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**DEVELOPMENT OF NOVEL GENE DELIVERY
SYSTEM FOR CANCER THERAPY**

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SCHOOL OF ELECTRICAL AND ELECTRONIC ENGINEERING

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DEVELOPMENT OF NOVEL GENE DELIVERY SYSTEM FOR CANCER THERAPY

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Abstract

In this thesis, novel biodegradable charged polyester-based vectors (BCPVs) were designed and synthesized as siRNA carrier for cancer treatment. Under optimized conditions, the BCPV can effectively protect siRNA from degradation by RNase in the serum, and would subsequently promote intracellular siRNA uptake via endocytosis. The formulation and usage of BCPV-based siRNA carriers cause significant inhibition of targeted abnormal proto-oncogenes expression in adherent growth cells (pancreatic cancer cell line) and non-adherent growth cells (leukemia cell line), resulting in obvious inhibition of cancer cell bioactivity. In addition, BCPV was able to simultaneously co-deliver K-ras and Notch1 siRNA into pancreatic cancer cells to overcome chemotherapeutic drug resistance (Gemcitabine) by reversing the epithelial-mesenchymal transition (EMT). Lastly, BCPV, as modifying polymer, was used to coat the surface of graphene quantum dots (GQDs) for fabrication of multifunctional siRNA and drug co-delivery systems, which realized real-time monitoring of the delivery efficacy and distribution of the drugs and siRNA.

Summary of abbreviations

γ -PGA	γ -Polyglutamic Acid
Abs	Absorbance
AIDS	Acquired Immunodeficiency Syndrome
APLAs	Allyl Functional Polylactides
AuNPs	Gold Nanoparticles
CD	Cyclodextrin
CML	Chronic Myeloid Leukaemia
Cy3	Cyanine 3
BCPV	Biodegradable Charged Polyester-based Vector
DAPI	4',6-Diamidino-2-Phenylindole
DCM	Dichloromethane
DEAET	2-(Diethylamino) ethanethiol hydrochloride
DI	Deionized
DLS	Dynamic light scattering
DMAEMA	Dimethylaminoethyl Methacrylate
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DOTAP	1,2-dioleoyl-3-trimethylammoniumpropane
DOX	Doxorubicin
EGF	Epidermal Growth Factor
EMT	Epithelia-Mesenchymal Transition
EPR	Enhanced Permeability and Retention
FBS	Fetal bovine serum
FDA	Food and Drug Administration

FITC	Fluorescein isothiocyanate
FWHM	Full width at half maximum
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GEM	Gemcitabine
GFP	Green Fluorescent Protein
GNPs	Gelatin Nanoparticles
GPC	Gel Permeation Chromatography
GQDs	Graphene Quantum Dots
HSP47	Heat Shock Protein 47
iNOS	Inducible Nitric Oxide Synthase
Lip2000	Lipofectamine2000™
MDR	Multidrug resistance
MMP-2	Matrix Metalloproteinase-2
MnMEIO	Manganese-doped Magnetism-engineered Iron Oxide Nanoparticles
MNP	Magnetic Nanoparticle
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MSNs	Mesoporous Silica Nanoparticles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide
NCA	N-carboxy anhydrides
NIR	Near Infrared
NMR	Nuclear Magnetic Resonance
NSC-PLL-PA	N-succinyl chitosan-poly-l-lysine-palmitic Acid
Oligo	Oligofectamine™
PAH	Poly(allylamine hydrochloride)
PBS	Phosphate-buffered Saline
PDEAEMA	Poly (2-(diethylamino)ethylmethacrylate)

PDI	Polydispersity Index
PDMAEMA	Poly (2-(dimethylamino)ethylmethacrylate)
PDT	Photodynamic Therapy
PEI	Polyethylenimine
PEG	Polyethylene Glycol
PGMA	Poly (glycidyl methacrylate)
PI	Propidium iodide
PL	Photoluminescence
PLA	Poly (L-lactide)
PLL	Poly-L-lysine
PLGA	Poly (lactic-co-glycolic acid)
PNIPMAm	Poly (<i>N</i> -isopropylmethacrylamide)
PS	Phosphatidylserine
PSE	Proton-sponge Effect
PTT	Photothermal Therapy
PVCL	Poly (N-vinylcaprolactam)
QDs	Quantum Dots
RES	Reticuloendothelial System
RISC	RNA-induced silencing complex
RNAi	RNA Interference
RNase	Ribonuclease
ROP	Ring-opening Polymerization
RT-PCR	Reverse Transcription Polymerase Chain Reaction
siRNA	Small Interfering RNA
TEM	Transmission Electron Microscopy
VEGF	Vascular Endothelial Growth Factor

Chapter 1 Introduction

Cancer is one of the leading causes of death in the world [1]. According to the World Health Organization report, it is estimated that tens of millions of people are diagnosed with malignant tumors globally each year, and more than half of the diagnosed patients eventually die [2]. Despite the continuous efforts devoted to identify efficient approaches and outstanding advances in cancer therapy, it still remains a challenge to fully remove the tumors from cancer patients especially after the onset of metastasis. Among the various cancer therapeutic approaches, chemotherapy is the main method which provided improved survival rates in the last decades. Nevertheless, significant side effects and systemic toxicity of anticancer drugs often cause failure of chemotherapy as a result of lack of specificity against cancer cells. The chemotherapy treatment usually induces the development of drug resistance which can occur against individual cytotoxic drugs or many different drugs, with various mechanisms of action and chemical structures, usually caused by increased rates of drug efflux and mutation of targets of these drugs [3]. The improved delivery of conventional chemotherapy drugs and development of novel anti-cancer agents are the main approaches presently used to overcome cancer drug resistance. The recent advances in nanomaterial technology have great promise for improving cancer treatment by generating new therapeutic strategies which may allow a more efficient passive or active targeted delivery of anticancer agents while minimizing non-specific distribution in healthy tissues [4]. Nanotechnology also developed both new imaging agents for diagnosis of various cancers and therapeutic agents. Novel nanotechnology platforms, such as synthetic biodegradable polymers, liposome, micelles, dendrimers, MSNs, magnetic nanoparticles, carbon nanotubes, quantum dots, and biodegradable polymers, have been applied to the delivery of anticancer agents, including small molecular weight drugs and macromolecules such as proteins, peptides or genes [5, 6].

In the last decades, the RNAi (RNA interference) technology targeting oncogene mutation appeared and proved to be a powerful and attractive tool for cancer treatment [7]. Its greatest challenge is that the naked siRNA is unable to cross the cell membrane phospholipid bilayer due

to its hydrophilic properties, large molecular weight and negative charges [8]. For these reasons, viral vectors have been developed for siRNA delivery and have been considered as a promising candidate because of their high gene transfection efficiency. However, due to the size limitation of the inserted genetic materials, the possible immune response and the biosafety risk, the application of viral vectors has been limited [9]. Non-viral vectors, such as lipids, peptides, aptamers, and various nanoparticles have thus been intensely investigated. Although some show promising results, their applications are often limited by low transfection efficiency, high cytotoxicity and metabolic toxicity. In order to overcome these challenges, we developed a novel transmembrane transport nanomaterial formulation, namely a biodegradable charged polyester-based vector (BCPV), as siRNA delivery carrier for cancer treatment.

In chapter 2, the mechanism and challenges of siRNA based RNAi technology on cancer treatment are briefly introduced. The recent advances of siRNA vectors including traditional lipid delivery systems, non-biodegradable polymers, biodegradable polymers and polymer hybrid nanoparticles were elaborately reviewed.

Chapter 3 presents how the BCPV with amino groups was synthesized through living ring-opening polymerization and thiol-ene click functionalization. The BCPV was investigated as siRNA carrier for pancreatic cancer treatment, which is one of the human malignancies with the highest mortality rates. Our results have shown that BCPVs can effectively absorb negative siRNA to form a capsular-like structure under optimized conditions, preventing siRNA from nuclease digestion in the serum. Moreover, these vectors are able to transfect siRNAs targeting mutant K-ras into MiaPaCa-2 cells (a pancreatic cancer cell line with K-ras gene mutation) with high transfection and inhibition efficiency of K-ras gene expression. More importantly, after treatment with the BCPV-siRNA^{K-ras} nanoplexes, the cell growth, migration and invasion of the MiaPaCa-2 cells were significantly reduced and the cell apoptosis was also promoted.

In chapter 4, the BCPV was employed to treat chronic myelogenous leukemia (CML) cells, which are well-known as hard-to-transfect cells due to the lower probability of contact between polyplexes and the non-adherent leukemic cells. CMLs are associated with an abnormal

chromosome BCR-ABL gene mutation. The results demonstrated that BCPV was able to form stable nanoplexes with siRNA molecules, and successfully assisted *in vitro* siRNA transfection in non-adherent K562 cells (a leukemic cell line), even in the presence of fetal bovine serum (FBS). As a consequence of downregulation of BCR-ABL, BCPV-siRNA nanoplexes successfully inhibited cell proliferation and promoted cell apoptosis.

Chapter 5 details how we further detected and used the potential of BCPV as siRNA carrier to reverse the drug resistance of pancreatic cancer cells by specifically targeting abnormal proto-oncogenes. BCPVs were used to co-deliver K-ras and Notch1 siRNA into MiaPaCa-2 cells to overcome drug resistance to gemcitabine (GEM), a first-line chemotherapeutic drug used in the clinic. Through K-ras and Notch1 gene silencing in MiaPaCa-2 cells, BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes effectively reversed the epithelial-mesenchymal transition (EMT) in MiaPaCa-2 cells, thereby greatly enhancing their sensitivity to GEM. MiaPaCa-2 cell proliferation, migration, and invasion were effectively inhibited, and cell apoptosis was also significantly enhanced by the synergistic antitumor effect of BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes and GEM. These results suggest that this combinatorial RNAi therapy can be used to enhance cancer cell sensitivity to chemotherapeutic drugs and improve the therapy effect.

In chapter 6, the BCPV was used to modify graphene quantum dots (GQDs) into multifunctional nanocomposites as a siRNA and drug co-loading platform. BCPV functionalized-GQDs nanocomplexes were able to simultaneously co-deliver siRNA^{K-ras} and doxorubicin (DOX) combined with NIR light-mediated biomolecules release for pancreatic cancer treatment. After treatment with GQD/DOX/BCPV/siRNA nanocomplexes, the proliferation, migration and invasion of pancreatic cancer cells were significantly suppressed and cell apoptosis was also promoted. Additionally, the photothermal properties of the GQDs exhibited enhanced intracellular release of DOX and siRNA from the nanocomplexes under irradiation with near-infrared (NIR) laser light, thus augmenting their therapeutic effect. These results indicate that nitrogen-doped GQDs can be considered as a novel PTT agent, simultaneously allowing imaging and remotely controlled drug release by NIR, and providing an efficient cancer therapeutic strategy.

Finally, the results of this Thesis are summarized in chapter 7. In brief, this Thesis covers the synthesis, characterization, cytotoxicity assessment of BCPV, demonstrates the potential of using BCPV as siRNA carrier for cancer treatment, and also indicates the potential of BCPV as coating polymer to modify nanoparticles for construction of multifunctional siRNA delivery system.

Chapter 2 Literature Review

RNA interference (RNAi) technology can inhibit specific gene expression induced by short interference RNA (siRNA) in mammalian cells, and has wide potential for disease treatment. However, the properties of siRNA, such as its negative charge, macromolecular structure and easily-degradable nature, block it to cross cell membrane in order to exert its function inside cells. To achieve this desired functionality, different kinds of nanoparticle delivery systems have been developed to overcome siRNA transport barriers. Non-viral carriers have been exploited extensively because of some disadvantages of viral vectors. Liposomes, cationic polymer and inorganic nanoparticles are designed to overcome siRNA transport challenges, and some of them have shown promising results in many preclinical studies due to their stability, high efficiency, and low cytotoxicity *in vitro* or *in vivo*. Even so, siRNA technology has not achieved the desired objective in clinical applications. Here, we discuss the intracellular trafficking and release mechanism of siRNA, and summarize the recent advances on siRNA delivery system.

2.1 RNAi technology and its challenges

Since RNA interference (RNAi) regulatory pathway was discovered in 1998, it has been developed as a useful tool to silence or downregulate the post-transcriptional expression of various specific genes [10, 11]. The promising silencing effect of RNAi to specific mutated genes makes it a valuable treating strategy for a variety of diseases such as hereditary diseases, neural disorders, viral infection and cancer [12-17]. The gene-specific therapeutics and target validation in RNAi technology are achieved by nucleotide sequence-specific complementary pairing [7, 18]. Particularly, due to the recognition of mutated genes that promote oncogenesis, RNAi has been applied as an effective treating modality for combating cancer-related diseases [19]. Mechanistically, cancer therapies based on the regulation of target genes exploit the process of nucleotide sequence-specific complementary pairing [8]. In this process, exogenous double-stranded RNA molecules (dsRNA) resulting from the biological functions are recognized by Dicer enzymes, a ribonuclease protein, followed by being cleaved into small double-stranded RNA

consisting of 21 to 23 nucleotides (nts) along with 2-nucleotide overhanging at the 3' ends (**Figure 2.1**). The resulting Dicer products are referred to as small interfering RNAs (siRNAs). In addition to the endogenous pathway, siRNAs can also be created via a synthetic approach. Such synthetic

Figure 2.1 The mechanism of RNA interference mediated gene silencing. In the cytoplasm, long dsRNA precursors are processed and cleaved to 22 nucleotides (nt) short pieces dsRNA. The synthetic siRNA can mimic the structure of a Dicer product. The siRNAs are loaded onto and activated RNA-induced silencing complex (RISC), which contains Argonaute-2 (Ago-2). Then, the Ago-2 cleaves the passenger strand, resulting in RISC and a single strand RNA, form the activated RISC-guide-strand complex, which directs the specific recognition and cleavage of the target mRNA that is perfect complementary to bases pairing to guide siRNA strand. The mRNA is cleaved precisely at the site of 10 nts from the 5'-end of the guide strand. The cleavage of mRNA prevents the translation of the gene, thereby inducing gene silencing.

siRNA exhibits a sequence structure mimicking that of the Dicer products, and thus has the same downstream enzymatic machinery as that of the Dicer products. In the cytoplasm, siRNAs are bound by the RNA-induced silencing complex (RISC). Due to the function of Argonaute 2 (Ago-2) protein in RISC, the sense (or passenger) strand of siRNA is eliminated from RISC and degraded [20, 21]. Meanwhile, the antisense (or guide) strand of siRNA is directed to its complementary messenger RNA, resulting in the degradation of the targeted mRNA [22-25]. Based on the mentioned mechanism, siRNA therapy via RNAi therefore has been regarded as a new hope for treating cancer.

To enhance the treating efficacy of siRNA therapy, siRNA molecules need to be delivered into cytoplasm and activate the required RNAi machinery. However, there are still several hurdles associated with siRNA delivery when combating cancer via siRNA therapy [9, 24]. Such hurdles can be classified into extracellular barriers and cellular barriers. In the extracellular barriers, siRNA molecules encounter problems regarding the attack of serum nucleases and the elimination via renal and hepatic clearance. The naked siRNA is easily degraded by nucleases in plasma, and has a half-life of less than an hour in the bloodstream [8, 26]. *In vivo*, naked siRNA can be rapidly filtered from the glomerulus and easily excreted from the kidney [11]. In addition, siRNA delivery is also challengeable when dealing with the issues about the off-target problems, the response of immune systems, the penetration to tumor tissues and the cell uptake of cancer cells. In the cellular barriers, hydrophilic siRNA molecules that are anionic in nature cannot be readily diffused across cell membranes. After being taken up by tumor cells, siRNA molecules also have to solve the endosomal escape problems. Following the escape from endosome compartments, siRNA molecules are required to be incorporated into the relevant RNAi machinery for silencing the aberrant genes relating to cancer diseases [9]. Hence, the development of biodegradable and efficient siRNA delivery carriers is necessary and urgent [27, 28].

To address the previously mentioned hurdles in successfully activating the RNAi pathway in siRNA therapy, a variety of delivery systems have been developed for protecting fragile siRNA molecules, delivering them into target cells, and even conferring them additional functions. Based

on the abovementioned considerations, an ideal delivery system essentially should possess a great ability to protect siRNA molecules from nuclease attack, conceal siRNA molecules from immune recognition and promote siRNA molecules to be readily taken up by cancer cells. With desired particulate characteristics, newly developed delivery systems are capable of enhancing the therapeutic efficiency of their loaded siRNA molecules. These desired characteristics such size, charge, shape, surface property, shielding coatings and targeting moieties are allowed to be rationally incorporated into the delivery systems through elegant chemistry approaches [29]. Moreover, siRNA therapy via delivery systems can also be endowed with beneficial optical, magnetic and electrical-related properties through the use of functional nanoparticles as the delivery system [30]. In this context, various types of materials have been utilized for preparing siRNA delivery systems for combating cancer. These materials can be basically categorized into three groups, including lipids, polymers and inorganic particles. The progress and development of the lipid-based delivery systems for siRNA delivery in terms of the chemical structure will be reviewed. For the polymer-based siRNA delivery systems, recent advances on conventional/biodegradable polymeric vectors as well as polymeric nanoparticles will be highlighted. Finally, polymer-coated inorganic particulate systems for siRNA delivery such as gold nanoparticle [31], quantum dots[32], carbon nanotube [33] and mesoporous silica[34, 35] will also be reviewed.

2.2 Lipid-based siRNA delivery systems in cancer therapy

Since the ability of liposomes to transport the preproinsulin gene into the liver cells was demonstrated [36], liposomes have probably been the most common nanoparticle system for siRNA delivery *in vitro* and *in vivo*. A phospholipid bilayer enclosing an aqueous compartment of liposomes can fuse with cell membranes, and enhance siRNA delivery into cells. The siRNA entrapped by lipids form amorphous lipid/siRNA nanoparticles known as lipoplexes which can prevent siRNA from RNase degradation. Lipoplexes deliver siRNA into cytoplasm by interacting with the negative charged lipid bilayer of cell membrane. Some commercially available liposome

have been proposed as siRNA transfection agents, such as Lipofectamine 2000 [37], Lipofectamine RNAiMAX [38], Oligofectamine™ [39], 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) [40], TransIT-TKO [41], RNAiFect [42], and TransIT-siQUEST [43]. More importantly, some lipids used as siRNA loading carriers are in Phase I clinical trials. However, siRNA delivery using lipids has a few important drawbacks. A major disadvantage of the lipid-based siRNA delivery system is that lipids can activate the complementary system of innate immune response after administration [44]. For example, DOTAP did not only induce a severe interferon response, but also up-regulated the expression of STAT1 (a cell signaling protein) [45]. To reduce the immunological reaction, some studies have developed various kinds of methods such as polyethyleneglycol (PEG) modification (nanoparticle surface with a layer of PEG) [46]. As an alternative method, one study showed that acute inflammation responses can be significantly reduced by treatment with dexamethasone (an anti-inflammatory drug), which did not influence siRNA knockdown efficiency mediated by lipid nanoparticles [47].

