA^{FAM} (siKras^{FAM}) and (D) free HDAC1-siRNA^{Cyc3} (siHDAC1^{Cyc3}) as negative control, the single delivery of Kras-siRNA^{FAM} (E) or HDAC1-siRNA^{Cyc3} (F) by MoS₂/PEG/PAH/FA (FA/MoS₂), and the co-delivery of Kras-siRNA^{FAM} and HDAC1-siRNA^{Cyc3} by (G) MoS₂/PEG/PAH/FA (MoS₂/FA) or (H) MoS₂/PEG/PAH (MoS₂). The cell nucleus was stained with DAPI (pseudo-colored in blue) and signals from FAM and Cyc3 are assigned in green and red, respectively.

According to the previous studies, HDAC1 plays an essential role in maintaining the pluripotency of embryonic stem cells and cancer stem cells. Furthermore, HDAC1 is overexpressed in most malignant tumours (pancreatic cancer, colorectal cancer, lung cancer, etc.), which regulates cell transformation, survival, invasion and metastasis. Therefore, in our study, we utilized PEGylated MoS₂ nanosheets as the gene delivery system for co-delivery of HDAC1 siRNA and K-Ras siRNA (the G12D mutant K-Ras gene, special mutant siRNA for Panc-1 cells) to Panc-1 cancer cells. The cellular uptake efficiency and intracellular distribution of HDAC1 siRNA^{Cyc3} and K-Ras siRNA^{FAM} were monitored using fluorescence microscopy

as shown in Figure 7.5. The Panc-1 cells were treated with various MoS₂ nanoformulations for 4 hours before examining them under the microscope. The observed fluorescence signals of Cyc3 (red) in Figure 7.5E and FAM (green) in Figure 7.5F demonstrated that two kinds of siRNA were successfully delivered into Panc-1 cells by FA/MoS₂ nanosheets separately. As shown in Figure 7.5G, both HDAC1 siRNA and K-Ras siRNA were successfully delivered into Panc-1 cells by FA/MoS₂ nanoformulations. As a comparison, weaker fluorescence signals were observed from Panc-1 cells treated with non-FA conjugated MoS₂ nanoformulations (Figure 7.5H). As expected, no fluorescence signals were observed from the cells treated with PBS (Figure 7.5A), FA/MoS₂ (Figure 7.5B), free HDAC1 siRNA^{Cyc3} (Figure 7.5C) and free K-Ras siRNA^{FAM} (Figure 7.5D). These fluorescent images indicate that the use of MoS₂ nanoformulations were able to successfully transport siRNAs into cells and the cellular uptake of MoS₂ nanoformulations could be effectively improved by the conjugation of FA.

To further quantitatively evaluate the delivery efficiency of HDAC1 siRNA^{Cyc3} and K-Ras siRNA^{FAM} by MoS₂ nanoformulations, flow cytometry analysis was performed. Figure 7.6A shows the representative fluorescence plots of Panc-1 cells treated with different nanoformulations. The cells treated with PBS (Blank), FA/MoS₂, free HDAC1 siRNA^{Cyc3} or free K-Ras siRNA^{FAM} were used as negative control. Figure 7.6B and 7.6C show the corresponding transfection efficiency and average fluorescence signals, respectively. Panc-1 cells treated with FA/MoS₂/siRNA^{Cyc3+FAM} nanoformulations exhibit strongest FAM and Cyc3 fluorescence signals and their corresponding transfection efficiency was estimated to be 65% and 90%, respectively. The fluorescence signals were much higher than that of the cells treated with MoS₂/siRNA^{Cyc3+FAM} nanoformulations, which transfection efficiency was 35% and 60%, respectively. The differences demonstrate the successful enhancement of cellular uptake of these MoS₂ nanoformulations by the conjugation of folic acid. As negative controls, the cells treated with PBS, FA/MoS₂, free HDAC1 siRNA^{Cyc3} or free K-Ras siRNA^{FAM} showed almost

no fluorescence signals from this analysis study. These results are consistent with the cell fluorescence imaging analysis in Figure 7.5.