Warashina *et al.* described a novel cationic lipid nanoparticles, named as YSK12-MEND (YSK12-C4 multifunctional envelope type nano device), which greatly promoted gene silencing in mouse DCs (dendritic cells) when loaded with siRNA, and at the same time exhibiting very low cytotoxicity at the same time [48]. The gene silencing efficiency of YSK12-MEND can be excess of 90% with a median effective dose of 1.5 nM, in comparison with the maximum gene silencing efficiency of the commercial transfection reagent Lipofectamine RNAiMAX which is less than 60% for the effective dose of 25 nM. YSK12-MEND is fabricated using the t-BuOH (*tert*-Butyl alcohol) dilution method, which is able to form small and uniform particles that efficiently encapsulate siRNA. To enhance siRNA transduction *in vitro*, YSK12-C4 (a ionizable cationic lipid) was designed with unsaturated carbon chains and a cationic property, based on YSK05 (**Fig 2.2**). YSK05 is a pH-sensitive cationic lipid, which enhances the ability of the YSK12-MEND to escape from endosomes to achieve high efficient gene silencing in the liver *in vivo* [48]. Even without any specific targeting protein of liver cell, liposome nanoparticles normally accumulate and

deposit in liver [49]. For instance, a lipid-based siRNA delivery system consists of positive cationic lipids with hydrophobic linoleyl and tertiary amine groups for ishepatocyte-specific delivery [49]. Tao *et al.* developed cholesterol-based PEGylated lipid nanoparticles containing luciferase that targeted siRNA, providing a peak of 90% gene knockdown efficiency in the liver, and which were sustained for 10 days [50]. A large number of lipid-based materials were screened

Figure 2.2 Structure and functional analysis of the YSK12-MEND [48]. (a) Chemical structural formula of YSK12-C4. (b) Conceptual structure of YSK12-MEND. YSK12-MEND was consisted of YSK12-C4, cholesterol and 1,2-Dimyristoyl-sn-glycerol methoxyethyleneglycol 2000 ether (PEG-DMG) with 85/15/1 mass ratio. (c) TEM image of the YSK12-MEND. (d) Gene silencing efficiency against the SR-B1 gene with different dose in mouse bone-marrow derived DCs (BMDCs). BMDCs were respectively transfected with the YSK12-MEND, R8/GALA-MEND_{SUV} or RNAiMAX, and the mRNA transcriptional levels of SR-B1 gene were detected by quantitative RT-PCR. (e) Cell viability of BMDCs. BMDCs were co-cultured with YSK12-MEND, R8/GALA-MEND_{SUV} or RNAiMAX with different doses of siRNA (3, 10, 100 nM), and the cell viability was measured at 2 h after the treatment.

to look for the optimal liposome nanoparticle component for siRNA delivery. In a recent report, Jayaraman *et al.* discovered that the lipid pK_a is an important parameter to measure the delivery efficiency, and demonstrated that lipids with pK_a (acid dissociation constant, known as acidity constant, is a quantitative measure of the strength of an acid in solution. It is the equilibrium constant for a dissociation in the context of acid–base chemical reactions) from 6.2 to 6.5 present the high siRNA delivery efficiency. By optimizing liposome/siRNA nanoparticle based on the pK_a , the liposome complex down-regulated 50% gene silencing *in vivo* [51]. To form protonated environment in endosome, the pK_a of ionized lipids should be high enough such that they can escape from lysosome to cytosol. Meanwhile, the pK_a should also be low enough to ensure high delivery efficiency, low toxicity and longer circulation time [8]. Alabi *et al.* identified the relationship of structure and function on activity of lipid nanoparticle–regulated siRNA delivery *in vivo* by using of a multi-parametric approach [52]. From these studies, we know that the gene silencing efficacy can be better predicted by referring to multiple parameters, such as *in vivo* and *in vitro* stability, loading efficiency and uptake rather than only examining the knockdown efficiency *in vitro*. On the other hand, because lipid nanoparticles tend to accumulate in the liver, their potential risk of inducing hepatotoxicity should also be considered when designing or using lipid structures.

2.3 Non-biodegradable polymers-based siRNA delivery systems

Polymeric systems have been widely applied to siRNA delivery for cancer treatment (**Table 2.1**). In contrast with lipid-based siRNA delivery systems, polymer-based siRNA delivery systems normally include a cationic component as a core component. The operating mechanism of such polymer-based DNA delivery systems has been explained in literature [53-55]. Both plasmid DNA and siRNA are nucleic acids, and share some similar characteristics. Hence, substantial knowledge of previous polymers for delivery of plasmid DNA can help to develop and design polymer structures for siRNA delivery. However, the siRNA molecule is different from plasmid DNA in terms of stability, charge ratio, molecular weight, method of action and activity

Table 2.1 Summary of siRNA delivery polymers and their modifications.

Property	Name	Modification	Targeting gene	Assay conditions	Cancer	Reference
Non-biodegradable	PEI	PEG, targeting peptide	VEGF, BCL2, Her-2	<i>In vitro</i> and <i>in vivo</i>	Breast, prostate, ovarian	[56-58]
	DMAEMA	PEG, PAA, PCL	VEGF, GAPDH,	<i>In vitro</i> and <i>in vivo</i>	Breast, cervical	[59-61]
	PAMAM (poly(amid oamine))	PEG, RGD	Hsp27, BCL2, GFP	<i>In vitro</i>	Prostate, ovarian, malignant glioma	[62-64]
Biodegradable	PLL (poly (l-lysine))	PEG, cholesterol	Luciferase, BCL2	<i>In vitro</i>	Colon, liver, breast	[65-67]
	Chitosan	PEG, PLL	P-gp, survivin	<i>In vitro</i> and <i>in vivo</i>	Liver, breast, lung	[68-71]
	Cyclodextrin	PEG, PLL, PEI, folic acid, transferrin	VEGF, EWS-FLI, MMP-9	<i>In vitro</i> and <i>in vivo</i>	Ewing's sarcoma, nasopharyngeal, cervical	[72-75]
	Gelatin	Thiolated	RFP, STAT6	<i>In vitro</i> and <i>in vivo</i>	Melanoma, lung	[76, 77]
	CPP (cell penetrating peptides)	TAT, VEGFR1	GAPDH, BCR-ABL	<i>In vitro</i> and <i>in vivo</i>	Liver, leukaemia	[78, 79]

site in cell. Hence, the same polymer will exhibit different delivery efficiencies for plasmid DNA and siRNA. Normally, cationic polymers can be classified as non-biodegradable and biodegradable. For instance, chitosan-based polymers, cyclodextrin-based nanoparticles and gelatin are biodegradable polymers, while non-biodegradable polymers include linear and branched polyethyleneimine (PEI), and polydimethylaminoethyl methacrylate (DMAEMA). Compared with cationic liposomes which require complicated processing steps before forming lipo-siRNA capsule-like structures, cationic polymers can form a complex with negative siRNA based on electrostatic interaction upon simple mixing.

2.3.1 PEI siRNA delivery system

An extensively used non-biodegradable polymer is PEI (poly-ethyleneimine), either in the branched or in the linear forms which consists of a variety of protonated amino groups and wide-range of molecular weights (MWs) [80-82]. These amino groups of PEI possess a relatively high positive charge density, such that upon electrostatic interaction, the negatively charged phosphate groups of siRNA and positively charged amino groups of PEI achieve complexation. Due to the proton sponge effect (PSE) and lots of positive charged amino groups, the PEI disturbs the pH in

Figure 2.3 Cationic polyplexes escape from endosome through the proton-sponge effect. The polymer with the positive amine groups disturbs the pH of the endosome. H^+ are pumped into endosome to balance this disturbance, and Cl^- also go into endosome by passive diffusion at the same time, causing an increased concentration of ions. Consequently, H_2O also diffuses into the endosome, leading to swelling and rupture of the endosomal membrane that results in the polyplex release [55].

the endosome, ultimately inducing the rupture of late endosome, and thus releasing the siRNA into the cytoplasm (**Fig 2.3**). Although PEI possesses some advantages for siRNA delivery, it suffers from obvious cytotoxicity that causes inflammation and its rapid absorption and clearance by macrophages [83, 84]. Additionally, unwanted cellular mechanisms induced by PEI such as necrosis and apoptosis, cell death have also been observed in various kinds of cell lines. Furthermore, the PEI/siRNA complex possesses very weak colloidal stability in serum-containing media. With the help of intravital real-time confocal laser scanning microscopy, Takahiro *et al.* observed that the positively charged nanoparticles tend to rapidly form aggregation complexes upon intravenous injection [85].

To address this issue and to reduce the cytotoxicity induced by PEI, a wide range of PEGs were conjugated to PEI in order to synthesize a block copolymer [86, 87]. Aggregation was significantly reduced in the PEGylated PEI/siRNA nanocomplex. Meanwhile, a shield block layer

formed by PEG moieties prevents the polyplex structure from undesirable interactions with proteins or degradation enzymes in *in vivo* environments [88]. The siRNA condensation and stability can be regulated by the graft density and chain length of PEG on the PEGylated PEI/siRNA nanoparticles. Previous reports showed that PEGylated PEI/siRNA nanoparticles have a lower deposition rate in liver after intravenous injection. Moreover, no obvious degree of complement activation was induced by PEGylated PEI/siRNA nanoparticles when compared with PEI/siRNA particles [83]. However, it should be cautioned that both PEI and PEGylated PEI nanoparticles induced complementary activation-related pseudoallergy in swines, as shown by cardiopulmonary reactions. PEI/siRNA nanoparticles and PEG-grafted PEI/siRNA nanoparticles with hydrophobic fatty acid modifications were intratracheally injected for gene knockdown in a rodent model. In this study, both nanoparticles elicited up-regulated levels of IgM in the bronchoalveolar lavage fluid and inflammatory responses [89]. These studies highlighted that it is necessary to develop novel PEI-based siRNA carriers with low cytotoxicity, reduced immunotoxicity, and improved gene knockdown efficiency. To direct the PEI-based polyplexes to a targeting site of action, the distribution of polyplexes can be oriented by conjugating a targeting ligand in the polymer structure. PEIs have been modified using mannose, galactose, transferrin, folic acid, RGD(Arg-Gly-Asp) peptide, epidermal growth factor (EGF), TAT peptide, and trastuzumab for targeting delivery by ligand-receptor mediating pathways [90].

2.3.2 The DMAEMA siRNA delivery system

DMAEMA (dimethylaminoethyl methacrylate) has attracted lots of attention for siRNA delivery because of its relatively high buffer capacity and biocompatibility. Strayton's group developed a siRNA polymer carrier that combines DMAEMA as a siRNA-condensing block with a PAA(propylacrylic acid) containing block to endow endosome escape activity [60]. This diblock copolymer was synthesized via reversible addition fragmentation chain transfer polymerization (RAFT) to prepare a series of well-defined diblock copolymers that vary the composition of the PAA block which acts as both a shielding/stabilizing and pH-responsive block. The resulting polymer/siRNA complexes have sizes of 80–250 nm with slightly positive zeta potentials. Finally,

Figure 2.4 Synthetic pathway of PDMAEMA–PCL–PDMAEMA triblock copolymers[59].

siRNA delivery using the diblock copolymers effectively reduces the expression of GAPDH on the HeLa cells at both mRNA and protein levels. The vesicles and micelles with higher ordered chemical structures have the additional option of integrating multiple therapeutic agents. The hydrophobic cores of the micelles allow loading of poorly soluble drug payloads, while the hydrophilic cores of vesicles can accommodate water-soluble drugs. Zhu *et al.* reported a cationic micelles based on PDMAEMA–PCL–PDMAEMA triblock copolymers applied for the combinatorial co-delivery of siRNA and paclitaxel [59]. PDMAEMA–PCL–PDMAEMA copolymers were synthesized via RAFT polymerization of DMAEMA using CPADN–PCL–CPADN (4-cyanopentanoic acid dithionaphthalenoate) as a macro-RAFT agent (**Fig 2.4**). Gel retardation results indicated that copolymers could effectively encapsulate siRNA at the 4:1 and 2:1 nitrogen/phosphorus (N/P) weight ratios, respectively. The combinatorial delivery of VEGF siRNA and paclitaxel by PDMAEMA–PCL–PDMAEMA triblock copolymers showed an efficient 90% down-regulation of VEGF expression on prostate cancer cells, whereas branched PEI, as positive control, only exhibited a 75% down-regulation[59]. The group of Stayton has also developed a multi-therapeutic micellar system comprising of doxorubicin, a pH-responsive polymer (poly(styrene-*alt*-maleic anhydride), pSMA), and siRNA against plk1, which has been

shown to cause cell-cycle arrest and apoptosis in ovarian cancer cell line as well as increased sensitivity to chemotherapeutics [91]. The cationic micelles were firstly formed from diblock copolymers of DMAEMA and butyl methacrylate (pDbB), the latter polymers was synthesized via RAFT using dodecyl cyanovaleric trithiocarbonate (DCT) CTA and 2,2'-azobis(2-methylpropionitrile) (AIBN) radical source. To improve delivery efficacy via endosomal release, a pH responsive copolymer of pSMA was electrostatically complexed with the positively charged DMAEMA corona. The pH-responsive ternary complexes were able to effectively deliver siRNA to drug-resistant ovarian cancer cell lines (NCI/ADR-RES), resulting in the 50% reduction of *plkl* gene expression and sensitized the NCI/ADR-RES cells to doxorubicin and a decrease in cell viability. In order to increase siRNA delivery efficiency, Duvall and colleagues developed a poly[(ethylene glycol)-b-[(2-(dimethylamino)ethyl methacrylate)-co-(butyl methacrylate)]] diblock polymer, PEG-(DMAEMA-co-BMA), via addition-fragmentation chain transfer (RAFT) with different mol% BMA in the second polymer block [92]. By optimizing the relative ratio of DMAEMA and BMA, the cationic and hydrophobic contents of PEGylated siRNA-loaded polyplex nanoparticles were balanced to enhance siRNA packaging, endosomal escape and blood circulation time. Compared to the standard polymer, the polymer with 50:50 mol% of DMAEMA: BMA in the RAFT-polymerized block efficiently improved the blood circulation half-life by three-fold as result of reduced rate of renal clearance and enhanced stability. This optimized polymer improved siRNA bio-distribution in the liver and other organs and obviously increased gene silencing *in vivo* relative to standardized and PEG-DMAEMA diblock architecture.

2.4 Biodegradable polymer-based siRNA delivery system

The selection of a suitable vehicle for siRNA delivery is very important because an inappropriate choice can increase off-target effects and reduce the gene silencing activity. The factors of controlling the efficient delivery of siRNA into the cytoplasm of the target cell have been illustrated in order to avoid non-specific silencing toxicity during systemic cancer treatment [93]. Because of cytotoxicity of high molecular mass of non-biodegradable polymers, various

degradable polymers with low molecular weight have been developed to acquire a safe vector with higher siRNA transfection efficiency including poly-L-lysine (PLL), chitosan, gelatin, poly(lactic-co-glycolic acid) (PLGA), and cyclodextrin (CD).

2.4.1 PLL based delivery system

As a biodegradable cationic peptide, poly-L-lysine (PLL) has been the subject of wide studies as a starting material to realize nanostructured polymeric carriers for siRNA delivery. Different molecular weights of PLL were synthesized through polymerization of lysine N-carboxy anhydrides (NCA). As the formed simple nanoplexes of siRNA and PLL alone are unstable in media containing serum, several derivatives of PLL were developed to improve the efficacy of siRNA delivery. Kodama *et al.* developed a completely biodegradable dendrigraft poly-L-lysine (DGL) with favourable properties of polylysine and dendrimers. DGL was conjugated with luciferase siRNA and recharged with biodegradable γ -polyglutamic acid (γ -PGA) to reduce the cytotoxicity of the cationic polymer. The ternary nanoplexes could inhibit luciferase protein expression in the tumor without haematological toxicity and cytotoxicity after straight intratumoral injection into of mice bearing Colon26/Luc cells [65]. In order to further enhance the delivery efficiency of siRNA, cholesterol (Chol) moieties were grafted to the ϵ -termini of PLL segments to form MPEG-*b*-PCL-*b*-PLL/Chol (poly(ethylene glycol)-*block*-poly(ϵ -caprolactone)-*block*-poly(L-lysine)/Chol) micelleplex. The Chol conjugation would improve the hydrophobic interaction with both cell and endosomal membranes as Chol moieties are well-known components of such membranes and have very good affinity with phospholipid molecules. The results demonstrated that the final micelleplex is originated from MPEG-*b*-PCL-*b*-PLL have smaller size, higher particle density, higher siRNA transfection efficiency and gene knock-down efficiency when compared to mPEG-b-PLL alone based micelleplex. These interactions finally would enhance cellular endocytosis and improve the escape capability from the endosomes [66]. The group of Cai developed a PLL-based polypeptide cationic micelle for co-delivery of hydrophobic anticancer drugs and siRNA-Bcl-2 to synergistically treat tumors. The triblock

copolymer of poly(ethylene glycol)-*b*-poly(L-lysine)-*b*-poly(L-leucine) (PEG-PLL-PLLeu) was synthesized as an effective vector for co-delivery of docetaxel and Bcl-2 targeting siRNA, in which the PLLeu served as the hydrophobic core to entrap docetaxel, while negative siRNAs were condensed with PLL via electrostatic binding. The resulting formulation exhibited very good biocompatibility, stability and excellent passive targeting property. The improved antitumor activity in MCF-7 xenograft murine model was observed after treatment with co-delivery system of siRNA and docetaxel [67].

2.4.2 Chitosan-based siRNA delivery system

Chitosan is biocompatible, biodegradable, non-immunogenic, and contains both hydroxyl and amine groups, and it has been widely explored as siRNA carrier. The presence of primary amino groups (pKa6.5) in the chitosan backbone renders chitosan positively charged under slightly acidic conditions, which promotes polyplex formation with siRNA by electrostatic interactions [94, 95]. Despite the interesting profile of chitosan, the *in vitro* transfection efficiency for delivery systems based on chitosan is lower than that of other polymers such as PEI. The major restrictions for chitosan are the minimal solubility and low buffering capacity, which are attributed to partial protonation of the chitosan's amino group at physiological pH value. These limitations can influence the chitosan-siRNA binding capability and thus can affect the stability of the nanoparticles in the blood, and finally reduce the biological activity of the siRNA. Additionally, the components in blood can destabilize the chitosan nanocomplexes. For instance, parts of serum proteins are able to induce chitosan-siRNA nanocomplexes aggregation, causing vascular embolization and increased toxicity, or to replace siRNA and conjugate with chitosan, resulting in release of siRNA and nanoparticles, followed by nucleases degradation and eventual decrease in the curative effect of siRNA [69, 96]. In order to increase the stability and solubility of the chitosan nanoparticles, PEG or PEI grafting onto chitosan has been developed. PEGylated chitosan for siRNA delivery has been widely described in the past decade [68, 95, 97]. PEG, a hydrophilic polymer, has been described to improve water solubility of chitosan, enhance stability of the chitosan complex in the physiological environment, and has a positive effect on the siRNA

transfection efficiency. The unique hydration ability and formal electrostatic neutrality of PEG allow the construction of a compact hydration layer that decreases the interaction with proteins [70, 98]. Zheng *et al.* reported that over 7% PEG density in chitosan based nanoparticles is necessary for shielding against non-specific interactions with proteins, resulting in a steric stabilization effect [69, 99]. In Sun's study, the PEG molecule was grafted on the hydroxyl of chitosan for formulation of stable siRNA-loading nanoparticles (PEG-CS/siRNA) in physical environment. PEG-CS/siRNA with smaller size exhibits higher siRNA transfection efficiency by enhancing the solubility of chitosan. This PEG-CS/siRNA could effectively deliver siRNA (targeting surviving gene) in xenograft breast tumors *in vivo*, and has significantly reduce the growth and metastasis of tumors [69]. In another study, the triblock copolymer micelle of (N-succinyl chitosan-poly-l-lysine-palmitic acid, NSC-PLL-PA)-based micelles was synthesized for co-delivery of siRNA-P-glycoprotein (P-gp) and doxorubicin (DOX). The P-gp coded by the *mdr1* gene is an energy-dependent drug efflux transporter that contributes to multidrug resistance, which weakens the treatment effect in drug-resistant tumors by decreasing intracellular concentrations of chemotherapeutic drugs. A novel method for inhibiting MDR is to downregulate the expression of P-gp using RNAi technology [71, 100]. In this NSC-PLL-PA triblock polymer, the NSC was designed as a hydrophilic shell to improve the half-life of micelles and reduce the cytotoxicity of PLL. PLL as cationic backbone was used to absorb the negatively charged siRNA by electrostatic interaction. PA was designed as hydrophobic core to encapsulate DOX. This triblock polymer micelle showed an average particle size of around 170 nm. More importantly, the micelles were much more unstable in acidic environments than in alkaline ones, which resulted in rapid release of siRNA and DOX, as observed *in vitro*. No obvious polymer cell cytotoxicity was observed. The *in vitro* and *in vivo* antitumor efficiency of the micelles increased considerably, particularly in HepG2/ADM cells. Due to the downregulation of P-gp gene, most of the Dox in the micelles accumulated in tumor cells. This co-delivery system significantly repressed tumor growth with synergistic effects *in vivo* by bypassing the drug efflux transporters [101].

2.4.3 Cyclodextrin-based siRNA delivery system

As we know from the aforementioned reports, although the PLL-based siRNA delivery system forms a large part of polymer-regulated siRNA carrier, other as siRNA carriers have been proposed, using novel biodegradable, stable and biocompatible polymers. Among these polymers, the cyclodextrin polymers (CDP)-based siRNA delivery system has entered clinical trials (**Fig 2.5**), being the first reported nanoparticle as targeted siRNA delivery system for clinical cancer treatment [102]. CDP are polycationic polymers synthesized by a polymerization reaction between dimethyl suberimidate and cyclodextrin monomers with diamine, then forming oligomers with amidine functional groups. At CDP and siRNA at N/P ratios as low as 3, the strong basicity of amidine groups can efficiently regulate condensation of CDP/siRNA nanoparticles. Moreover, CDP can form 50 nm diameter colloidal nanoparticles with siRNA by self-assembly, in which terminal imidazole groups can promote intracellular trafficking and siRNA release from endosomes [103]. However, nanocomplexes composed of only CDP and siRNA were able to get efficient delivery *in vitro*, these complexes still need additional formulation components for efficacy and stabilization *in vivo*. CDs possess a hydrophilic outer shell with hydroxyl groups and a hydrophobic inner core which can be easily modified by amphiphilic, cationic polymers and targeting ligands to improve the siRNA delivery efficiency. In order to reduce aggregation of cationic CDs in physiological environment, further modification with stabilizing groups such as PEG is necessary. For *in vivo* experiments, CDP/siRNA colloidal nanoparticles can be stabilized by modification with PEG through inclusion complex formation between the cyclodextrins and adamantanes. Furthermore, in targeted *in vivo* delivery, some of the PEG chains were conjugated with targeting ligands such as folic acid to allow specific interaction with receptor on the cells surface [72]. Heidel *et al.* synthesized cyclodextrin-containing cationic particles that displayed promising biosafety property for siRNA delivery on a non-human primate animal model. In this study, CDP/PEG/siRNA nanoparticles modified with transferrin were intravenously injected in gradually increased doses. These nanoparticles were tolerated at concentrations as high as 9 mg siRNA/kg by cynomolgus monkeys. Even at these high siRNA concentrations, these particles

Figure 2.5 Cyclodextrin polymer nanoparticles (CDPs). Composition of the cyclodextrin-siRNA delivery system[8]. Both adamantane–PEG (AD–PEG) and adamantane–PEG–transferrin (AD–PEG–Tf) were incorporated to improve CDPs-based particle properties *in vivo*. Adamantane is a hydrophobic molecule that forms a stable inclusion complex with the cyclic core of the cyclodextrin structure.

invoked a comparatively mild immune activation, inducing an escalated level of IL-6 and IFN- γ . No obvious toxicity effect to the treatment was observed [73]. The group of Ma developed a new star-shaped CD derivative containing poly(L-lysine) dendron arms and β -cyclodextrin core (CD-PLLD/pMR3) for co-delivery of docetaxel (DOC) and MMP-9 siRNA plasmid (pMR3) into cancer cells. It was demonstrated that the CD-PLLD/pMR3 nanocomplex indicated a good gene transfection efficiency *in vitro*, and could effectively reduce the MMP-9 protein in HNE-1 cells. This vector could promote a more significant apoptosis than DOC or pMR3 used alone, and exhibited lower toxicity and better biocompatibility than 25 kDa PEI [74]. The (2-hydroxypropyl)- β -cyclodextrin (HP- β -CD) was cross-linked with PEI-600 and folic acid (FA) to develop a gene carrier for targeted delivery of siRNA into cancer cell lines [75]. This complex administered via tail vein injection resulted in obvious inhibition of tumor growth and down-regulation of VEGF protein expression of tumor cells in nude mouse.

2.4.4 Gelatin-based siRNA delivery system

Proteins normally display low cytotoxicity and high binding affinity for various drugs and genes. The aqueous steric barrier of protein-based carriers possesses low levels of reticuloendothelial system (RES) clearance, resulting in improved pharmacokinetic characteristics. According to these properties, natural proteins are attractive materials as a potential gene carrier to achieve safe and efficient gene delivery. Gelatin, a natural protein obtained from the hydrolysis of collagen, is an excellent biodegradable protein candidate for siRNA delivery which is biocompatible under physiological environment. Additionally, the gelatin has been approved for the clinical application by the FDA. There are various categories of gelatin differing in terms of production technique, source of collagen and extraction conditions. In general, the hydrolysis of collagen depends on the temperature and pH value of its environment [104]. Recently, gelatin nanoparticles (GNPs)-loaded iNOS (inducible nitric oxide synthase) siRNA was synthesized by Choi's group, and was applied intranasally in postischemic rat brain. By delivering of iNOS siRNA using gelatin, GNPs were observed in extracellular and intracellular compartments of brain, concluding the olfactory bulb, striatum and cerebral cortex. Significant inhibition and sustained knockdown of iNOS were detected in iNOS siRNA/GNPs treated groups compared to iNOS siRNA alone [105]. Obata *et al.* developed biodegradable cationized gelatin microspheres (CGMs) comprising HSP47 siRNA, in which ethylenediamine was used for cationization of the gelatin carboxyl groups. These CGMs with a degradation period of about 2 weeks were used to continuously release siRNA to inhibit expression of heat shock protein 47 (HSP47) in fibrotic peritoneum of mouse. A single injection of this complex incorporating HSP47 siRNA could reduce collagen expression and macrophage infiltration in mice, thus avoiding peritoneal fibrosis [106]. Lee *et al* synthesized a tumor-targeted siRNA delivery system using gelatin and polymerized siRNA [76]. The latter was prepared through polymerization of thiol groups at the 5'-end of siRNA strands, which then were encapsulated in the self-assembled thiolated gelatin (tGel) nanoparticles (NPs) via chemical cross-linking. The resulting polysiRNA-tGel (psi-tGel) nanoparticles could effectively protect siRNA from being degraded by RNase, and they can be

reversibly reduced to release siRNA in reductive environments. The psi-tGel NPs could efficiently inhibit the expression of red fluorescence protein in melanoma cells (RFP/B16F10), and present negligible cytotoxicity. Importantly, the psi-tGel NPs also obviously suppressed tumor RFP gene expression *in vivo* without significant toxicity. Using gelatin as siRNA carrier, Youngren *et al.* successfully delivered STAT6 siRNA into human adenocarcinoma A549 cells, resulting in obviously reduced expression of STAT6 protein and significantly decrease cell viability compared to native siRNA and siRNA-lipofectamine complex [77]

2.5 Polymer-hybrid multifunctional delivery system

2.5.1 Quantum dots (QDs) as carrier for siRNA delivery

Tan *et al.* firstly reported that QDs can be utilized as a carrier for siRNA delivery to silence the *HER2/neu* gene of breast cancer cells [107]. Following this study, QDs as outstanding light-emitting nanoparticles have been extensively developed for siRNA delivery [32, 108, 109]. In previous works, when QDs were used as siRNA carrier, the QDs had to be mixed with either an external chemical reagent such as chloroquine or conventional siRNA delivery agents, which can be utilized for endosome rupture for siRNA release [110, 111]. By taking advantage of semiconductor QDs and proton-absorbing polymeric coatings, Yezhelyev developed multifunctional nanoparticles as carriers for siRNA delivery and imaging. By balancing two chemical groups (tertiary amine and carboxylic acid) on the surface of QDs nanoparticles, various kinds of compound of polymer-encapsulated QDs were synthesized for safe siRNA delivery. A proton sponge effect arises from lots of weak conjugate bases, resulting in proton absorption in acid organelles leading to an increased influx of Cl⁻ ions and water, and an osmotic pressure formation across the lysosomal membrane. This osmotic pressure induces the collapse of the endosomes, releasing its contents into the cytoplasm. Compared with existing transfection agents (lipofectamine2000, TransIT and JetPEI) using the same proton sponge effect, this study exhibited not only significant improvement in gene silencing efficiency, but also simultaneous reduction in cellular toxicity. As these nanometer-sized particles were particularly designed to address cross-

barrier cellular penetration, carrier unpacking, endosomal release, and intracellular transport, they show very high delivery efficiency for siRNA. These particles can also act as dual-modality optical and electron-microscopy probes, allowing real-time tracking of the precise location of QDs during transfection processes [112]. HY Zhu *et al.* recently synthesized pH-responsive hybrid quantum dots based on water-soluble cadmium (Cd) tellurium (Te) quantum dots (CdTe QDs), which can deliver HIF-1 α siRNA into cells to treat hypoxic tumors [113]. These nanoparticles with CdTe quantum dots (QDs) as a core include a functional shell composed of 2-deoxyglucose (DG)-polyethylene glycol (PEG) linked with a compound of lipoic acid, lysine and 9-poly-d-arginine (LA-Lys-9R) by a hydrazone bond. The pH-responsive CdTe QDs with hydrazine functionalization of DG-PEG-LA-Lys-9R could efficiently deliver HIF-1 α -siRNA into the cytoplasm without receptor-regulated endosomal recycling. Within the condition of hypoxic tumor microenvironment, DG-PEG was released after breakdown of the hydrazone bond in the low endosomal pH. Meanwhile, remaining 9R-QDs showed a high cell-penetrating ability as a result of degradation of the DG-PEG shell, which enhanced cellular uptake and efficient intracellular delivery of siHIF-1 α for gene silencing. The same QDs also enabled real-time dynamic tracking of the whole siRNA delivery process *in vitro* and *in vivo*. Wang *et al.* synthesized and characterized Mn:ZnSe/ZnS/ZnMnS sandwiched QDs in a facile aqueous condition for multimodal imaging and siRNA delivery [114]. In these nanoparticles, paramagnetic Mn²⁺ ions are introduced as core/shell dopants that provided phosphorescence which enhances the magnetic resonance signal. Mn phosphorescence can be maintained by the sandwiched ZnS shell as a result of high level shell doping. Meanwhile, the SQDs (sandwiched quantum dots) are free of toxic heavy metals, and no obvious cytotoxicity was detected *in vitro* [114, 115]. Meanwhile, the high delivery efficiency of SQDs as siRNA carrier in Panc-1 cells were confirmed by real-time PCR (Polymerase Chain Reaction) analysis [114].

The major disadvantage of using QDs as multifunctional imaging probes and siRNA delivery systems is their inherent cytotoxicity, as most of the well-fabricated QDs are composed of highly toxic chemical heavy metal elements, such as lead or cadmium. Functional modification

of these QDs with polymers may be the key to solve some concerns related to acute toxicity but this may diminish their intrinsic potential as delivery and imaging indicator. Although various kinds nontoxic QDs for siRNA delivery and imaging *in vitro* and *in vivo* have been exploited recently [116, 117], a systematic analysis of their toxicity is necessary before they enter biomedical applications *in vivo* [7]. For this reason, the toxicity of QDs was assessed recently on non-human primates [118]. In this study, Rhesus macaques as animal models were intravenously injected with phospholipid micelle-encapsulated CdSe/CdS/ZnS quantum dots. These results demonstrated that the acute cytotoxicity of these QDs nanoparticles was non-significant. Chemical analyses indicated that most of Cd accumulated in the kidneys and liver after three months, proving that the clearance of QDs *in vivo* was very slow, and suggesting that long-term studies are necessary to detect the ultimate metabolic pathway of these QDs. The influence of QDs on the immune, nervous and reproductive systems of animal models should also be meticulously studied before moving towards pre-clinical experiments.

2.5.2 Mesoporous silica nanoparticles for siRNA delivery

Mesoporous silica nanoparticles (MSNs) are another promising inorganic material for siRNA delivery due to the ease of controlling its size, large surface area, large pore volume, high synthesis scalability and low cellular toxicity. Several MSNs-based platforms for siRNA delivery have been developed. The siRNA and drug molecules can be loaded into the large surface area pores of MSNs. Xia *et al.* firstly demonstrated that MSNs coated with polyethyleneimine (PEI) can enhance the cellular uptake of MSNs for safe delivery of siRNA [119]. Subsequently, a series of MSNs-based systems for disease therapy have been observed to deliver siRNAs into cells, normally through endocytosis pathway. For example, Li *et al.* described the synthesis of MSNs functionalized with a fusogenic peptide and a cationic polymer PEI, which increased the endosomal escape of siRNA and consequently improved siRNA-induced silencing gene expression both *in vitro* and *in vivo* [120]. However, the modified MSNs platform without PEG to shield them from RES (reticuloendothelial system) recognition showed no effect on inhibiting

the tumor growth. Thus, PEG can provide a steric blocking effect to prevent the siRNA from enzymatic degradation, and can also reduce binding of serum proteins to the MSNs. Hence, the layer of PEG on the nanocomplex can reduce immune response, prevent aggregation and improve the blood compatibility. Shen *et al.* developed MSNs functionalized with cyclodextrin-grafted PEI (CP) as a siRNA delivery carrier [121]. The positive charge of CP gave the MSNs the ability of loading siRNA through electrostatic binding and allowed effective endosomal escape of siRNA. The delivery of siRNA by CP-MSN/siRNA particles specifically targeting the M2 isoform of the glycolytic enzyme pyruvate kinase (PKM2) resulted in effective knockdown of gene expression *in vitro* and *in vivo*. Subsequently, the suppression of PKM2 induced by CP-MSN/siRNA particles effectively inhibited cell growth, invasion, and migration of the MDA-MB-231 cell line. The surface of synthesized MSNs was functionalized with PEG and poly (2-(diethylamino)ethylmethacrylate) (PDEAEMA) or poly(2-(dimethylamino) ethylmethacrylate) (PDMAEMA). The modified MSNs were then used for co-loading lysosomotropic agent chloroquine (CQ) and plasmid DNA or siRNA. The biological studies demonstrated that polycation-modified MSN were able to simultaneously deliver CQ with DNA or siRNA. The transfection and silencing effectiveness of the complexes with CQ and the nucleic acids were obviously increased compared with MSN not loaded with CQ [122]. Another important application of MSNs is that they can be used to enhance therapeutic effect of chemotherapy. In a co-delivery system using the PEI-coated MSNPs, a synergistic effect in inhibiting drug-resistant cell growth was validated. PEI-coated MSNPs that delivered siRNA to inhibit the P-glycoprotein gene expression of KB-V1 cell line and simultaneously release encapsulated DOX achieved high efficiency of tumor cell killing [123]. Multiple drug resistance (MDR) in cancer cells is attributed to the overexpression of drug efflux transporters such as P-glycoprotein (Pgp) protein, which is a well-known contributor to the formation of a drug efflux pump that prevents the intracellular accumulation of chemotherapeutic agents. The phosphonate functionalization on the surface of MSNs allowed them to be further coated with PEI, which endowed MSNs to co-load DOX and

the Pgp siRNA resulting in a significantly improved cell sensitivity to DOX, and thus enhanced therapeutic effect.

The group of Min synthesized sponge-like structured porous silica particles with pores of average diameter of 23 nm that were subsequently decorated with PEG by MeO-PEG-NH₂. These particles, upon loading with siRNA, efficiently down-regulated the expression of the vascular endothelial growth factor (VEGF) in Hela cell xenografts [124]. A novel general method for siRNA delivery was developed combining porous core-shell silica nanoparticles with a multifunctional block polymer [34, 125]. Möller *et al.* reported a highly efficient siRNA delivery platform based on MSNs with medium-sized pores of 4 nm. The negatively charged MSN shell enabled the adsorption of a block copolymer comprising of positively charged artificial amino acids, which acts simultaneously as capping and endosomal release agent. The extremely high siRNA loading capacities of up to 380 µg per mg siRNA/MSN were achieved with charge-matched amino-functionalized core-shell nanoparticles. This multifunctional delivery platform was highly effective in cell transfection and siRNA delivery.

The large capacity of MSNs was developed to efficiently co-load a cancer chemotherapeutic, such as DOX together with Bcl2-siRNAs. The study indicated that both DOX and Bcl2-siRNA were delivered into cells. By silencing the multi-drug resistant pump of Bcl2 gene expression, they enhanced cell sensitivity to DOX [126]. Recently, MSNs coated with PEI and PEG (PEG-PEI-MSNs) were used to overcome DOX resistance on a human breast cancer in a xenograft nude mice model by co-delivering DOX and siRNA that targeted the P-glycoprotein (drug exporter protein). This co-delivery system caused synergistic inhibition of tumor cells growth *in vivo* when compared to free DOX or the carrier loaded with either siRNA or DOX alone. Assessment of some xenograft biopsies indicated that apparently P-gp was down-regulated at heterogeneous tumor parts corresponding to the regions where DOX was released from MSNs nanocomplex and induced cell death [127]. Based on previous reports, Ngamcherdtrakul *et al.* synthesized 47nm-MSNs coated with a crosslinked PEI and PEG copolymer to overcome the delivery barrier, minimize toxicity, and to enable scaled-up production [35]. This nanocomplex

not only carried siRNA against the HER2 (human epidermal growth factor receptor type 2) oncogene, but also was coupled with trastuzemab (anti-HER2 monoclonal antibody) to enhance tumor-specific cellular uptake. While the HER2 positive breast cancer cells were effectively inhibited by this complex, it had no apparent effect on HER2 negative cells. When exposed to PBMC (human peripheral blood mononuclear cell), this siHER2-PEG-PEI-MSNs complex induced very low secretion of cytokine, which means that this nanoplex exhibited very low immunotoxicity.

All in all, the research advances in MSN delivery systems are promising and hold great potential for future clinical applications. However, there are still several significant challenges that should be overcome and investigated to enable the clinical applications in the treatment of human diseases. For instance, innovative methods for fabrication of multifunctional MSNs with efficient and regulated drug release should be developed to allow real-time monitoring of delivery process via bioimaging. More particular knowledge about siRNA delivery systems and bioimaging based on MSNs is necessary to investigate more comprehensive diagnostics and therapeutics in the field of nanomedicine. It is still too early to use MSNs as carriers for clinical diagnostics and treatment even though their toxicity *in vitro* and *in vivo* has been assessed. The studies of detailed toxicity, metabolism, bio-distribution and clearance rate are imperative before MSNs are used in patients. The toxicity induced by different administration routes, MSN particle sizes, and modifications also are needed to be addressed. A recent study suggests that the pore size of MSNs plays critical roles in the knockdown of gene expression by siRNA, possibly because it is directly related to the siRNA loading capacity. However, a more detailed mechanistic study of the loading and delivery process has to be carried out further [128]. These studies tell us that the MSN-based siRNA delivery systems have not yet achieved the expected goals, as their operation mechanism needs to be further studied in more details.