Figure 7.6 Flow cytometry evaluations on the transfection efficiencies of Panc-1 cells treated with different MoS₂/siRNA nanoparticle formulations. Panc-1 cells were treated with PBS (as blank), MoS₂/PEG/PAH/FA (FA/MoS₂), free Kras-siRNA^{FAM}, free HDAC1-siRNA^{Cyc3}, single delivery of Kras-siRNA^{FAM} (FA/MoS₂/siKras^{FAM}) or HDAC1-siRNA^{Cyc3} (FA/MoS₂/siHDAC1^{Cyc3}) by FA/MoS₂, co-delivery of Kras-siRNA^{FAM} and HDAC1-siRNA^{Cyc3} by FA/MoS₂ or MoS₂. (A) Representative dots plot of flow cytometry assays, where the x-axis and the y-axis show the fluorescent intensities of FAM and Cyc3, respectively. (B) and (C) Transfection efficiency and average fluorescence intensity from experiments showed in (A). Values are means $\pm$ SEM, n = 3; **, P < 0.01 vs Control, FA/MoS₂, siRNA^{FAM} and siRNA^{Cyc3}.

Figure 7.7 Gene expression evaluations of Panc-1 cells treated with different MoS₂-based nanoparticle formulations. Panc-1 cells were treated with PBS, MoS₂/FA, FA/MoS₂/scramble siRNA, free Kras siRNA, free HDAC1 siRNA, FA/MoS₂/siKras, FA/MoS₂/siHDAC1 and FA/MoS₂/si(Kras+HDAC1) for 4 hours, then all the cells were washed with PBS and re-incubated in fresh cell medium for additional 68 hours. (A) mRNA relative expression levels detected by RT-PCR. (B) Protein relative expression levels detected by Western Blotting. Actin was used as the protein loading control for samples. Values are means $\pm$ SEM, n = 3; **, P < 0.01 vs Control, MoS₂/FA and free siRNA.

Due to the critical roles of HDAC1 and mutant K-Ras in pancreatic cancer, in our studies the specific siRNAs which can target HDAC1 gene or mutant K-Ras gene were delivered into Panc-1 cells by MoS₂ nanoformulations for pancreatic cancer gene therapy. Following the flow cytometry analysis, the results in Figure 7.7 demonstrated the degree of gene knockdown of HDAC1 or K-Ras at both mRNA level and protein level in MoS₂ nanoformulations treated Panc-1 cells. Compared with PBS-treated cells (as the blank control), cells treated with FA/MoS₂, FA/MoS₂/scramble siRNA or free siRNA exhibit no obvious changes of HDAC1 or K-Ras mRNA and protein levels. On the other hand, cells treated with FA/MoS₂/HDAC1 siRNA, FA/MoS₂/K-Ras siRNA or FA/MoS₂/HDAC1+K-Ras siRNAs showed remarkable inhibition rate of HDAC1 or K-Ras gene expression. According to the RT-PCR analysis and Western Blotting analysis, MoS₂-based nanoformulations possess great potential as an effective siRNA delivery system to deliver siRNA in cells and induce target gene knockdown for gene therapy in cancer and potentially other diseases.

Figure 7.8 Cytotoxicity study of MoS₂/PEG/FA/PAH on Panc-1 cells. Panc-1 cells were treated with different concentrations of MoS₂/PEG/FA/PAH for 72 hours.

HDAC1 is overexpressed in most malignant tumours, which plays essential roles in maintaining the pluripotency of cancer stem cells, regulating vital movements of cancer cells (survival, invasion, metastasis, etc.). Meanwhile, the mutational oncogene K-Ras exists in almost all pancreatic cancers, which regulates cell proliferation, transformation and apoptosis. Based on the gene knockdown results in Figure 7.7, the functionalized MoS₂ nanocarriers were