2.5.3 Gold nanoparticles siRNA delivery system

Due to facile synthesis, monodispersity, biocompatible, optical properties and easy functionalization with chemical polymers via thiol groups, gold nanoparticles (AuNPs) have been

used as imaging agents, biosensors, and even as therapeutic agents carriers for cancer treatment. Thanks to the nanoscale size of AuNPs, they can passively target tumors via the enhanced permeability and retention (EPR) effect, or can be functionalized with tumor-specific ligand molecules for active targeting [129, 130]. Recently, several studies have reported the engineered AuNPs coated with PEG, PEI, or a biodegradable polymer widely designed for gene delivery for cancer therapy [7]. Anderson and co-workers synthesized AuNPs functionalized with PEG and siRNA, then coated them with end-modified poly(β -amino ester)s (PBAEs) to facilitate intracellular siRNA delivery. These AuNPs formulations showed high effectiveness for *in vitro* siRNA delivery, when compared with the commercially transfection reagent, Lipofectamine 2000 [131]. Bishop *et al.* synthesized layer-by-layer degradable polymer-coated AuNPs for simultaneous co-delivery of siRNA and DNA [132]. The reducible disulfide-containing poly (amido amine) and the hydrolytically degradable poly (beta-amino ester) facilitated the particles' internalization by cells, resulting in effective siRNA-regulated gene knockdown. The group of Kataoka developed cyclin RGD-installed unimer polyion complex-assembled gold nanoparticle (c-RGD-uPIC-AuNPs) for active targeting delivery siRNA into HeLa cells (cervical cancer cell line), in which the cyclic Arg-Gly-Asp (cRGD) peptide ligand was able to selectively bind to $\alpha v \beta 3 / \alpha v \beta 5$ integrin receptors on the cancer cell surface to enhance cellular internalization [133]. The group firstly synthesized the monodispersed uPIC formation consisting of a block cationer, namely cyclic RGD (cRGD) peptide-installed poly(ethylene glycol)-block-poly(L-lysine), modified with lipoic acid (LA) at the ω -end (cRGD-PEG-PLL-LA), and therapeutic siRNA. Then, the cRGD-uPICs was conjugated to AuNPs through double Au-S bonds, followed by grafting of additional PEG for production of uniform dispersed cRGD-uPIC-AuNPs (**Fig 2.6**). Eventually, the targeted delivery of siRNA for silencing of papilloma virus-derived E6 oncogene by cRGD-uPIC-AuNPs obviously inhibited the growth of subcutaneous HeLa tumor via intravenous administration. Recently, Yin *et al.* reported a gold nanorods (AuNRs)-based nanoplex formulation that co-delivered siRNA and DOX for pancreatic cancer treatment [134]. In this study, the AuNRs were functionalized with PSS and PAH to form a multilayer structure after which both

the siRNA and the drug were adsorbed on the surface of AuNRs nanocomplexes by electrostatic interaction. In xenograft animal model, the AuNRs/PSS/DOX/PAH/siRNA nanoparticles significantly down-regulated the targeted gene expression and reduced tumor volume.

Although these Au nanoparticles present a promising application on siRNA delivery, there are still important issues that need to be addressed, such as how siRNA nanoparticles can be accurately tracked following incubation/injection/implantation *in vivo*, and in particular how long-lasting circulation and clearance of these materials take place in the body. Their metabolic processing mechanisms also need to be understood before these inorganic nanoparticles enter clinical experiments.

Figure 2.6 Schematic illustration of cRGD-uPIC-AuNP nanoparticle synthesis. The cRGD-installed uPICs were consisted of a single pair of siRNA and cRGD-PEG-PLL-LA, in which LA and c RGD moieties were respectively employed for AuNP conjugation and cancer targeting. Secondly, the uPICs were coated onto a 20 nm AuNP core via double Au–S bonds, then, short PEG was grafted resulting in monodispersed 40 nm-sized cRGD-uPIC-AuNP [133].

2.5.4 Carbon dots siRNA delivery system

Carbon dots demonstrated excellent gene loading capability due to their large surface area and easy conjugation with polymers. Carbon dots (CDs) can load gene molecules/anticancer drugs through π - π stacking interaction, hydrophobic interaction, or electrostatic attraction. Liu and

colleagues demonstrated that PEI-functionalized carbon dots (PEI-CDs) displayed great potential for gene delivery and bio-imaging in COS-7 and HepG2 cells [135]. PEI molecules, as a nitrogen-rich compound, passivated the surface of CDs and thus enabled them to exhibit new properties, e.g. strong photoluminescence or act as a polyelectrolyte to condense DNA for gene transfection. Wu *et al.* synthesized the folate-conjugated reducible PEI passivated carbon dots (fc-rPEI-CDs) for targeted gene delivery [136]. The fc-rPEI-CDs are highly biocompatible with low cytotoxicity, and able to effectively encapsulate double siRNAs (EGFR and cyclin B1). These elaborately fabricated nanocomposites selectively accumulated in lung cancer cells through receptor mediated endocytosis, resulting in silencing of EGFR and cyclin B1 gene expression and obviously inhibiting the cell viability. Recently, Feng *et al.* reported a cisplatin (IV) prodrug-loaded charge convertible CDs (CDs-Pt(IV)@PEG-(PAH/DMMA)) for imaging-guided anticancer drug delivery with enhanced internalization and activation of highly cytotoxic cisplatin (IV) in the reductive cytosol (pH 7.4) [137]. As shown in **Fig 2.7**, the anionic polymer with dimethylmaleic acid (PEG-(PAH/DMMA)) on the functionalized CDs-Pt(IV)@PEG-(PAH/DMMA) could undergo fascinating charge conversion to a cationic polymer in mildly acidic tumor extracellular microenvironment (pH 6.8). The experimental results *in vivo* further confirmed high inhibition effectiveness in tumor growth with low side effects [137].

Figure 2.7 Schematic graphic for the synthesis of charge-convertible CDs-based drug nano vectors CDs Pt(IV)@PEG-(PAH/DMMA). pH_e means tumor extracellular pH [137].

Photoluminescent (PL) graphene quantum dots (GQDs) is the next generation of carbon-based nanomaterials. Due to superior mechanical flexibility and large surface area, GQDs exhibit fascinating physical and chemical properties, and possess great promise in biomedical application. The nanosized zero dimensional photoluminescent GQDs possess outstanding optical properties due to the quantum confinement and edge effects which have generated great excitement for bio-imaging [138]. Dong *et al.* synthesized a multifunctional fluorescent nanocarrier of biodegradable poly(L-lactide) (PLA) and biocompatible PEG-grafted GQDs (f-GQDs) for simultaneous microRNAs (miRNAs) imaging analysis and combined gene delivery [139]. The f-GQDs effectively delivered a miRNA probe for intracellular miRNA imaging analysis, and the large surface of GQDs was also capable of simultaneous delivery agents targeting miRNA-21 and the survivin gene for enhanced therapeutic efficiency, respectively. The combined conjugation of targeting agents induced a larger apoptotic cell population of cancer cells when compared with conjugation of agents targeting miRNA-21 or survivin alone. These results address the promise of the versatile multifunctional GQDs nanocomposite in biomedical application of intracellular molecules imaging and clinical gene therapy.

2.5.5 Magnetic nanoparticles siRNA delivery system

The magnetic nanoparticles, particularly magnetite (Fe_3O_4) and/or maghemite ($\gamma\text{-Fe}_2\text{O}_3$), have been promising candidates in developing a variety of functional nanoparticles for biomedical applications, including diagnostics, biosensors, drug delivery and magnetic resonance imaging (MRI). The simple fabrication of magnetic nanoparticles and subsequent surface functionalization to graft extra diagnostic and therapeutic functionalities has enabled these systems to be engaged as smart nanomedicines that combine different components, without losing the individual properties of each component. After surface functionalization by coating with poly(L-lysine), PEI, or their derivatives, magnetic nanoparticles become highly useful carriers for siRNA delivery system. The group of Cheon synthesized multifunctional magnetic nanoparticles that can simultaneously perform siRNA delivery and multimodal imaging [140] (**Fig 2.8**). They

Figure 2.8 The synthesis of multimodal MnMEIO-siGFP-Cy5/PEG-RGD[140].

synthesized manganese-doped magnetism-engineered iron oxide nanoparticles (MnMEIO) coated with bovine serum albumin (BSA) that were further modified with N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) as a linker. Then, SPDP-decorated MnMEIO were conjugated with RGD peptide (Arg-Gly-Asp), polyethylene glycol (PEG, MW 3400), and siRNA through degradable disulfide bonds to form MnMEIO-siRNA-Cy5/PEG-RGD multimodal nanoparticles. RGD-conjugated MnMEIO were internalized through receptor-mediated endocytosis by $\alpha_v\beta_3$ integrin positive MDA-MB-435 cells. The distribution of MnMEIO in the tumor cells was observed by Cy5 fluorescence imaging MRI. Furthermore, the down-regulation of the specific green fluorescent protein (GFP) gene was demonstrated by confocal imaging. Wang *et al.* synthesized PEI modified magnetic nanoparticles (PEI-MNPs) as carriers for glioblastoma multiform treatment by combined intracellular imaging and siRNA delivery [141]. These synthesized PEI-MNPs presented very low cytotoxicity in a large range of concentrations, and could form stable complex with siRNA (siRNA-PEI-MNPs) in the serum. The resulting siRNA-PEI-MNPs very effectively entered the intracellular matrix and approach the nuclei, which were observed by fluorescent magnetic labeling. Consequently, siRNA-PEI-MNPs efficiently suppressed cell proliferation, and promoted cell apoptosis and autophagy of glioblastoma U251 cells [121]. Lee *et al.* developed chitosan-deoxycholic acid nanoparticles consisting of iron oxide and perfluoropentane with a hydrodynamic diameter of 257.6 ± 10.9 nm. Then, AllStars Hs cell death siRNA bound to the particle surface by electrostatic interaction and delivered to breast cancer cells (MCF-7 cells) and lung cancer cell (A549 cells) was investigated under ultrasound

exposure. The siRNA functionality was not adversely affected by the treatment protocol and that the nanodroplets were able to successfully promote siRNA uptake, leading to significant apoptosis after ultrasound treatment. In this context, the magnetic particles were introduced into the droplets to increase vaporization efficiency, and also visualize droplet localization for targeted delivery using an externally applied magnetic field [142].

2.5.6 Nano-gels and Hydrogels siRNA delivery system

Nanogels have high payload capability, a high degree of porosity, and can be selectively surface-functionalized with biomolecules to enable tumor-specific targeting delivery, and thus have been developed to delivery anti-cancer drugs [143, 144]. In 2007, Lee *et al.* firstly explored hyaluronic acid (HA) nanogels with disulfide crosslinking by inverse emulsion method. The anti-green fluorescent protein (GFP) siRNA was entrapped inside the self-crosslinked HA nanogels in oil phase under ultra-sonication condition. The HA/siRNA nanogels were effectively uptaken by HA-specific receptor positive cells (HCT-116 cells) via ligand-receptor mediated endocytosis manner, and the reported GFP gene expression was obviously inhibited [145]. Later, the group of Lyon reported the application of peptide-labeled nanogels with high loading capability of siRNA to selectively target ovarian carcinomas by ligand-receptor binding [146]. This core/shell nanogels consisting of poly(*N*-isopropylmethacrylamide) (pNIPMAm), YSA peptide mimicking the ephrin-A1 and siRNA targeting-EGFR effectively delivered siRNA into cytoplasm of ovarian cancer cells in the presence of serum, resulting in silencing of EGFR expression and increasing sensitivity of the SK-OV-3 cells to docetaxel chemotherapy [147]. Raemdonck *et al.* developed a kind of biodegradable dextran nanogels siRNA delivery system for time-controlled release in the intracellular environment. The biodegradable property was attributed to the coupled HEMA (hydroxyethyl methacrylate) in the dextran backbone by carbonate ester that can be hydrolyzed under physiological conditions. These nanogels helped siRNA to effectively escape from endosome and successfully knock-down the expression of luciferase gene of hepatoma cells with negligible cytotoxicity [148]. Based on ethylenediamine (ED)-functionalized low-molecular-weight PGMA (aminated poly (glycidyl methacrylate)), Li *et al.* developed flexible well-defined

Figure 2.9 Schematic diagram illustrating the preparation of PGED-NG, morphologies of PGED-NG/pDNA and PGED-NG/siRNA complexes, and nucleic acid delivery processes [149].

reducible cationic nanogels (denoted by PGED-NGs) with α -lipoic acid (LA) as crosslinking reagents for highly efficient encapsulation of DNA/plasmid and siRNA (**Fig 2.9**). The PGED-NGs could not only efficiently transport MALAT1 siRNA into hepatoma cells, but also obviously inhibited the HepG2 cell (liver cancer cell line) proliferation and migration [149]. In other reports, pH-dependent charge-reversal nanogels were developed to sustain a negative surface charge to increase their stability under physiological conditions (pH 7.4), and then reverse their surface charge to promote cell uptake at the tumor lesion due to the lower pH value [150]. For instance,

Tasciotti *et al.* described a one-pot synthesis method to produce uniform disperse nanogels through coating of cationic poly(2-diethylaminoethyl methacrylate) hydrogel around the fluorescently-doped monomeric silica nanoparticle cores by oil-in-water emulsion process. The siRNA was loaded into the cationic hydrogel shell via electrostatic binding [151]. The siRNA was controlled-released in an acidic pH environment as a result of protonation of the tertiary amine groups of the poly (2-dimethylamino) ethyl methacrylate shell when the pH changed from 7 to 5, which resulted in the nanogels volume swelling and porous size increasing. Moreover, the human breast cancer cell line treated with these nanogels loaded with anti-CXCR4 membrane receptor siRNA demonstrated a significantly decreased expression of CXCR4 protein. After intravenously injection of these siRNA-loaded nanogels, athymic nude mice showed an obvious reduction of CXCR4 expression at the tumor site when compared to mice treated with free siRNA. Forcada *et al.* designed a core-shell nanogels siRNA delivery system with temperature and pH dual-response[152]. These core-shell nanogels consisting of a poly(2-diethylaminoethyl) methacrylate (PDEAEMA)-based core and poly(N-vinylcaprolactam) (PVCL)-based shells were synthesized by batch seeded emulsion polymerization. The siRNA was conjugated into the formed nanogels through electrostatic interactions. In this nanocomposite, PVCL displayed a volume phase transition when temperature changes in water from 32°C to 38°C, and PDEAEMA presents volume phase transition with respect to the pH change (5.0–7.4). Hence, the siRNA release from the core-shell nanogel/siRNA complexes was controllable by pH of the solution and the temperature which both determine the crosslinking density of the PVCL-based shell.

2.6 The barrier and future of siRNA delivery system

The barriers of siRNA delivery are multiple according to the administration routes and targeted organs. In common, locoregional delivery of siRNA has fewer barriers when compared with systemic delivery. For instance, intranasal inhalation of siRNA treat respiratory virus infection, in which naked siRNA and encapsulated in polycationic liposomes have about equally effective in reducing the viral infection[153]. The intravenous injection of siRNA faces more

serious challenges. By intravenous injection, siRNA is carried to organs through the blood circulation and undergoes immunological elimination. The nano-sized vehicles are quickly distributed to organs in the reticuloendothelial system (RES) and phagocytosed by the mononuclear phagocyte system (liver Kupffer cells and macrophages)[9]. These clearance processes regulated by the interaction of particles with blood components (immunoglobulins of the complement system) can caused higher particle accumulations in RES organs, such as liver and spleen[154]. This property can be used to target the siRNA delivery to the RES organs, and should be avoided for targeting non-RES organs. Meanwhile, the positively charged of carrier tend to aggregate in biological fluid and serum-containing media[8]. For this reason, PEG coating the strategy have been developed for enhancing greater stability of siRNA carriers in the blood[99]. After reach an organ, siRNA leaves the intravascular space within a blood vessel to enter the interstitium. Then, siRNA is transported across the interstitial space to the target cells[11, 22]. In order to improve the targeting and cellular uptake of siRNA nanocomplexes, some tumor cell specific ligands were installed on the surface of delivery vehicles, such as folic acid, RGD peptides and transferrin[75, 133, 155]. Within the target cell, siRNA need to be internalized via endocytosis, a process that involves siRNA being encapsulated in endocytic vesicles that fuse with endosomes. After entering the endosome, siRNA must escape and be released from the vehicle to the cytosol in order to form RISC[9]. In this process, the proton-sponge effect plays important role for the positive charge vehicles of siRNA delivery[11].

According to all the reports reviewed in this chapter, gold nanorods (AuNRs), gold nanoparticles, magnetic nanoparticle nanogels, and carbon dots all showed promising results and high efficiency for siRNA delivery [140, 156-159]. Nanoparticle-based siRNAs delivery systems have shown to be less susceptible to degradation by nuclease enzyme when compared to conventional transfection agents [7]. These inorganic nanoparticles need to be functionalized with various polymers depending on their core material and expected target, which is then conjugated to siRNA through specific approaches including, electrostatic absorption, covalent binding and encapsulation depending on the material. Additionally, inorganic nanoparticles can also be used

to construct layer by layer structures with different polymer coatings for siRNA and drug co-loading, which also displayed tremendous potential and opportunities in therapeutics.

To date, while important progress has been made in nanoparticles-based siRNA delivery systems, several key areas, including the design of multifunctional biocompatible nanocomposite and further understanding the uptake mechanism of nanoparticles in cells, still need to be addressed. Despite the promising achievements in many reports, the results of initial clinical studies have yet to meet the expected goals. These initial results indicated that there are still some challenges for us to overcome. Much more knowledge on the interaction between blood components and nanoparticle/siRNA complexes is required as it is very important for controlling the nanoparticle/siRNA stability in the body circulation system. Different imaging methods also need to be developed for siRNA transport tracking and real-time access to the targeting efficiency *in vitro* or *in vivo*. Hence, such physiological and physical factors should be studied to ensure increased biocompatibility for the design of next generation of nanoparticle. Although there are some challenges on the road ahead, the future for the development of novel therapeutics based on RNAi identified in the next section is bright as novel nanocarriers will be continually designed to surmount the existing challenges.

Chapter 3 The synthesis of biodegradable polymer and its potential as siRNA carrier on pancreatic cancer therapy

RNA interference (RNAi) targeting oncogene mutations in cancer mediated by small interfering RNA (siRNA) transfection is a very promising treatment. However, the rapid degradation and negative charge of naked siRNAs restrict their direct delivery into cells. In this Thesis, we propose a safe and effective transmembrane transport nanocarrier formulation based on a newly developed biodegradable charged polyester-based vector (BCPV) for siRNA delivery into cancer cells. Our results have shown that these biodegradable and biocompatible vectors are able to transfect mutant K-ras siRNAs into the MiaPaCa-2 cells with high transfection and knockdown efficiency. More importantly, the RNAi process has initiated a cascade gene regulation of the downstream proteins of K-ras associated with cell proliferation, invasion and apoptosis. We observed that after the mutant K-ras siRNA transfection, the growth and invasion of MiaPaCa-2 cells have been significantly reduced, and the apoptosis of the pancreatic cancer cells has also been promoted. Although *in vivo* testing data are limited, we propose that the BCPV-based nanoparticle formulation could be a promising candidate as non-viral vector for gene therapy in clinical settings.

3.1 Introduction

Among gene therapy strategies, RNA interference (RNAi) has received great interest in the past decade. RNAi is a process of post-transcriptional gene silencing where a sequence-specific double-stranded RNA is introduced to trigger the degradation of the targeted messenger RNA (mRNA) homologous [19]. It can be initiated by endogenous microRNAs (miRNAs) or synthetic small interfering RNAs (siRNAs), of which the latter mimics the structure of Dicer products and is incorporated in the pathway downstream of the Dicer enzyme [8]. When delivered into the cytoplasm, siRNAs are incorporated into a nuclease complex, which then identifies and binds to the target mRNA [21]. Consequently, the bound mRNA is rapidly degraded and interrupted from being translated into proteins [22-25]. In recent years, siRNAs have been widely

used for targeted gene silencing in pancreatic cancer therapy. For example, siRNA targeting HIF-1 α suppressed pancreatic cancer cell proliferation and induced cell apoptosis [160]. Zhi *et al.* have used siRNAs against matrix metalloproteinase-2 (MMP-2) to decrease the pancreatic cancer cell adhesion and invasion [161]. Another study has demonstrated that the silencing of sphingosine kinase-1 can sensitize the pancreatic cancer cells to chemotherapy drugs [162]. All these results have shown great promise towards a breakthrough in pancreatic cancer therapy.

Although RNAi initiated by siRNAs has great potential for gene therapy, the application of siRNA faces a series of challenges. Naked siRNAs are not stable due to nuclease-mediated degradation and they are negatively charged. Without proper carriers, they cannot enter a cell by themselves. On the other hand, the carriers need to be nontoxic and able to deliver siRNA with high efficiency. Most importantly, the carriers should be able to efficiently release the siRNAs so that they can interact with the RNAi machinery [163]. Furthermore, naked siRNAs are possibly immunogenic in the bloodstream and not able to cross biological barriers to reach the lesion site [164]. Viral vectors have been considered as a promising candidate because of their high gene transfection efficiency. However, due to the size limitation of the inserted genetic materials, the possible immune response and the biosafety risk, the application of viral vectors has been restricted [165]. Non-viral vectors have thus been greatly investigated, such as lipids, polymers, peptides, antibodies, aptamers, nanoparticles and small molecules [8, 42, 166, 167]. Of these vectors, although some show promising results, their applications are often limited by low transfection efficiency and/or cytotoxicity. For example, cationic lipid complexes, which have been the most widely used carrier for delivery of siRNA [42], may cause severe membrane damages, reduced number of mitoses, cell shrinking and vacuolization of the cytoplasm [168].

Pancreatic cancer is listed as the fourth leading cause of cancer related death, yet its prognosis has not been improved over the past 40 years [169]. Due to the lack of specific early symptoms and early detection methods, it is usually diagnosed at advanced and incurable stages [170] and usually results in fatalities even after treatment with existing therapies [171]. Gene sequencing analysis revealed that K-ras mutations are very common genetic mutations in

approximately 90% of all pancreatic cancers and occur mostly in the early stages of pancreatic cell transformations and tumor progression [172]. The activated K-ras oncoproteins are thought to alter the cellular signal transduction pathways and enhance the neoplastic growth of the pancreatic cancers [173, 174]. Reports have shown that the down regulated K-ras gene expression causes reduced proliferation and migration, and increase of apoptosis in MiaPaCa-2 cells [175]. Therefore, the mutant K-ras gene transcripts can be used as potential targets of specific gene modulation for suppressing the development of the pancreatic cancer.

In our previous work, the cationic polylactides, which can be referred to as biodegradable charged polyester-based vectors (BCPVs) have shown great promise as nanocarriers for siRNAs. In our previous study, the high complexation ability and well-defined structure of BCPV have led to the application for siRNA delivery [13]. More importantly, BCPV also exhibited very low toxicity, high biocompatibility and considerable hydrolytic degradability. To further advance the applicability of BCPV for gene delivery in clinical settings, transfection conditions need to be carefully optimized. The physiological characteristics of cancer cells are also important before preclinical animal experiments. In this study, we have optimized the BCPV-siRNA formulation for efficient delivery of siRNAs into cancer cell lines. All these results suggest that the BCPV-based nanoparticle formulation has a bright prospect as a non-viral vector for clinical cancer gene therapy.

3.2 Experimental Methods

3.2.1 Synthesis of BCPVs and characterization of polyplexes

As for the synthesis of BCPVs, allyl functional lactide monomers were first synthesized using the process previously reported by our group (**Figure 3.1**) [13, 176]. Subsequently, **ALA** (720 mg; 5 mmol) and lactide monomers (850 mg; 5 mmol) were introduced into a 10 mL polymerization flask, and treated with vacuum and nitrogen purge for three cycles. After the treatment, 10 mL dry dichloromethane was added into the flask and heated up to 35°C. Until the temperature was 35°C, the initiator benzyl alcohol (10.8 mg; 0.1mmol) and catalyst 4-(Dimethylamino) pyridine (48.9 mg; 0.4mmol) were added into the flask with a mild stirring. The

polymerization was conducted for 3 weeks to have a comonomer conversion of ~90%. The resulting polymer was precipitated in 10 ml cold methanol in order to separate impurities and unreacted monomers, resulting in allyl functional polylactides (APLAs). APLAs were further characterized by $^1\text{H-NMR}$ (500 MHz, CDCl_3), and their M_n^{NMR} was obtained to be 19.3 kDa. After the synthesis of APLAs, the allyl functionality on the APLA was partially converted into tertiary amines by thiol-ene reaction to prepare BCPVs. First, in a 10 mL flask, APLA (200 mg), 2-(Diethylamino)ethanethiol hydrochloride (DEAET, 54 mg) and the initiator 2,2'-Dimethoxy-2-phenylacetophenone (DMPA, 32 mg) were dissolved in 5 mL chloroform, resulting in a molar ratio of $[\text{ene of APLA}]_0:[\text{SH of DEAET}]_0:[\text{DMPA}]_0 = 1:0.5:0.2$. After removing the remaining oxygen, the solution was exposed to UV ($\lambda_{\text{max}} = 365 \text{ nm}$) for 30 min in order to conduct thiol-ene reaction. After the reaction, the resulting polymer was treated by dialysis (against 200 ml acetone) to remove any impurities. In the dialysis process, the solution is changed every 24 hours for 5 consecutive days. Following dialysis, the solution was completely dried by vacuum to give BCPVs at 90% yield. After that, the BCPV is in powder form, the mass was confirmed by weighing directly. Then, BCPVs were analyzed through $^1\text{H-NMR}$, and their charge density relative to the PLA backbone was calculated to be 26% by the $^1\text{H-NMR}$ results. The degradation property of BCPVs in physiologically mimicking media such as PBS buffer has been evaluated before by our collaborators [176]. The results showed that BCPVs degraded into small molecules (M.W. smaller than 1 kDa) in one week in various physiologically mimicking media at 37°C . This demonstrated that BCPVs exhibited great biodegradability, thereby hugely benefiting and broadening their biomedical applications.

3.2.2 Cell cytotoxicity on different cancer cell lines

The cell lines used were MiaPaCa-2 (pancreatic cancer cell line), A549 (lung cancer), HepG2 (liver hepatocellular carcinoma), and HeLa cells (cervical cancer), obtained from American Type Culture Collection, which were used in this study. The cells were maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM, Hyclone), supplemented with 10%

fetal bovine serum (FBS, Hyclone), 100 units/mL penicillin (Gibco) and 100 µg/mL streptomycin (Gibco). The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂, and maintained as described previously [12]. Cell viability was measured by the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay kit as previously described [12]. The MTT assay is a colorimetric assay for assessing cell cytotoxicity against biomaterials or drugs. Under defined conditions, NAD(P)H-dependent cellular oxidoreductase enzymes can reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple color in the dimethylsulfoxide (DMSO) solution[177]. Briefly, in each assay, 5×10³ cells were dispensed into each well of a 96-well flat-bottom Microtiter plate and cultured overnight. Eight sets were treated with different concentrations of BCPVs and one set was treated with DI water as the non-treated control. The cells were subsequently incubated for 24 h or 48 h before the assay. To perform the assay, 20 µL of MTT reagent was added to each well and incubated for another four hours. 150 µL of dimethylsulfoxide (DMSO, Sigma) was then added to dissolve the precipitate with 5 min gentle shaking. Absorbance was then measured with a microplate reader (Bio-Rad) at the wavelength of 490 nm. The cell viability was obtained by normalizing the absorbance (Abs) of the sample against the control well and expressed as percentage, assigning the viability of non-treated cells as 100%. Assays were performed in triplicate and the results were averaged.

3.2.3 The formation of BCPV-siRNA polyplexes

Nanoplexes of siRNA and BCPVs were prepared by mixing custom-synthesized negative control siRNA^{FAM} (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') or K-ras siRNA(sense: 5'-FAM-GUUGGAGCUUGUGGCGUAGUU-3'; Antisense: 5'-CUACGCCACAAGCUCCAACUU-3') with BCPV (1mg/ml) at different weight ratios of BCPV:siRNA (0.5:1, 1:1, 2:1, 4:1, 8:1) followed by gentle vortexing for 10 s. The solutions were left undisturbed for 30 min to enable the formation of stable BCPV-siRNA nanoplexes. The hydrodynamic size and zeta-potential of BCPV/siRNA polyplexes with different weight ratios were measured with Brookhaven 90Plus Particle Size

Analyzer. The structure of the BCPV-siRNA nanoplexes was observed under JEM-2010 Transmission Electron Microscope (TEM) (JEOL USA, Inc., Peabody, MA, US). Meanwhile, the formation of stable complexation was verified by measurements of hydrodynamic diameters and zeta potential (surface potential) of BCPV-siRNA nanoplexes in ultrapure DI water at 25°C using a 90-Plus particle size analyzer (Brookhaven Instrument Corp., Holtsville, NY, USA).

3.2.4 Gel retarding assay

To verify the effective encapsulation of the siRNA with BCPVs and determine the optimum dosage of BCPVs required to fully interact with siRNA, agarose gel electrophoresis was performed. After 20 minutes of incubation at room temperature, the BCPV-siRNA nanoplexes with different weight ratios were loaded on a 1.2% agarose gel and electrophoresed at 100V for 15 minutes. Gels were observed under UV transilluminator (Bio-Rad).

3.2.5 Serum enzymatic protection assay

According to the previous reports [15, 35], free siRNA and BCPV-siRNA nanoplexes at a 8:1 optimized weight ratio (8:1) were treated with FBS at a final concentration of 50% in PBS for a specified time (0, 0.5, 1, 1.5, 2, 2.5, 3, 6, 12, 24, 48, and 72 h) at 25°C for evaluating the stability of siRNA. The sample was mixed with 200 g/ml proteinase K solution and stored at -80°C to stop the enzymatic reaction. In order to extract siRNA from the nanoplexes, the samples were mixed with 1 wt% SDS (sodium dodecyl sulfate) for 3 minutes, then the 6× loading buffer was added and immediately loaded on a 1.2% agarose gel followed by electrophoresis at 110V for 10 minutes. The gel was viewed under the UV chamber.

3.2.6 Cell transfection

As cancer cells model, the MiaPaCa-2 cells (1×10^4) were seeded onto 6-well plates in DMEM medium without antibiotics to give 30%-50% confluence at the time of transfections. 20 μ l BCPV (1mg/ml) dispersion was mixed with siRNA^{FAM} at different weight ratios with gentle vortex and incubated for 20 minutes. Before transfection, the culture medium was replaced with OPTI-MEM (Invitrogen), and the above mentioned BCPV-siRNA^{FAM} mixture was then added to

the 6-well plate wells and the cells were continuously cultured for four hours. After that, the cells from each treated group were prepared for imaging using an inverted fluorescence microscope and for measuring the transfection efficiency by flow cytometry. The 500 μ L DMEM medium with 30% FBS were added in the dishes with the remaining cells of each group. Free siRNA^{FAM} was also used in another parallel experiment at the same dosage level. A commercial transfection reagent OligofectamineTM (Invitrogen) coupled siRNA^{FAM} was used as positive control. The gene expression was monitored at 48 hours post-transfection. For transfection efficiency examination, fluorescence imaging was performed at 4 hours post-treatment.

3.2.7 Transfection efficiency

In vitro fluorescence microscopy images were obtained using an inverted fluorescence microscope (Eclipse-Ti, Nikon). After 4 h of incubation, the transfected cells were washed twice with 1ml PBS, and fixed with 4% formaldehyde. The cells were incubated with DAPI (4',6-Diamidino-2-Phenylindole, Sigma) for nuclear counterstaining before performing the imaging experiments. To image the cells, filter sets for DAPI (excitation at 405 nm, and emission was collected with a band pass filter 525/50 nm (the peak wavelength at 450 nm and full width at half maximum (FWHM) of 50 nm) and FITC (excited with 488nm 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. For the flow cytometry experiments, the cells were washed twice with 1ml PBS and harvested by 500 μ l trypsin. 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).

3.2.8 Semi-Quantitative PCR

48 hours after transfection, total RNA was extracted from MIAPaCa-2 cells using an E.Z.N.AtM Total RNA Kit (OMEGA) and the amount was quantitated by a spectrophotometer (Nano-Drop ND-1000). Reverse transcription was conducted in a 20 μ l reaction mixture and

cDNA was synthesized from 2 µg of total RNA using the reverse transcriptase kit (Promega) as described in a previous report [178]. The K-ras relative mRNA expression level was determined using semi-quantitative 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 a previous report [12], 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 1.2% ethidium bromide-stained agarose gel and observed under a UV transilluminator (Bio-Rad).

3.2.9 Cell proliferation, apoptosis, migration and invasion Assays

The effect of BCPV-siRNA on the MiaPaCa-2 cell proliferation was detected by MTT assay as 1.3 sector described above. A wound-healing assay was conducted to examine the capacity of cell migration, as described previously [179, 180]. Briefly, MiaPaCa-2 cells (total cell number is 1×10^4) were seeded into each well of 24-well plates, then transfected with BCPV-siRNA (weight ratio is 8:1) for 48h, in which the cells reached almost confluent. The wound was generated by scratching the surface of the plates with a sterile middle pipette tip. Then, the cells were incubated in a complete culture medium and photographed with a Nikon Eclipse-Ti microscope. Cellular migration toward the scratched area was respectively assessed at 0, 24, 48, and 72 h (0 h is time of scratching line). The distances of migration from the cellular monolayer to the wounded area during these time points were measured by NIS Elements Br Analysis 4.0 software. The invasive capacity of MiaPaCa-2 cells treated with siRNA transfection or after different treatments were tested by using 8 µm BD BioCoat Matrigel Assay System invasion chambers (BD Biosciences), according to the manufacturer's protocol with minor modifications. Briefly, MiaPaCa-2 cells were transfected with BCPV-siRNA for 48 h as described above. Furthermore, transfected MiaPaCa-2 cells (5×10^4) with free FBS medium were seeded into the upper chamber of the system. Bottom chamber in the system were filled with 10% FBS medium

as a chemoattractant. After 48 h 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 buffered saline 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 10^6 MiaPaCa-2 cells were seeded in a six-well plate. Then, the cells were treated with the BCPV-siRNA (the weight ratio is 8:1) for 72 hours. The cells were then collected and washed twice with 1 ml cold PBS and suspended in 500 μ l Binding Buffer. Subsequently, the externalization of phosphatidylserine was measured as FITC labeled AnnexinV. The cell nucleus was stained with PI. The stained cells were analyzed by flow cytometry to determine the apoptotic cells.

3.2.10 Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA). All statistical calculations were performed with the SPSS 11.0 software package. Data are presented as mean $\pm$ SD. When two groups were compared, a *p*-value less than 0.01 or 0.05 is regarded as statistically significant.

3.3 Results and discussion

Nanomaterials have been a powerful tool to overcome many biomedical obstacles due to their unique physical and chemical properties. In order to overcome the challenge that naked siRNA is easily degraded and cannot penetrate the cell membrane by itself, we developed a novel siRNA delivery system based on biodegradable charged polyester-based vector (BCPV). The BCPV with positive charge was synthesized by ring-opening polymerization (ROP) and thiol-ene click functionalization using BnOH as the initiator and DMAP as the organocatalyst. The ROP process and thiol-ene click reaction were chosen because these processes can be operated under mild conditions, without using toxic metal-based chemical agents. For inducing the “proton sponge” effect, a tertiary amine group was selected as the cationic group for the BCPV, and

DEAET was chosen as the thiol functionalization agent. The tertiary amine functionalities were introduced on a well-defined APLA using a thiol–ene click reaction. BCPV denoting 28 % of amine group grafted to the PLA backbone was synthesized. The schematic drawing of the BCPV chemical structure is shown in **Fig 3.1a**. ^1H NMR analysis was performed to characterize the BCPV composition (**Figure 3.1b**). Its molar fraction of ALA was 22%, and that of the tertiary amine-based unit was 28% as resulted from comparison of the resonance intensities of allyl CH protons ($\text{CH} = \text{CH}_2$) of the monomer unit of ALA (at 5.77–5.79 ppm) and the methyl protons of the tertiary amine-based unit (at 1.21–1.43 ppm). By comparing the resonance intensities of the CH protons from the comonomer units and the allyl CH_2 protons ($\text{CH} = \text{CH}_2$) from monomer unit of ALA at 5.14–5.23 ppm with the resonance intensities of the C_6H_5 protons from BnOH at 7.35 ppm, the number-average degree of polymerization (DP_n) of 95 was calculated and acquired.

Figure 3.1 (a) Synthesis of BCPV via ROP (ring-open polymerization) and thiol–ene functionalization. (b) The chemical structure of the BCPV was confirmed from the NMR spectra (500 MHz ^1H NMR spectra of BCPV in CDCl_3).

In our previous report [176], we have demonstrated that the degradation of BCPV happened at the main chain cleavage. The cleavage not only was induced by the hydrolysable ester bonds, but also caused by the nucleophilic attack of the tertiary amines. The synthesized BCPV is a degradable vector with high surface charge ($+62.68 \pm 9.46$ mV) attributed to the presence of tertiary amine groups grafted to the polylactide backbone. Before forming the nanoplexes with siRNAs, the hydrodynamic diameter of BCPV was measured to be 13.64 ± 4.96 nm. Prior to further applications, the intrinsic cytotoxicity of BCPVs was evaluated by an MTT assay. As shown in **Fig. 3.2**, BCPVs showed no significant cytotoxicity after 48 h of incubation on four different cancer cell lines, namely a pancreatic cancer line (MiaPaCa-2), a lung cancer line (A549), a hepatocellular carcinoma line (HepG2), and a cervical cell line (HeLa cell), even at a dose as high as 256 $\mu\text{g/mL}$. This result indicates that BCPVs exhibit negligible cytotoxicity for various cell lines, and thus, they can be used in large amounts as siRNA delivery carriers.

For efficient delivery, the ratio between the siRNAs and the carriers is an important parameter that uses to be optimized. To investigate the influence of the BCPV:siRNA weight ratio on the formation of BCPV-siRNA nanocomplexes, BCPVs and siRNA were mixed at seven

Figure 3.2 Cytotoxic effect of BCPVs on four cancer cell lines. The cells were treated with different concentrations of BCPVs, and the intrinsic cytotoxicity was evaluated by MTT assay 48 h post treatment with BCPVs. Results are represented as means $\pm$ SD, n = 5.

different BCPV:siRNA weight ratios, where the weight of siRNA molecules is fixed at 1.25 μg . The hydrodynamic size and zeta potential of the seven conjugates were monitored. As shown in **Fig. 3.3a** and **3.3c**, the size of BCPVs alone was 7 ± 3.5 nm, while the average hydrodynamic diameter of BCPV-siRNA nanocomplexes increased with increasing BCPV:siRNA weight ratio. **Figure 3.3b** shows that the zeta potential of BCPV-siRNA nanocomplexes decreased with decreasing BCPV:siRNA weight ratio due to the negatively charged nature of siRNA. The change in the zeta potential is more obvious. A clear drop in the zeta potential of the positively charged BCPV is identified upon loading of siRNAs. As the ratio of siRNAs gets higher, a constant decrease in the zeta potential is observed due to higher loading ratio of the negatively charged siRNAs. These results indicate that the zeta potential and size of BCPV-siRNA nanocomplexes can be effectively tuned by altering the BCPV:siRNA weight ratio.