able to deliver HDAC1 siRNA or K-Ras siRNA into the cells and were able to sufficiently suppress the expression of HDAC1 gene or K-Ras gene. Subsequently, we have further examined the growth inhibition of the cells treated by MoS₂ nanoformulations (Figure 7.8 and 7.9). To assess the toxicity of naked-FA/MoS₂, Panc-1 cells were treated with different concentrations of FA/MoS₂/PEG/PAH ranging from 5 µg mL⁻¹ to 200 µg mL⁻¹ for 72 hours (Figure 7.8). The cell viability was maintained around 80% even at the highest concentration (200 µg mL⁻¹), which strongly demonstrates the biocompatibility and low toxicity of PEGylated MoS₂-based nanocarriers. As shown in Figure 7.9, compared with PBS-treated cells (as the blank control), there was negligible inhibition of the cell growth in these cells treated with FA/MoS₂ or FA/MoS₂/scramble siRNA for 72 hours. On the contrary, obvious inhibition was observed in cases of FA/MoS₂/HDAC1 siRNA (62.15±1.23%), FA/MoS₂/K-Ras siRNA (58.34±3.54%), and FA/MoS₂/HDAC1+K-Ras siRNA (53.34±4.71%). It is worth noting that, the co-delivery of HDAC1 siRNA and K-Ras siRNA caused a stronger inhibition rate of Panc-1 cells growth. In addition, due to the strong NIR absorbance and optical properties of MoS₂ nanosheets,¹⁴⁵ which endow them as a potent agent for photothermal therapy, we have subsequently investigated the anti-proliferative effects of the combination of MoS₂ nanoformulations and NIR light. In this study, Panc-1 cells were treated with FA/MoS₂ or FA/MoS₂/HDAC1+K-Ras siRNA nanoformulations for 4 hours, and were then exposed under 808 nm NIR light for 5 minutes. In the additional 72 hours, 40% of inhibition rate has been observed for NIR light with FA/MoS₂, and the strongest inhibition rate (over 70%) has been observed for the combination of FA/MoS₂/HDAC1+K-Ras siRNA nanoformulations and 808 nm NIR light. Previous evidence has also supported the possibility of enhanced MoS₂ release from endosome and release of genetic material from nanocarriers upon irradiation of NIR light, which might also contribute into the enhanced therapeutic effect of the MoS₂ nanosheets carriers.¹⁴⁴ These results have not only suggested both HDAC1 gene and K-Ras gene are the

prospective candidates for gene therapy in pancreatic cancer, but also demonstrate the synergistic effects of the photothermal effect and gene therapy to pancreatic cancer.

Figure 7.9 Cell viability tests of different MoS₂-based formulations. The growth of Panc-1 cells was inhibited by FA/MoS₂/siRNA nanocomplex with NIR light. Panc-1 cells were treated with PBS, MoS₂/FA, MoS₂/FA with NIR light, FA/MoS₂/scramble siRNA, FA/MoS₂/siKras, FA/MoS₂/siHDAC1, FA/MoS₂/si(Kras+HDAC1) and FA/MoS₂/si(Kras+HDAC1) with NIR light for 4 hours, then all the cells were washed with PBS and re-incubated in fresh cell medium for designated time. Phase contrast microscope images (A) and relative cell viabilities (B) of Panc-1 cells treated with different MoS₂/siRNA nanocomplex formulations without or with NIR light for 4, 24, 48, and 72 hours. Data are presented as the means±SEM of triplicate experiment. *, P < 0.05, **, P < 0.01 vs PBS (as blank) and FA/MoS₂.

Figure 7.10 Wound healing migration assay in Panc-1 cell culture treated with different MoS₂-based nanoparticle formulations. (A) Phase contrast microscope images of the wound healing process monitored Panc-1 cells treated with PBS, MoS₂/FA, MoS₂/FA with NIR light, FA/MoS₂/scramble siRNA, FA/MoS₂/siKras, FA/MoS₂/siHDAC1, FA/MoS₂/si(Kras+HDAC1) and FA/MoS₂/si(Kras+HDAC1) with NIR light for 4 and 72 hours. The gap was produced by scraping of the cell monolayer with a 200- μ m micropipette tip. (B) Quantitative evaluation by measuring the width of the gap distance after treatment, values were normalized by the 4 hours wound gap width (as initial width). Data are presented as the means $\pm$ SEM of triplicate experiments. **, P<0.01 vs PBS (as blank) and FA/MoS₂.

Collective cell migration is the process of a large scale coordinated movement of cells, which plays essential roles in invasion and metastasis of malignant tumours.²²¹ In the previous reports and our studies, HDAC1 and K-Ras are required to regulate cell proliferation, cell

invasion and metastasis of pancreatic cancer. To investigate the effectiveness of the gene therapy, we have performed the wound healing migration assay²²² to study the collective cell migration of Panc-1 cells treated with different MoS₂ and siRNA nanoformulations (Figure 7.10). In this study, Panc-1 cells were seeded in 6-well plates and were treated with PBS, FA/MoS₂, FA/MoS₂ with NIR light, FA/MoS₂/scramble siRNA, FA/MoS₂/HDAC1 siRNA, FA/MoS₂/K-Ras siRNA, FA/MoS₂/(HDAC1+K-Ras) siRNA or FA/MoS₂/(HDAC1+K-Ras) siRNA with NIR light, respectively. After 4 hours, cells were wounded by manual scraping to produce a gap and were incubated for additional 68 hours. Compared with the initial wound window width, the 72 hours wound closing percentage in cells treated with PBS, FA/MoS₂ or FA/MoS₂/scramble siRNA reached 86.37±6.43%, 81.59±2.71% or 80.25±2.05% respectively. On the other hand, cells treated with FA/MoS₂ with NIR light, FA/MoS₂/HDAC1 siRNA, FA/MoS₂/K-Ras siRNA, FA/MoS₂/(HDAC1+K-Ras) siRNA or FA/MoS₂/(HDAC1+K-Ras) siRNA with NIR light exhibited obvious reduction in the wound healing process. The wound healing process in cells treated with co-delivery of HDAC1 and K-Ras siRNA by MoS₂ nanocarriers was much slower than that of cells treated with single delivery of HDAC1 or K-Ras siRNA by MoS₂ nanocarriers. The most significantly suppressed healing process was observed in the cells treated with FA/MoS₂/(HDAC1+K-Ras) siRNA and NIR light. The 72 hours wound closed to around 16.31±2.49% of the initial wound window width. This wound healing assay indicate that the synergistic effect of photothermal therapy and co-delivery of HDAC1 and K-Ras siRNA by MoS₂-based nanocarriers can greatly reduce the migration ability of pancreatic cancer cells.

7.4. Conclusion

In this study, the novel kind of 2D nanomaterials-MoS₂ nanosheets were functionalized with FA-PEG as biocompatible gene delivery system for combinatorial therapy of pancreatic cancer. This is the first demonstration of multifunctional PEG-modified MoS₂ nanosheets as a gene delivery system for co-delivery of HDAC1 and K-Ras siRNA with photothermal effect for pancreatic cancer therapy. The cytotoxicity assessment has shown that the functionalized MoS₂ nanosheets exhibited low cytotoxicity and was shown to be biocompatible for *in vitro* application. Following the transfection of the nanocarriers, fluorescence microscopy images, flow cytometry analysis, RT-PCR and Western Blotting results indicate that the MoS₂ based nanocarrier successfully delivered both HDAC1 and K-Ras siRNA molecules into Panc-1 cells. The siRNAs were able to be released inside the cells and resulted in the down-regulation of gene expression of both genes. Following the successful suppression of HDAC1 and K-Ras genes expression, cell proliferation and migration were significantly inhibited. These results demonstrated that both HDAC1 and K-Ras play essential roles in regulating pancreatic cell proliferation, invasion and migration, which are consistent with previous reports.^{217-218, 223} Furthermore, the use of NIR light in the application of the nanocarriers have also shown to be advantageous towards the therapeutic efficacy, due to the unique optical properties of MoS₂ nanosheets. In summary, our studies displayed the novel and promising applications of TMDCs in cancer therapy with the combination of gene therapy and photothermal effect, which may expand a new era for the development of 2D-nanomaterials for cancer theranostics.

Chapter 8

Conclusions and Future Work

8.1. Conclusions

Until today, pancreatic cancer remains as one of the deadliest type of cancer with high mortality rates. This is partly contributed by diagnosis of cancer patients which have often reach advanced stage of pancreatic cancer. Despite advances in cancer theranostics over the past decades, early diagnosis of pancreatic cancer remains challenging and therapy options for pancreatic cancer patients are limited to surgical resection, chemotherapy, and radiotherapy. Furthermore, although chemotherapy is considered as preferred treatment option for advanced cancer stage, such treatment carries along heavy side effects due to the inefficient and nonspecific drug delivery to cancer cells which leads typically heavy drug dose being used. Many of these treatments were found only as palliative rather than a curative approach. These issues have rendered development of novel treatment approach for pancreatic cancers as a great necessity in cancer theranostics research. Among therapeutic techniques, novel approach such as RNA interference (RNAi) showed early promises for effective cancer treatment. However, it suffers from bottlenecks such as fragile nature of siRNA, ineffective transfection of naked siRNA, and difficulty of endosomal escape of siRNA for initiation of gene silencing. Current transfection agents such as viral vectors and lipid polymers suffer from high toxicity and immunogenic result, such that discovery of biocompatible transfection agent for effective gene therapy is necessary.