Figure 3.3 Hydrodynamic size and surface charge changes of the BCPV-siRNA nanoplexes. (a) The hydrodynamic size distribution of BCPV-siRNA nanocomplexes at different weight ratios as measured by DLS. The apparent change in the polydisperse hydrodynamic diameter of BCPVs alone and BCPV-siRNA nanocomplexes at different mass ratios was measured by a 90-Plus particle size analyzer, confirmed the formation of stable nanocomplexes. **(b)** Zeta potential changes of BCPVs loaded with different amounts of siRNA. The anionic DNA was encapsulated by cationic BCPV polymers through electrostatic interactions. **(c)** The size distribution of BCPV/siRNA polyplexes at different weight ratios.

In the gel retarding experiment, five different weight ratios of BCPV:siRNA (1:1, 2:1, 4:1, 8:1 and 16:1) were evaluated, which the weight of siRNA fixed at 1.25 μ g. The conjugation of siRNAs to the BCPV was identified by gel electrophoresis. Equal amount of siRNAs were mixed with BCPV at different weight ratios and loaded into a gel electrophoresis after this process, as shown in **Fig. 3.4**, the bright color associated with the ethidium bromide indicates that the siRNAs conjugated with BCPV were retained in the loading wells while the un-bounded siRNAs migrated to the opposite electrode. By comparison in the pure siRNA channel decreasing fluorescent intensities could be observed as the ratio of BCPV gets increased. When ethidium bromide is bound to a nucleic acid, its intensity will be amplified. In the cases of high BCPV:siRNA ratios, the excess amount of BCPV “buried” the siRNA and impeded the contact to ethidium bromide, which resulted in decreasing intensities. Nevertheless, this some result can also serve as a clear

Figure 3.4 (a) Agarose gel electrophoresis of BCPV/siRNA nanoplexes using Oligo-siRNA as a positive control group. As the concentration of BCPV was increased (up to 16:1 ratio of BCPV-siRNA), the stained intensity turned less bright and eventually disappeared, indicating that most of the siRNA macromolecules were encapsulated by BCPVs. **(b)** TEM image of BCPV-siRNA nanoplexes at 8:1 weight ratio. The capsule-like structures were assembled as the negatively charged siRNA was encapsulated with positively charged BCPV molecule to form stable polyplexes through electrostatic interaction.

indication of the siRNA-BCPV conjugation. Additionally, we found that the BCPV formulation is able to combine most of the siRNA when the BCPV-siRNA weight ratio is above 8:1. At 8:1 feeding ratio, the BCPV weight was estimated to be 10.25 μg , and that of siRNA was 1.25 μg . Based on these values, the drug loading content was calculated and resulted to be about 11.1 wt% at a 8:1 feeding ratio. The dynamic light scattering (DLS) results (**Fig. 3.3**) show that the BCPV-siRNA nanocomplexes at a BCPV-siRNA weight ratio of 8:1 had an average diameter of 104 ± 15 nm and a zeta potential of 40 ± 5 mV. The successful formation of BCPV-siRNA nanocomplexes at the 8:1 weight ratio was also verified by TEM, which revealed BCPV-siRNA nanocomplexes with a nanocapsule morphology and a particle size of 100 nm, which is fairly comparable to that measured by DLS (**Fig. 3.3b**).

Figure 3.5 Fluorescence microscopy images (20 $\times$ magnification) of MiaPaCa-2 cells treated with different materials. Cell nucleus are stained with DAPI and rendered in blue, and the fluorescent signal from the FAM labeled siRNA is rendered in green.

After characterization, BCPV-siRNA complexes of different weight ratios were used for cellular transfection, with the weight of siRNA fixed at 2.5 μ g. **Fig 3.5** shows the color coded fluorescence images of MiaPaCa-2 cells treated with different formulations at 4 hour post treatment. The siRNAs were labeled with fluorescent FAM and the signals were rendered in green while the cell nuclei were stained with DAPI and color coded in blue. To obtain a positive control, a commercialized siRNA transfection reagent Oligofectamine™ was introduced (Oligo-siRNA). As shown in **Fig. 3.5**, no FAM signal was observed in the cells treated with free siRNAs of same dosage and BCPV alone. For comparison, bright FAM signals could be observed in the cells treated with BCPV-siRNA formulations of different weight ratios from 2:1 to 16:1 (**Fig 3.5e-h**). This demonstrated that BCPV has successfully delivered siRNAs into the cells without rapid degradation. A closer examination could find slight differences on the fluorescence intensities in the BCPV-siRNA groups of various weight ratios. As the weight ratios of BCPV-siRNA changed from 2:1 to 8:1, the brightness of fluorescence gradually became stronger. When the weight ratio increases to 8:1, comparable fluorescence intensities could be found between the BCPV-siRNA formulation and the positive control of Oligo-siRNA.

To further quantify the transfection efficiency of siRNAs by BCPV, flow cytometry analysis was used and the results are shown in **Fig 3.6**. For all the treatment groups, a reference gate was chosen to identify the normal cell events (**Fig 3.6a**). Based on the intensity of the fluorescent signals, all the gated events were divided into two populations of M1 and M2 (**Fig 3.6b-h**): M1 cells have low fluorescent signals from autofluorescence while M2 cells express strong fluorescence from the FAM label. The transfection efficiency was defined as the fraction of M2 which is normalized by the total gated events. **Fig 3.6i** summarizes the statistical data of the results obtained from flow cytometry. It shows that the cells treated with free BCPV ($1.05 \pm 0.73\%$) and naked siRNA^{FAM} ($2.14 \pm 0.63\%$) have negligible fluorescent signals which are similar to those of the blank control ($0.35 \pm 0.01\%$). For comparison, the cells treated with BCPV-siRNA^{FAM} have shown remarkably high transfection efficiencies of $96.69 \pm 1.16\%$, $96.52 \pm 1.40\%$, and $96.15 \pm 2.19\%$, for weight ratio of 4:1, 8:1 and 16:1, respectively, which are comparable with

the positive control group Oligo-siRNA ($84.95 \pm 3.90\%$). In addition to the transfection efficiency, the average fluorescent intensity was also recorded, which indicates the averaged siRNAs delivery efficiency to cells. The result shows that although different weight ratios of BCPV share similar transfection efficiency with the positive control, the averaged siRNAs delivery efficiency varies for different BCPVs ratios. Since the siRNA loading capacity per BCPV is limited, there were

Figure 3.6 Transfection efficiency of MiaPaCa-2 cells evaluated by flow cytometry. (a) Dot plot of cells. The cells in the R1 region were selected for evaluating the transfection efficiency. (b)~(h) Statistical counts of cells treated with PBS, BCPV, siRNA^{FAM}, Oligo-siRNA^{FAM}, and BCPV/siRNA^{FAM} at different weight ratio of 4:1, 8:1 and 16:1, respectively. Accordingly, the concentrations of BCPV are estimated to be 10, 20 and 40 $\mu\text{g/ml}$ where the weight ratios of BCPV-siRNA complexes are determined to be 4:1, 8:1 and 16:1 respectively. (i) Quantitative evaluation of the results from (b)~(h) showing the average fluorescent intensity and transfection efficiency of each group. The average fluorescence intensity represents the FAM intensity from the labelled 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 means $\pm$ SD, n=3 (*: P<0.01 compared to Blank group, #: P<0.01 compared to the Oligo-siRNA group).

excess amounts of free siRNAs in the 4:1 group left unconjugated, which eventually were dissipated during the transfection process. Increasing the weight ratio of BCPVs led to a higher concentration of the effective transfection complex BCPV-siRNA. As a result, the 8:1 group shows a higher average fluorescent intensity than the 4:1 group. However, further increase of the BCPVs weight ratio results in an insufficient siRNA loading per nanoparticle and consequently there is no significant difference in the overall average fluorescent intensity between the 8:1 and 16:1 groups.

Figure 3.7 BCPVs protection of siRNA from serum degradation. (a) Residual siRNA after contact with 50% human serum after specified periods of time; conducted with free siRNA, or siRNA loaded on BCPV, all at room temperature with shaking, and (b) the corresponding siRNA quantification using ImageJ software. The ability of BCPV nanoplexes to protect siRNA from nuclease degradation by RNase enzymes was analyzed by measuring the remaining amount of siRNA after the incubation of the BCPV-siRNA nanoplexes in FBS (50% in PBS buffer) containing digestive RNase for 0-48 h at room temperature. Sodium dodecyl sulfate was further used to isolate the remaining siRNA after the incubation, followed by electrophoresis on a 1.2% agarose gel to observe the fluorescence intensity of the remaining siRNA. As in the previous experiments with BCPV-siRNA nanoplexes, free siRNA was added using the same test procedures.

In order to assess the stability of BCPV-siRNA nanoplexes against nucleases, free siRNA and BCPV-siRNA at 8:1 weight ratios nanoplexes were incubated with 50% FBS up to 48 h at room temperature. As shown in **Fig 3.7a**, intact siRNA that survived the ribonuclease (RNase) degradation could be identified by gel electrophoresis, and quantified based on its fluorescence

intensity by image processing with the ImageJ software. In the absence of BCPVs, naked siRNA was clearly degraded after 12 h and its half-life was approximately 18 h, indicating the instability of naked siRNA against nuclease. On the contrary, there was no significant degradation of siRNA in the BCPV-siRNA nanoplexes even after 48 h incubation, indicating that the siRNA was protected by BCPVs, and remained stable for longer than 48 h (**Fig. 3.7b**).

Figure 3.8. Gene expression of K-ras in MiaPaCa-2 cells of different treatment groups. (a) Agarose gel electrophoresis of K-ras relative expression. (b) Semi-quantitative relative gene expression of K-ras after siRNA transfection of different treatment groups. The quantified gene expression level based on its fluorescence intensity by image processing with the ImageJ software. The relative values shown are means $\pm$ SD, n=3 (*, P<0.01 compared to Blank group. #, P<0.01 compared to the Oligo-siRNA group)

To verify the knockdown efficiency of different formulations, the expression of mutated K-ras gene was evaluated at the mRNA level through PCR. Using the housekeeping gene GAPDH as reference, the mRNA levels of the mutant K-ras gene from different treatment groups were extracted and normalized to the control group. As shown in **Figure 3.8**, the relative gene

expression of mutant K-ras of the BCPV treated group and the free siRNA treated group share no obvious differences with the blank control. In contrast, the BCPV-siRNA treated groups have shown significant inhibition of K-ras gene transcription. Specifically, the groups with weight ratios of 8:1 and 16:1 have shown similar knockdown efficiencies of $44.24 \pm 2.01\%$ and $47.85 \pm 6.11\%$, respectively, comparable with the Oligo-siRNA treated positive control group ($46.67 \pm 6.23\%$), while the 4:1 weight ratio group has a slightly lower knockdown efficiency of $29.56 \pm 6.16\%$. This is because there were also unconjugated free siRNA in the 4:1 weight ratio group as mentioned earlier. According to these results, a BCPV:siRNA weight ratio of 8:1 can significantly inhibit the mutant K-ras gene expression in MiaPaCa-2 cells and this ratio was used in the following experiments.

The monomeric ras family G proteins act as “molecular switches” linking extracellular signals to intracellular signals through membrane receptors. As GTPases, they cycle from a GDP-bound “off” state to a GTP-bound “on” state in response to the activation from various receptors. Activated ras targets a number of downstream effectors including Raf kinase, Phosphoinositide 3'-kinase (PI3-K) and RalGEFs to produce pleiotropic cellular effects that affect cell growth, differentiation and survival[181]. The point mutation at codon 12 in the K-ras gene reduces the intrinsic GTPase activity of the protein and lock the protein to the GTP-bound “on” state, which leads to constitutive activation and eventually cancer progression [182, 183]. The inhibition of the mutant K-ras expression has been reported to promote cell apoptosis [172, 174, 184, 185] and also to inhibit cell proliferation [186, 187], migration [180] and invasion [175]. In this study, the cellular activities of MiaPaCa-2 cells were systematically evaluated after the mutant K-ras gene transfection by BCPV-siRNA complexes.

The **Fig 3.9** shows that the proliferation of MiaPaCa-2 cells after treating with different formulations. The BCPVs as well as the free siRNA treated groups have shown similar trends with the blank control group where the cell population doubled after 24 hours and proliferated four fold after 72 hours. In contrast, after the treatment with BCPV-siRNA, a significant inhibition in the cell proliferation could be observed. The proliferation rate has been greatly altered by the

successful delivery of siRNAs. In addition to the cell proliferation evaluation, the migration of the cells after treatments was also assessed. As shown in **Fig 3.10a**, a scratch wound in the monolayer cell culture of MiaPaCa-2 cells was generated for each of the different treatment groups. In the non-treated blank group, a decrease in the window width could be clearly observed after 24 hours. At 48 hours post treatment, the cells started to migrate to the open space in between the wound margins as the window closed further and the previous

Figure 3.9 Proliferation of MiaPaCa-2 cells after treatment with different formulations. Cells were first treated with BCPV, siRNA, BCPV-siRNA (the weight ratio is at 8:1) and Oligo-siRNA, then continued to culture for 24h, 48h, and 72h. Cell viability was determined using the MTT cell viability assay. Results are represented as means $\pm$ SD, n=3.

-ly straight wound boundaries became cluttered. After 72 hours, the wound had almost completely healed, and the boundaries were hardly identified. Similar observations could be found in the BCPVs and free siRNA treated groups. For comparison, a significantly suppressed healing process was observed in the cells treated with the BCPV-siRNA complex or Oligo-siRNA. The decrease of the window width was much slower than those in the other groups. More importantly, it took the cells 72 h to migrate to the open space in-between the wound boundaries. It is 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 cell-cell contacts and an increased concentration of growth

factors [188, 189]. It is a commonly used method to study the cell migration and the underlying biology [190, 191]. As shown in **Figure 3.10b**, the healing process of each group was further quantified and expressed as the normalized percentage of the wound closure. It clearly shows the difference between the BCPV-siRNA treated group and the other three groups, where the wound in the BCPV-siRNA treated group closed at almost half the speed of the other three groups. These results indicate that the gene expression regulation of mutant K-ras in MiaPaCa-2 cells mediated

Figure 3.10 Wound healing assay in MiaPaCa-2 cell culture treated with different formulations. (A) Phase contrast microscope images (10×magnification) of the wound healing process monitored for 72 hours post treatment. (B) Quantitative evaluation on the percentage of window close after treatment. The values were normalized to the initial wound window width.

by the BCPV-siRNA complex have successfully changed the expression pattern of the downstream gene associated with the cell proliferation and migration. Moreover, there is no significant difference among the BCPV-siRNA and Oligo-siRNA group. Cell migration is a complex process which can be viewed as a process regulated matrix-degrading proteinases, integrins and other cell adhesion molecules [192]. It is a critical event in cancer progression and especially metastasis [193]. The inhibition of the cell migration by silencing the mutant K-ras gene in MiaPaCa-2 cells has provided a potential therapeutic way for the treatment of pancreatic cancer.

Figure 3.11 Cell invasion assay based on a transwell chamber model of MiaPaCa cells after treatment with different formulations. (a) Bright field microscope images (10×magnification) of cells that penetrated through the Matrigel-coated membrane in the transwell chamber. Cells were stained with trypan blue. (b) Quantitative cell counts on the cells penetrated through the Matrigel-coated membrane. Each bar represents the average for the results from five random microscopic fields under 20× magnification. Error bars indicate SD, *P<0.01 compared with Blank group, n=5.

Metastatic tumors spreading to different distant organs are the primary cause of death in pancreatic cancer. Metastasis is a complex and multi-step process which starts with the migration and invasion of primary tumor cells to the adjacent tissue [194, 195]. As a result, the ability to block the migratory and invasive capacity of cancer cells is a main approach to treat patients with malignant cancer [196, 197]. To assess the change in the invasion capacity of MiaPaCa-2 cells treated by different formulations, an *in vitro* Matrigel invasion chambers assay was conducted [198, 199]. The main component laminin and type IV collagen of Matrigel has provided it the structural and biochemical properties of basement membranes[200]. The capability of cells penetrating through the membrane is a good measurement of their invasiveness and metastatic potential [201]. As shown in **Figure 3.11**, the population of penetrated cells from the BCPV-siRNA and Oligo-siRNA treated group is significantly smaller than those of the other three control groups. In the quantitative measurement, the normalized data show that the cells able to penetrate through the matrigel in the BCPV-siRNA treated group is over 60% less than these in the blank control group. These results clearly suggest that the knockdown of K-ras by BCPV-siRNA, but not free siRNAs, could retard the invasion and subsequently the metastasis of pancreatic cancer cells.

Finally, the apoptosis of MiaPaCa-2 cells treated with different formulations were assessed by FITC labeled annexin V and propidium iodide (PI) staining. Annexin V has a high affinity for the membrane phospholipid phosphatidylserine (PS), which is exposed to the external cellular environment in apoptotic cells. It has been widely used as an early apoptosis indicator. PI is a vital dye which will be excluded by viable cells with intact membranes. It is commonly used to label the dead and damaged cells. As shown in **Figure 3.12**, after 72 hours treatment, the FITC and PI positive apoptotic cell counts in the Q2 zone of the BCPV-siRNA treated group show a conspicuous difference with the other three groups. The quantitative data indicates no significant difference between the blank control group ($17.19 \pm 2.72\%$) and BCPV ($16.11 \pm 2.79\%$) or free siRNAs ($17.47 \pm 2.44\%$) treated groups, while the apoptotic cell population in the BCPV-siRNA

treated group is doubled ($37.52 \pm 3.15\%$). When compare to the Oligo-siRNA group ($32.67 \pm 4.11\%$), no statistically significant differences are observed for the BCPV-siRNA group.

To date, 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, the 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 demonstrated that the inactivation of the mutated K-ras gene by BCPV-siRNA leads to a significant inhibition of cellular proliferation, migration, invasion and anti-apoptosis in MiaPaCa-2 cells. To the best of our knowledge, this is

Figure 3.12 FITC Annexin V and Propidium Iodide (PI) co-stained apoptosis assay on 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 by flow cytometry. (b) Late apoptotic cell counts in the upper right quadrant (Q2) of different treatment groups. Error bar indicate SD, *P<0.01 compared with Blank group, n=5.

the first report that demonstrates the comprehensive effects of K-ras gene knockdown on pancreatic cancer cells mediated by biocompatible and biodegradable nanoparticle conjugated siRNAs. According to these results, the BCPV-siRNA delivery system is far-reaching in gene therapy for pancreatic cancer, which successfully overcomes some challenges in RNA interference and will serve as a promising candidate for gene therapy in pancreatic cancer treatment. Yet it is also worth noting that before entering into 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 tumor models also needs to be assessed.

3.4 Conclusion

We have demonstrated the use of a biocompatible and biodegradable cationic polymer-based nanoparticle formulation for the delivery of siRNAs targeting the mutant K-ras oncogene in pancreatic cancer cells. BCPVs are capable of preventing siRNA from degradation in the FBS-containing medium. We have shown that the expression of mutant K-ras gene was efficiently suppressed through fluorescent imaging and quantitative flow cytometry assessment. In addition, we have monitored the consequence of the K-ras inhibition in MiaPaCa-2 cells. Our results have shown that the RNA interference process has initiated the change in the expression pattern of the downstream gene that regulates the cell proliferation, migration and apoptosis. With an optimized weight ratio between the nanocarriers and siRNAs, we have significantly reduced the growth, migration, invasion of MiaPaCa-2 cells, and the apoptosis of the pancreatic cancer cells has also been promoted. Taken together, all these *in vitro* results have demonstrated the great potential of the BCPV in gene therapy of pancreatic cancer. We envision that it could serve as an excellent nanocarrier platform for genetic materials in clinical settings of gene therapy.

Chapter 4 Biodegradable polymer as a siRNA vector for gene knockdown of BCR-ABL hybrid oncogene in human leukemia cell line

First-line therapy of chronic myelogenous leukemia (CML) has always involved the use of BCR-ABL tyrosine-kinase inhibitors which is associated with an abnormal chromosome called Philadelphia chromosome. Although the overall survival rate has been improved by the current therapeutic regime, however, the presence of resistance has resulted in limited efficacy. In this study, RNA interference (RNAi)-based therapeutic regime is proposed with the aim to knockdown the BCR-ABL hybrid oncogene using small interfering RNA (siRNA). The siRNA transfection rates have usually been limited due to the declining contact probability among polyplexes and the non-adherent nature of leukemic cells. Our work aims at addressing this limitation by using biodegradable charged polyester-based vector (BCPV) as a nanocarrier for the delivery of BCR-ABL-specific siRNA to the suspension culture of K562 CML cell line. In this study, BCR-ABL siRNAs were encapsulated in the BCPVs by electrostatic force. We observed that BCPV successfully assisted *in vitro* siRNA transfection in the non-adherent K562 cells. As a consequence of downregulation of BCR-ABL, BCPV-siRNA nanoplexes inhibited cell proliferation and promoted cell apoptosis. More importantly, this class of non-viral vector exhibits biodegradable features and negligible cytotoxicity, thus providing a versatile platform to deliver siRNA to non-adherent leukemia cells with high transfection efficiency by effectively overcoming extra- and intra-cellular barriers.

4.1 Introduction

Leukemia is one of the most aggressive cancer diseases which is mostly caused by chromosomal translocations between chromosomes 9 and 22 that results in the fusion of BCR and ABL genes to form an oncogenic hybrid gene, BCR-ABL [202]. The BCR-ABL chimeric protein is known for its increased active tyrosine kinase activity which leads to oncogenic growth and reduction of apoptosis [203-205], thus promoting CML. As reported in literature, activated

tyrosine kinase activity plays an important role in abnormal proliferation of CML progenitors including increased proliferation in progenitor culture, reduced adhesion to fibronectin, and reduced chemotaxis to stroma-derived factor-1 α [206]. Several critical phenotypic abnormalities observed in primary CML cells, including larger myeloid colony-forming units and delayed apoptosis are mainly caused by the overexpression of the BCR-ABL oncoprotein [207]. A BCR-ABL inhibitor, imatinib mesylate (or STI 571), an orally administered drug, has been widely used for the suppression of BCR-ABL expressing cells and induction of apoptotic cell death. However, various clinical reports have highlighted the induced resistance of CML cells to STI 571 [208-212].

Due to increasing knowledge on molecular changes in CML, RNA interference (RNAi) technology is becoming a promising approach since it can specifically target the mutated gene. siRNA can induce sequence-specific silencing of target genes by complementary base pairing [10]. This new regime revolutionized the therapy strategies for combating various types of deadly diseases like cancers [213, 214]. Although lots of potential targets have been recognized for gene therapy in CML cells, a functional carrier is required for effective intracellular siRNA delivery as a result of the anionic siRNA can't traverse plasma membrane by itself. Electroporation is a traditional physical method for siRNA delivery on Leukemia cells, and it can reach 26% reduction of BCR-ABL gene expression on K-562 cell (Leukemia cell line). However, electroporation method caused high cell death and cannot be used *in vivo* [215]. Viral vectors have been developed for siRNA delivery of leukemic cells, but they have a potential bio-safety risk due to their capacity to react with the host's genome and induce immune toxicity [216]. In general, commercially available transfection agents and synthetic cationic polymers are safer alternative to viruses, such as Lipofectamine2000TM, TransMessenger, polyethylenimine (PEI), poly-L-lysine (PLL) and poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA) can result in a high transfection efficiency of about 70% in non-adherent leukemia cells [217-220]. The triblock copolymer polybutadiene-block-poly(methacrylic acid)-blockpoly(2-(dimethylamino)ethyl methacrylate) (BMAAD) that can form well-defined micelle structures has been reported to display superior

transfection capabilities for adherent cells and human leukemia cells [221]. Recently, SNALP-like lipids with different compositions have been explored for siRNA transfection in suspension cells, and the optimal composition of a SNALP-like lipid was determined based on its transfection results [222]. However, its long-term physiological toxicity and transfection efficiency need to be further assessed *in vivo*. Juliana *et al.* explored the efficacy of lipid-modified polyethylenimines (PEIs) for siRNA delivery on K562 cells as a CML model[223]. The structural modifications of lipid-substituted PEIs influenced the siRNA delivery efficiency. Palmitic acid (PA)-substituted 1.2 kDa PEI (~2 lipids/PEI) has proven to be highly efficient in delivery siRNA and silencing of the reporter gene green fluorescent protein (GFP) and BCR-ABL fusion oncoprotein. Moreover, when BCR-ABL protein was targeted in K562 cells, an induction of early and late stage apoptosis was detected. Cell penetrating peptides (CPPs) which stretch up to 30 amino acids long were used to deliver different carriers on various cells[224]. The cationic nature of CPPs has the ability to translocate cell membranes through electrostatically bind to the phosphate backbone of siRNA to form a stable complex in which siRNA can be protected from degradation. Arthanari *et al.* provided evidence that the fusion complex of the HIV-Tat (a kind of CPP) and the membrane lytic peptide (LK15), named as Tat-LK15, mediates high transfection efficiency in delivering siRNA targeting the BCR-ABL fusion oncoprotein in K562 cells[225]. Additionally, silencing of the BCR-ABL oncoprotein by Tat-LK15 is more efficient and longer lasting than that achieved using Lipofectamine. Although acceptable transfection efficiency was obtained on these synthetic delivery carrier, their long-term cytotoxicity, non-degradable properties, and lack of reactive sites for further chemical modifications are still major concerns for further investigations for clinical setting. Based on these reasons, biodegradable polymeric based carriers have been developed for siRNA delivery in suspension cells.

Degradable cationic polymers with insignificant cytotoxicity have presented far-reaching applications as gene carriers and were synthesized using step polymerization and living polymerization techniques. In this study, BCPVs, safe and degradable cationic polymers, were used as carriers of BCR-ABL siRNA for transfection into non-adherent BCR-ABL-expressing

myeloblastic cell-line (K562) as depicted in **Fig 4.1**. The transfection efficiency of BCR-ABL siRNA-loaded BCPVs, and cytotoxicity of BCPVs in non-adherent K562 cells were thoroughly investigated. The gene silencing effect of BCPVs, both in FBS-present and FBS-absent cell culture media have been presented and compared with Lipofectamine2000TM. The results indicated that the downregulation of BCR-ABL oncoproteins by RNAi can lead to decreased leukemic proliferation associated with enhanced cell-apoptotic effects in CML cells. These significant findings highlight the potential of BCPV as an appropriate siRNA delivery vector for leukemia cell line, and that BCPV may be further tailored as a novel drug formulation for siRNA-based leukemia treatment.

Figure 4.1 Overview of the BCPV-siRNA nanoplex formation and the ABL-BCR fusion gene knockdown by siRNA transfection of leukemia cells.

4.2 Experimental Methods

4.2.1 Synthesis and characterization of BCPV-siRNA nanoplexes

The BCPV was synthesized by ring-opening polymerization (ROP) of allyl functionalized lactide (LA) and L-LA monomers followed by a thiol-ene modification, as described in **3.2.1** section.

4.2.2 Cell culture and siRNA transfection

The human CML cell line, K562, obtained from American Type Culture Collection (ATCC), was grown in culture with Roswell Park Memorial Institute medium (RMPI, Hyclone), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin (Gibco) and 100 µg/ml streptomycin (Gibco). Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂, and maintained routinely. MiaPaCa-2, PC-3, SMMC7721, T47D and T98G cell lines were also purchased from ATCC, and maintained in DMEM culture medium. The other conditions for culturing these cells were the same as K562. A custom-synthesized BCR-ABL siRNA was obtained from Shanghai GenePharma Co., Ltd (Shanghai, P.R.China). The sense and antisense sequences were as following – sense: 5'-GCAGAGUUCAAAAGCCCTT-3'; antisense: 5'-GGGCUUUUGAACUCUGCTT-3', in accordance with a previous report [226]. Negative control siRNA (NC-siRNA, sense: 5'-UUCUCCGAACGUGUCACGUTT-3; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') was purchased from Sigma (All stars negative control, Sigma, UK). K562 cells (1×10^4) were seeded in 6-well plates in the RMPI medium without antibiotics (FBS) to give 30% - 50% confluence at the time of transfections. For experiments with high levels of FBS, the culture medium was supplemented with 30% FBS, with other culture conditions remaining the same as the normal culture process of K562 cells. The culture medium was replaced with OPTI-MEM (Invitrogen) prior to transfection. BCPV-siRNA nanoplexes were prepared (ratio 8:1) by mixing BCPVs with Cy3-labeled BCR-ABL-siRNA^{Cy3} (20 µl, 10 µM) or NC-siRNA^{Cy3} (20 µl, 10 µM), and incubated for 20 minutes. The assembled nanoplexes were then added to the 6-well plate wells and the cells were continuously cultured for eight hours. The commercial transfection reagent Lipofectamine2000™ (Invitrogen) coupled-siRNA^{Cy3} (Lip2000-siRNA^{Cy3}) was used as a positive control. Additionally, blank K562 cells (Blank) and cells transfected with free-siRNA^{Cy3} (siRNA^{Cy3}) were also used as controls. Subsequently, parts of cells were prepared for imaging under an inverted fluorescence microscope and flow cytometry for detection of transfection efficiency. Transfection efficiency examination and fluorescence

imaging were performed 8 hours post-treatment. The gene expression was monitored at 48 hours post-transfection.

4.2.3 Cytotoxicity studies

K562 cells were seeded at a number of 5×10^3 cells per well in 96-well plates and allowed to incubate for 48 h with different concentrations of BCPV. After incubation, cell viability was measured in terms of cell counts, which was observed under the Eclipse Ti-U Inverted Microscope System (Nikon, Japan) after 48 h under the BPCV environment. It should be highlighted here that the conventional MTT assay for evaluating the cell viability could not be used in our experiments, as the K562 CML cell line is essentially non-adherent, and it is not feasible to fix the cells on the bottom of the wells. As a result, the MTT assay cannot be used for observing absorbance and determining cell viability. Hence, the cell viability was obtained by normalizing the cell counts of the sample well against that from the control well and expressed as a percentage of non-treated cells (Blank).

4.2.4 siRNA transient transfection

For the visual inspection of the proportion of cells that may have succeeded in siRNA transfection, *in vitro* fluorescence microscopy images were obtained using an Eclipse Ti-U Inverted Microscope System (Nikon, Singapore). After 8 hours of incubation, the transfected cells were washed with PBS twice, and fixed with 4% formaldehyde. Prior to imaging, the cells were mixed with DAPI (Sigma) for nuclear counterstaining. Filter sets for DAPI (405/450 nm) and Cy3 (550/570 nm) were applied for cell imaging. (DAPI and Cy3 are fluorescent dyes that are abbreviated forms of (4',6-diamidino-2-phenylindole) and Cyanine3 respectively.) Quantitative determination of transfection efficiency of the K562 cells that were successfully transfected with BCR-ABL siRNA was performed using flow cytometry. The cells were washed twice with PBS and harvested by trypsinization. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Mississauga, CA), using Cy3 as the luminescent marker. Transfection efficiency was estimated in terms of Cy3-fluorescent intensity signal obtained from flow cytometry results, and a statistical comparison of different groups was performed subsequently.

4.2.5 Post-transfection gene expression analysis

After 48 hours of transfection, the BCR-ABL gene expression was detected by real-time RT-PCR in terms of mRNA levels. First, total RNA was extracted from K562 cells using TRIzol reagent (Invitrogen) and the amount was quantitated by a NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA). Next, 1 µg of RNA was reverse transcribed to cDNA in a 20 µl reaction mixture following the procedure described in a previous report [227] using the M-MLV reverse transcriptase kit (Promega). The cDNA products were then used as templates for subsequent real-time PCR with gene-specific primers to determine the relative BCR-ABL mRNA expression level. Specific forward and reverse primers' sequences were – BCR-ABL: 5'-CATTCCGCTGACCATCAATAAG-3'; 5'-GATGCTACTGGCCGCTGAAG-3', β-actin (housekeeping endogenous gene): 5'-CCACCCCACTTCTCTCTAAGGA-3' and 5'-GATGCTACTGGCCGCTGAAG-3', as mentioned previously by Valencia-Serna *et al.* [223] . Real-time RT-PCR was performed using SYBR Select Master Mix (Invitrogen), with the following cycling conditions: 94°C for 2 min, followed by 40 cycles of denaturation (95°C for 15 s) and an annealing/elongation step (60°C for 15 s). The difference in gene expression was estimated as $2^{-\Delta\Delta CT}$, and the gene expression of BCR-ABL was normalized against the internal control (β-actin).

4.2.6 Antitumor effects assessment

All the transfected cell groups, control samples, blank and free-siRNA formulations were incubated for 72 hours and counted under the microscope for estimation of cell proliferation. The effect of BCPV-siRNA transfection on the induction of K562 cell apoptosis was realized by Annexin V : FITC Apoptosis Detection Kit I (BD Pharmingen™, BD Biosciences, Singapore) according to the manufacturer. Briefly, approximately 1×10^6 K562 cells were seeded in a six-well plate. Then, the cells were incubated for 72 hours after treatment with the BCPV-siRNA (8:1) nanoplex and other formulations. The cells were then washed twice with cold PBS and resuspended in binding buffer. The onset of the apoptosis process enables the FITC-labeled

Annexin V to bind with phosphatidylserine (PS) as it externalizes from the inner boundary of the cell membrane. The cell nuclei were stained with propidium iodide (PI). The stained cells were analyzed by flow cytometry to determine the apoptotic cell count. Positive fluorescent intensity signals for both FITC and PI were representative of the total apoptotic cell count.

4.3 Results and Discussion

RNAi technology, initiated by a double-stranded siRNA molecule targeting a sequence-specific gene at the post-transcriptional level, has attracted great interest for leukemia treatment in the past decade [218, 228-230]. However, the application of RNAi for leukemia treatment faces bottlenecks because siRNA is easily degraded and cannot cross the cell membrane by itself. For these reasons, various nanoparticles have been developed as carriers to deliver siRNA into cells and only few of them showed promising results, primarily for adherent cell lines. There were rare reports that outlined desired siRNA transfection efficiency by nonviral transfection agents on the suspension cells, such as K562, a model cell line of human leukemia. A major underlying reason is the reduced contact probability between nanoplexes and cells due to 3D cultivation condition as compared to the adherent cells [231]. Hence, designing polymers that can efficiently deliver siRNA into suspension cells would accelerate the development of novel treatment for leukemia, and even for acquired immunodeficiency syndrome (AIDS). Recently, we successfully synthesized an outstanding polymer, BCPV, as a potential candidate for siRNA delivery in pancreatic cancer cells and demonstrated high transfection performance on these adhesive cells [14]. Following these studies, it would be worth exploring if BCPVs can overcome the challenges of siRNA transfection in suspension cells.

BCPVs, a class of cationic polylactides, are synthesized by ring-opening polymerization technique and thiol-ene click functionalization, where the mol fraction of charged moieties can be precisely controlled for facilitating the loading of interested siRNA cargos. BCPVs interact with negatively charged siRNA to form stable nanoplexes and the net positively charged-surface facilitates endocytosis of resulting nanoplexes by interacting with the negatively charged cell

membrane. The positive surface charge of BCPV enables siRNA to readily permeate the negatively charged cell membrane of targeted cells. The aforementioned information demonstrates that an optimum condition was obtained at the 8:1 weight ratio of BCPV-siRNA. TEM images further proved this assertion, revealing the spherical morphology of the assembled BCPV-siRNA nanoplexes. The capsule-like structure was assembled as the negatively charged siRNA was encapsulated with positively charged BCPV molecule to form stable polyplexes through electrostatic interaction. This encapsulation of BCPV-siRNA could provide continuous release of siRNA over longer time periods and prevent siRNA degradation from hydrolase [4].

Figure 4.2 BCPV as an effective transfection agent for adherent cells. Various types of adherent cancer cells, namely MiaPaCa-2 (pancreatic cancer), PC-3 (prostate cancer), SMMC7721 (hepatocellular carcinoma), T47D (ductal carcinoma), and T98G (glioblastoma multiforme) were transfected by BCPV-siRNA^{FAM}-siRNA^{Cy3}. The appearance of green (FAM), red (Cy3) and yellow colors (Overlay of FAM and Cy3) in the images obtained with an inverted fluorescence microscope (20×magnification) confirm the BCPV-assisted transfection of siRNA into these cell lines.

Figure 4.3 Cytotoxic effect of BCPV on K562 leukemic cells. The cells were treated with different concentrations of BCPV, and the intrinsic cytotoxicity was evaluated by counting cell numbers under a microscope post 48 hours treatment with BCPV. Blank cells were untreated. Results are represented as means $\pm$ SD, n=3.

Before challenging the suspension cells, we first investigated the capacity of BCPVs to deliver siRNA into adhesive cell lines. FAM- and Cy3-labeled siRNA were mixed with BCPV at 8:1 weight ratio (where the weight of the siRNAs was fixed at 2.5 µg) for 30 min, and were then cultured with various adherent cancerous cell lines (MiaPaCa-2, PC-3, SMMC7721, T47D and T98G) for 4 hours, following which they were observed under inverted fluorescence microscopy to assess the ratio of transfected cells. Substantial and strong green (FAM), red (Cy3) and yellow (overlay) fluorescence signals were observed, revealing the high transfection ratios of selected cells (**Fig 4.2**) [14, 232]. Although the BCPVs have presented very high efficiency for siRNA delivery in adherent cells, BCPV-assisted siRNA transection in non-adherent cells has not been reported to date. K562 CML is a suspension cell line and represents a unique source of malignant leukemic cells overexpressing the BCR-ABL oncogene due to t(9;22) chromosome aberrations [233]. Hence, this is a perfect model to demonstrate a successful gene therapy of CML by knockdown this fusion oncogene. Moreover, K562 CML cells are well known to be difficult-to-transfect with current available non-viral carriers [222, 228, 234, 235]. Aggressive methods such

as lentiviral vectors, lipid-based transfection reagents and electroporation have been implemented for the siRNA delivery in the K562 cells [228, 236, 237]. Herein, we propose BCPVs as nanocarriers for BCR-ABL siRNA delivery into K562 cells. Prior to the complexation of BCR-ABL with siRNA, innate cytotoxic effects of BCPVs on K562 cells were evaluated by counting the cell numbers under an inverted microscope (**Fig 4.3**). It was observed that more than 90% of cells numbers were viable over a wide dosage range (up to 80 $\mu\text{g/ml}$) 48 hours post treatment, and no significant difference was observed among these groups. In addition, BCPVs also showed negligible cytotoxicity even at dosages higher than 160 $\mu\text{g/ml}$. The results indicated that BCPVs are biocompatible and have insignificant cytotoxicity to K562 cells at a wide dosage range of BCPV.