Throughout the works in this thesis, we investigated development and applications of nanomaterials for cancer theranostics, in particular RNAi therapy and imaging towards

pancreatic cancer. We investigated the role of IL-8 oncogene in pancreatic cancer cell lines and found that the gene expression was varied among pancreatic cancer cell lines. This oncogene might contribute to different aggressiveness of the cells and were observed to affect cancer cell viability if its expression was inhibited. We then demonstrated the application of cationic polylactides (CPLAs) as organic nanomaterials with customizable formation and biodegradable nature as novel gene therapy agent. CPLAs were able to form nanoplexes with IL-8 targeting siRNA and entered pancreatic cancer cells, showing no significant difference in transfection efficiency to commercial transfection agent. These complexes were able to release siRNA in cells and inhibit expression of IL-8 gene. These CPLAs were biocompatible, displaying cell viability of over 80% even after exposure at dose as high as $160\text{ }\mu\text{g ml}^{-1}$ for 48 hours. This early study demonstrates the potential of CPLAs as biodegradable gene nanocarrier for gene therapy of pancreatic cancer cells.

We further analyse the therapeutic efficacy of BCPV for delivery of siRNA targeting K-ras oncogene in Mia PaCa-2 cells. In this study, complexation of BCPV-siRNA at 8:1 ratio was sufficient for binding of all siRNAs onto BCPVs and exhibited extremely high transfection efficiency. Having shown the gene knockdown capability of the BCPV-siRNA nanocomplex, we investigated whether this can be translated to therapeutic effect in the cancer cells. Significant inhibition in properties such as cell proliferation and migration of cells treated with BCPV-siRNA were observed in multiple assays such as cell proliferation assay and wound healing assay. Cell invasion assay also showed close to 40% cell penetration in these cells, similar to the level in Oligofectamine-siRNA treated cells. Efficacy of K-ras gene therapy was also evaluated in apoptotic cell assay, in which BCPV-siRNA has shown doubled apoptotic cell numbers as compared to untreated cells. Altogether, these results show the effectiveness of BCPVs as nanocarrier for gene therapy by RNAi as a pancreatic cancer therapy approach with massive potential.

Carbon nanotubes are composed of intrinsically nontoxic carbon atoms which exhibit extremely high surface area to volume ratio owing to their 1-dimensional morphology. In our work, we utilized the large surface area of carbon nanotubes for loading and transfection of siRNAs for RNAi therapy. Noncovalent functionalization with amphiphilic phospholipids and cationic polymers allow water solubility and positive surface charge for siRNA loading by electrostatic interaction. Functionalized SWNTs were biocompatible and suitable for gene delivery application. We were also able to confirm the release of siRNA to initiate subsequent cellular process upon their uptake into cells by evaluating gene knockdown induced from siRNAs in cells. These functionalized SWNTs showed comparative transfection efficiency and gene knockdown capability with Oligofectamine. We believe that with further development in noncovalent functionalization strategy, these SWNTs might be able to provide a multifunctional platform for targeted cancer therapy with high efficiency.

Graphene quantum dots exhibited intrinsic photoluminescence and photostability not observed in graphene. Due to these reasons, we believe that GQDs can be applied as simultaneous imaging and therapeutic agent for cancer therapy. Synthesis of GQDs were carried out by acidic exfoliation followed by hydrothermal treatment with graphene nanosheets as precursor. The GQDs exhibited negative zeta potential due to the oxygenated functional groups such as hydroxyl, carboxyl, and carbonyl groups on their surface. The GQDs exhibited excitation dependent photoluminescence with maximum emission intensity with peak at 505 nm at 420 nm excitation and stable photoluminescence even after 24 hours of continuous excitation. We also conducted investigation in properties of the GQD in various pH and temperature. The GQDs can be readily used for bioimaging application in multiple cell lines. Furthermore, we examined the biocompatibility of the as-prepared GQDs by cell viability assays at extremely high concentration. The results signified that the GQDs posed relatively low toxicity at high concentrations. Finally, the GQDs can be complexed with cationic polymer

such as PAH to tune the surface charge of the nanoparticles. This can potentially allow their use as simultaneous drug or gene delivery and imaging application.

Transition metal dichalcogenides (TMDCs) are considered as graphene analogue due to their 2-dimensional morphology and low thickness of 3 atomic layers. Their 2-dimensional structure, similar to graphene, means that they share similarity in high surface area to volume ratio. With few investigations in this area, we looked into functionalization and application of MoS₂ nanosheets for application in simultaneous delivery of siRNA molecules targeting 2 different oncogenes, HDAC1 and K-ras genes. Functionalization with lipoic acid-PEG and lipoic acid-PEG-folic acid improved their water solubility and biocompatibility and also added targeting moiety into the nanosheets for specific delivery of therapeutic materials. We modified the MoS₂ nanosheets with PAH cationic polymer to modify their surface charge and allow conjugation with siRNA by electrostatic interactions. The nanosheets were able to deliver both siRNA into pancreatic cancer cell Panc-1 successfully, and induced the gene knockdown of both genes for successful gene therapy. It was also observed that NIR exposure during application of these nanomaterials improved the therapeutic efficacy of the treatment by exploiting photothermal property of MoS₂ as well as promoting endosomal escape of the nanomaterials. Cell viability assay and wound healing assay have confirmed the lowered survival and cell migration capability of the cells treated with the folic acid conjugated MoS₂-siRNA nanocomplex, in particular upon the exposure of NIR light. We believe that our study in this TMDC application may expand a new era for the development of these nanomaterials for cancer theranostics and other diseases.

Our studies explore and investigate interesting set of nanomaterials and their application for cancer theranostics. Due to distinctive properties of these nanomaterials, they provide advantageous platforms in cancer theranostics applications. In bioapplications, organic nanomaterials such as CPLAs offer advantages such as customizable composition of these

nanomaterials and degradability in biological environment. Inorganic nanomaterials in general provide robust structures for conjugation of therapeutic molecules. Nanomaterials with unique morphology such as 1-D CNTs, 0-D GQDs, and 2-D graphenes and TMDCs exhibit extremely high surface area to volume ratio and provides a major advantage in terms of high loading efficiency. Furthermore, such materials possess intrinsic properties applicable for simultaneous applications in drug & gene delivery with combinations in fluorescent imaging, Raman spectroscopy imaging, or photothermal therapy. However, appropriate functionalization of these nanomaterials is required to ensure they are biocompatible and effective for the proposed application. Further development for functionalization of such nanomaterials to improve their biocompatibility and add functionalities such as targeted drug and gene delivery is an important step for their application in cancer theranostics. We believe that our works provide a positive outlook in nanomaterials functionalization and application for cancer theranostics applications.

8.2. Recommendations for Future works

In our works, we have shown that these CPLAs were biocompatible and acted as efficient gene transfection agent *in vitro*. Low toxicity and biodegradability of CPLAs has made them a promising candidate for safe and biocompatible transport agent against pancreatic cancer in the immediate future. Further studies in this area should involve a thorough *in vivo* investigation which is necessary to evaluate the pharmacological profile and their therapeutic efficacy *in vivo*. These *in vivo* evaluations may also provide insights for optimized design of CPLA structure with improved biocompatibility and efficacy.

In general, inorganic nanomaterials exhibit interesting intrinsic properties by virtue of their chemical composition and structure. We have shown that noncovalent functionalization of SWNTs can achieve successful gene therapy in pancreatic cancer cells. We believe that this

non-destructive approach maintains the optoelectronic properties of SWNTs. This allows further investigation in utilizing the optoelectronic properties such as strong Raman peaks of these SWNTs as imaging contrast simultaneously.

We have synthesized GQDs with visible photoluminescence which exhibited relatively low cytotoxicity even at high concentration. We believe that further functionalization by utilizing functional groups on the as-synthesized GQDs as sites for biomolecules attachment can modulate properties of GQDs. These strategies can be applied to alter their photoluminescence, improve biocompatibility of these GQDs, attach cationic polymer for gene delivery, or even targeting moiety to improve specificity of these nanomaterials.

Studies of TMDCs in bioapplications were still at initial stage. Despite early promising results so far, efforts in this area are still lacking. One major stumbling block is the lack of surface functionalization options for these nanomaterials. In order to accelerate studies on the bioapplication of these materials, studies on novel functionalization approaches are necessary. Breakthrough in this area might bring to light the potential application of this material. Furthermore, studies in optoelectronics of TMDC may also uncover similar phenomenon as GQDs.

We have shown that RNAi therapy on oncogenes such as IL-8 and K-ras is an encouraging option for pancreatic cancer therapy. Enhanced efficacy was observed in simultaneous multigene therapy targeting HDAC1 and K-ras genes. It is possible to investigate whether this enhanced efficacy can be observed in combination of specific oncogenes or whether even more enhanced results might be attained. Furthermore, similar approach by combination of drug and multigene therapy can also be explored to evaluate the therapeutic effect and potentially lower the dose of drug application. Such investigation can also probe into

efficacy of delivery strategies by sequential or simultaneous treatment. In such applications, development of biocompatible multifunctional nanomaterials with large surface area and high loading efficiency plays an important role towards the success of this therapy.

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List of Publications

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