Figure 4.4 BCPV-assisted transfection of Cy3-labelled siRNA into K562 cells were observed under inverted microscopic fluorescence (20 $\times$ magnification). The Blank and siRNA^{Cy3} group show only blue fluorescence, indicating the DAPI-stained cell nuclei without siRNA transfection. The BCPV-siRNA^{Cy3} and BCPV-siRNA^{Cy3}+FBS, exhibit both blue and red fluorescence indicating siRNA transfection (Cy3-stained) into the cells. The images are compared with the standard transfection agent Lipofectamine2000TM (Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS).

Based on the previous experience on adhesive cells, the K562 suspension cells were transfected with BCPV-siRNA^{Cy3} and other groups (Blank, free-siRNA^{Cy3}, BCPV-siRNA^{Cy3}, and Lip2000-siRNA^{Cy3}), and left for 8 h incubation. In the BCPV-siRNA group, the weight ratio was fixed at 8:1, with the siRNA weight being 2.5 µg. Because the total volume of one well was 1 ml, 20 µg/ml of BCPV was chosen as a safe loading concentration. On the other hand, when compared with the solid tumor treatment in which siRNA has to survive in the blood stream and go through the complicated extracellular matrix before it can reach the tumor tissue, the treatment of leukemia presents an obvious advantage as siRNA can be directly exposed to leukemia cells in the blood vessels by intravenous injections. For mimicking the blood stream conditions, the K562 cells were treated with BCPV in the culture medium with 10% FBS (the main component of serum), and the attempts were denoted as BCPV-siRNA^{Cy3}-FBS. To obtain a positive control, a commercialized siRNA transfection reagent Lipofectamine2000TM was used under the same condition of FBS (Lip2000-siRNA^{Cy3}-FBS). The fluorescence microscopic images showed a relatively bright Cy3 fluorescence (red color) of BCPV-siRNA^{Cy3} and BCPV-siRNA^{Cy3}-FBS in K562 cells, as compared with the positive control groups (**Fig 4.4**), indicating that BCPVs can successfully deliver siRNA into K562 cells, and prevent siRNA from rapid degradation by RNase present in FBS medium. The un-transfected cell group (“Blank”) displayed only blue fluorescence arising from the DAPI stained-cell nuclei.

To further quantify the transfection efficiency of the siRNAs by the BCPVs, flow cytometry was employed to determine transfection efficiency of BCPV- and control-assisted transfection in non-adherent K562 cells. Quantitative values regarding transfection in terms of the Cy3-fluorescent signal strength were obtained by flow cytometry assessments (**Fig 4.5a**). Extremely high transfection rates (**Fig 4.5b**) were obtained via BCPV-siRNA^{Cy3} ($96.7 \pm 3.13\%$), which was larger than that of Lip2000-siRNA^{Cy3} ($88.46 \pm 2.42\%$). Notably, there also was a significant difference in the average fluorescent intensity per cell count (**Fig 4.5c**) between the BCPV-siRNA^{Cy3} (>46000) and Lip2000-siRNA^{Cy3} (3400) groups. These results indicate the enhanced siRNA transfection efficiency of BCPV in non-adherent K562 cells. Additionally, the

transfection efficiency of BCPV-siRNA^{Cy3}+FBS ($97.8\pm2.55\%$) was very close to that of BCPV-siRNA^{Cy3} ($96.7\pm3.13\%$), implying that the transfection efficiency of BCPVs was not influenced by the antibiotics in the cell culture medium. In contrast to BCPVs, Lip2000-siRNA^{Cy3}+FBS exhibited a considerably decreased transfection efficiency ($52.67\pm0.83\%$) as compared with Lip2000-siRNA^{Cy3} ($88.46\pm2.42\%$), suggesting that Lipofectamine2000TM is quite sensitive to the serum components and thereby weakened its gene delivery functions.

Figure 4.5 Estimation of transfection efficiency in K562 cells by flow cytometry. (a) Cell groups treated with different formulations (siRNA^{Cy3}, BCPV, BCPV-siRNA^{Cy3}, BCPV-siRNA^{Cy3}+FBS, Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS) whose fluorescent signal intensities showed positive staining for Cy3-labeled siRNA were representative of siRNA transfection into the cells. The higher fluorescent signals for groups BCPV-siRNA^{Cy3} and BCPV-siRNA^{Cy3}+FBS indicate higher transfection efficiencies. Blank cells were untreated. (b) Statistics of transfection efficiency. The ratio of cells in P3 to the total P2+P3, yielded the transfection efficiency. (c) The average fluorescent signal intensities resulting from (a), depicting the performance of BCPV as an outstanding transfection agent for non-adherent leukemic cells. Results are represented as means $\pm$ SD, n=3; *, P<0.05; **, P<0.01.

Figure 4.6 Downregulation of BCR-ABL mRNA post siRNA transfection. The cell groups were treated with different formulations and the gene knockdown of the BCR-ABL oncogene was evaluated by real time RT-PCR assay 48h post treatment. BCPV-siRNA-treated cell group showed considerable gene suppression, down to 20%, compared to 51% in the case of commercial transfection agent, Lipofectamine2000™. These results correlate with the high transfection efficiency of BCPV-siRNA nanoplexes. Results are represented as means±SD, n=3; **, P<0.01.

The efficacy of cancer gene therapy through RNAi mechanism is generally assessed by the expression of the targeted mRNA. The BCR-ABL mRNA was targeted to be suppressed by the exogenously-mediated siRNA. In this study, the treatments of K562 cell line with BCPV-siRNA and other control groups (Blank, free siRNA^{Cy3}, BCPV, BCPV-siRNA^{Cy3}+FBS, Lip2000-siRNA^{Cy3} and Lip2000-siRNA^{Cy3}+FBS) were subjected to RT-PCR 48 hours post transfection for the quantitative determination of BCR-ABL mRNA levels. **Fig 4.6** shows that all the negative control groups including untransfected cells, BCPVs and free siRNA exhibit minimal effect on knocking down BCR-ABL mRNA expression (100±11.58%, 100.35±17.36% and 93.25±8.47%), suggesting that a potential vector is required for the uptake of therapeutic siRNA in K562 cells, and subsequent gene silencing. In comparison with the positive control group Lip2000-siRNA^{Cy3} (51.38±8.07% mRNA expression), the BCPV-siRNA^{Cy3} group has demonstrated a substantial enhancement in gene knockdown efficiency (21.83±7.14% mRNA expression) post transfection (**Fig 4.6**). Additionally, the Lip2000-siRNA^{Cy3}-FBS group demonstrated a significant decrease in

the gene knockdown efficiency ($75.7\% \pm 11.61$ mRNA expression) due to the strong interference by the FBS. In contrast to Lip2000-siRNA^{Cy3}-FBS, the BCPV-siRNA^{Cy3}+FBS group exhibited $30.7 \pm 8.54\%$ mRNA expression, which describes the ability of BCPVs to withstand the reduced knockdown efficiency caused by the presence of FBS in the culture medium.

The abnormal gene expression of BCR-ABL prevented leukemia cells from apoptosis and promoted cell proliferation by activating the cell survival signaling such as STAT5 and Erk signal pathway. In this study, the cellular activities of the K562 cells were systematically evaluated after the downregulation of BCR-ABL mRNA expression post BCPV-siRNA transfection. We examined the cell proliferation based on the total cell count 72 hours after transfection with treatments of BCPV-siRNA^{Cy3} and other positive and negative control groups. As expected, the cell count was considerably reduced to around 2.78×10^4 by BCPV-siRNA^{Cy3} and to 3.23×10^4 by BCPV-siRNA^{Cy3}+FBS (**Fig 4.7**), much lower than that inhibited by Lip2000-siRNA^{Cy3} (3.54×10^4) and Lip2000-siRNA+FBS (4.9×10^4).

Figure 4.7 Control of cell proliferation as a consequence of BCR-ABL oncogene suppression. The cell groups were treated with different formulations and the cell numbers were counted under a microscope post 72 hours of incubation. Consistent with the gene knockdown results in Figure 6, BCPV-siRNA-treated cell group yielded the lowest cell count, revealing the efficient performance of BCPV-assisted siRNA transfection in non-adherent leukemic cells. Blank cells were untreated. Results are represented as means $\pm$ SD, n=3; *, $P < 0.05$; **, $P < 0.01$.

The results showed that after treatments with BCPV-siRNA and BCPV-siRNA-FBS, the viable cell percentages (relative to untreated cells) were decreased to 44.76% and 52.02%, while the posttreatment Lip2000-siRNA and Lip2000-siRNA^{Cy3}-FBS samples had 57.06% and 79.04% viable cell percentages. Consequently, BCPV had a larger effect on suppressing the proliferation of K562 cells than Lipofectamine2000TM due to its higher transfection efficiency. These results demonstrate the active role of BCPVs (even in FBS) in transfecting more siRNA in the K562 cells, which positively correlates to greater inhibition of cell growth, and considerable suppression in cell proliferation.

Figure 4.8 Enhanced cell apoptosis as a consequence of high BCPV-siRNA transfection. (a) The cell groups were treated with different formulations and subjected to Annexin V: FITC Apoptosis assay, 72 hours post treatment. Cells presenting higher fluorescent signals for both FITC and PI staining in the flow cytometry micrographs were representative of the apoptotic cell count. (b) The BCPV-siRNA-treated cell group records the highest proportion of apoptotic cells. Blank cells were untreated. Results are represented as means $\pm$ SD, n=3; **, P<0.01.

Indicative results regarding the induction of apoptosis resulting from knockdown of BCR-ABL siRNA expression in K562 cells were assessed by FITC labeled Annexin V and Propidium Iodide (PI) staining. As shown in **Fig4.8a**, the FITC- and PI-positive apoptotic cell counts in the Q2 and Q4 zone represent the number of early apoptosis cells of BCPV-siRNA treated group and other control groups. The quantitative data (**Fig4.8b**) indicate that all negative control groups, including untransfected cells, siRNA and BCPVs, have no influence on induction of apoptosis. However, BCPV-siRNA treatments (78.17% gene knockdown efficiency) result in 12.4% apoptotic cells while Lip2000-siRNA^{Cy3} treatment (48.63% gene knockdown efficiency) exhibit 5% apoptotic cells. These results indicate that the cell apoptosis in K562 cells can be induced by knockdown of mRNA expression, and the proportion of apoptotic cells is positively correlated with the corresponding transfection efficiency. Moreover, in the presence of FBS, BCPV-siRNA^{Cy3}-FBS and Lip2000-siRNA^{Cy3}-FBS decreased their portions of apoptotic cells to 6.3% and 2.6%, implying that the reduced gene transfection efficiency arising from FBS has a direct effect on the decrease in apoptosis. The decrease in cell proliferation and apoptosis can be attributed to a decrease in cyclin D expression as a direct consequence of BCR-ABL mRNA silencing, which leads to cell cycle arrest in G1 phase [238]. The downregulation of the anti-apoptotic BCR-ABL gene activates the required signaling components for re-opening of the apoptotic pathway [239].

Previous reports stated fairly low transfection results for targeting BCR-ABL mRNA in K562 suspension cells [217, 222, 228, 237]. In this report, we have demonstrated the knockdown of BCR-ABL fusion oncogene by RNAi using BCPVs as vectors with outstanding siRNA transfection efficiency, followed by subsequent inhibition of cell proliferation and induction of cell apoptosis in K562 cells *in vitro*. This is the first report describing the use of BCPVs as vectors to both transfect BCR-ABL siRNA into non-adherent K562 cells and induce gene silencing. BCPVs possess numerous favorable material features, such as tunable charge density, low cytotoxicity and high transfection efficiency, which all make them a very promising platform to siRNA to non-adherent leukemia cells. Eventually, using RNAi technique for treating cancers

can be more effective, more reliable and safer. Moreover, the discovered siRNA delivery mechanism envisions a roadmap for accelerating RNAi-based therapeutic research for targeting suspension blood cancer cells apart from solid tumors.

4.4 Conclusion

In conclusion, we have demonstrated that the biodegradable cationic polymers BCPVs can interact and form stable nanoplexes with BCR-ABL siRNA, and efficiently transfect targeted mRNA into non-adherent K562 leukemia cells with insignificant cytotoxicity. The transfection and release of BCR-ABL siRNA from BCPV-siRNA nanoplexes have been confirmed through fluorescent imaging and quantitative flow cytometry. High transfection rates of up to 96% have been observed. As a consequence of transfection, the expression of BCR-ABL oncogene has been downregulated considerably. Successful siRNA transfection and gene silencing has resulted in inhibition of cell proliferation and induction of apoptosis process in the CML cell line. BCPVs are postulated to contribute towards newer technologies to deliver siRNA in a non-toxic and degradable way, rendering the RNAi phenomenon a more effective, bio-compatible, and sustainable approach to gene-based cancer therapy. Our work presented a systematic study of using BPCVs to transfect BCR-ABL siRNA in the non-adherent K562 leukemia cells. Moreover, the results in this study can be regarded as a breakthrough for treating leukemia via gene therapy.

Chapter 5 Biodegradable polymer for siRNA co-delivery strategy increasing the chemo-sensitivity of pancreatic cancer cells to Gemcitabine

For the treatment of pancreatic cancer, targeting only one mutant proto-oncogene by RNAi is not sufficient to provide the desired therapeutic efficiency. Both the K-ras gene mutation and the Notch1 over-expression that are common syndromes in pancreatic cancer patients, play a vital role in regulating the drug resistivity of pancreatic cancer cells. In this study, biodegradable charged polyester-based vectors (BCPVs) were synthesized for co-delivery of K-ras and Notch1 siRNA into MiaPaCa-2 cells to overcome the drug resistance to Gemcitabine (GEM), a first line chemotherapeutic drug used in clinical therapies. BCPVs can effectively promote siRNA cell internalization and endosomal escape. Through K-ras and Notch1 gene silencing in MiaPaCa-2 cells, BCPV-siRNA^{K-ras}siRNA^{Notch1} nanocomplexes effectively reversed the epithelial-mesenchymal transition (EMT) in MiaPaCa-2 cells, thereby greatly enhancing the sensitivity of MiaPaCa-2 cells to GEM. MiaPaCa-2 cell proliferation, migration, and invasion were effectively inhibited, and cell apoptosis was also significantly enhanced by the synergistic antitumor effect of BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes and GEM. These results suggest that this combination RNAi therapy can be used to improve cancer cell sensitivity to chemotherapeutic drugs

5.1 Introduction

The number of fatalities in patients with pancreatic cancer is almost equal to the number of diagnosed patients [1]. Pancreatic cancer has a very poor prognosis, as most of the diagnosed patients have metastatic or locally advanced cancer. The progression of pancreatic cancer is the result of a series of genetic mutations, especially in the K-ras proto-oncogene and Notch1. The K-ras mutation is the most frequently encountered pathogenic genetic variation, accounting for approximately 95% of the gene mutations found in pancreatic cancer [240]. Accordingly, K-ras has been considered an attractive target in the relevant gene therapy [14, 241]. However, directly

targeting K-ras has not yielded the desired results in the clinical treatment of pancreatic cancer and the therapeutic strategies have shifted from directly targeting K-ras to combinatorial gene therapies. Notch1 signaling pathway is one such additional target, which plays an important role in maintaining the neoplastic phenotype of Ras-transformed human cells [242]. The multiple components of the Notch1 signaling pathway, including ligands, receptors, and downstream targets, are upregulated in pancreatic cancer cells compared with normal tissue cells [243]. Previous studies have demonstrated that Notch1 signaling is activated during pancreatic cancer development, indicating that Notch1 promotes the development of pancreatic cancer [242-244].

The epithelial-mesenchymal transition (EMT) plays an important role in the progression of pancreatic cancer, and is responsible for the drug-resistant phenotype of cells in response to chemotherapy [16]. When acquiring EMT characteristics, tumor cells lose the expression of cell-cell contact proteins, such as γ -catenin and E-cadherin, and begin to express mesenchymal markers, such as fibronectin and vimentin, which were also reported to promote cancer cell migration and invasion [245-248]. Conversely, the epithelial phenotype in cancer cell lines results in less invasive and migratory characteristics, and renders the cells more susceptible to chemotherapeutics. The process of EMT reversal involves acquiring a reformatted of epithelial cell-cell junction, actin cytoskeleton reorganization, and down-regulation of mesenchymal molecular markers such as ZEB1, fibronectin, vimentin, and α -smooth muscle actin, and vimentin. A reassembly of the cell-cell junction, including up-regulation and relocation of E-cadherin from cell membrane to nucleus, results in the induction of EMT reversal [16, 249, 250]. It was reported that Notch1 over-expression contributes to the acquisition of the epithelial-mesenchymal transition (EMT) phenotype by pancreatic cancer cells, and EMT is associated with the development of chemotherapy drug resistance, thereby resulting in tumor recurrence [251]. Moreover, Notch1 has an oncogenic function since induced coactivation of Notch1 and K-ras^{G12D} in mature acinar cells leads to a clearly higher number of pancreatic intraepithelial neoplasia lesions than activation of K-ras^{G12D} only. It should be noted that the sole expression of Notch1 without oncogenic K-ras is unable to induce acinar cell differentiation and pancreatic cancer

development, indicating that activation of Notch1 only is not sufficient to promote tumorigenesis[252]. Hence, co-delivery of K-ras and Notch1 siRNA for pancreatic cancer treatment should be a promising strategy as both genes are significant tumor growth factors capable of inducing pancreatic tumorigenesis [241, 251-257].

To date, although chemotherapy remains the most widely used clinical treatment for pancreatic cancer, it does not effectively improve patient rate survival. In most cases, chemotherapy failure can be attributed to drug resistance developed by the cancer cells. Gemcitabine (GEM), an anticancer drug approved for pancreatic cancer treatment in 1997 based on a randomized trial, functions by arresting cancer cells at the S-phase of the cell cycle and inhibiting deoxyribonucleic acid (DNA) synthesis [258]. However, based on the clinical data, only 23.8% of patients treated with GEM have experienced satisfying therapeutic benefits. Patients suffering from drug resistance to GEM have a median survival time of merely 6 months [259, 260]. To overcome the drug resistance, multidrug combinations including cisplatin, oxaliplatin, and irinotecan, together with GEM, have been tested in pancreatic cancer clinical trials. The antitumor efficacy of various drug combinations has been greatly improved based on the results of phase II trials [261, 262]. Although combination therapies in the presence of GEM showed an obvious improvement in their antitumor activity, there is still significant concern for the emergence of chemotherapy drug resistance after a prolonged treatment.

RNA interference (RNAi) mediated by siRNA has recently emerged as a promising therapeutic strategy for treating pancreatic cancer [12-16] that can also enhance the antitumor activity of chemotherapy drugs by reducing drug resistance. Previous reports showed that the delivery of functional siRNA via biodegradable lipid nanocomplexes can significantly improve the chemo-sensitivity of colon cancer cell stem cells to paclitaxel [263]. Although the RNAi technology has been demonstrated as a powerful tool for treating cancer by silencing the expression of mutated genes, [7, 18], it is very difficult for naked siRNA, which is highly hydrophilic, has a high molecular weight, and is negatively charged, to cross the phospholipid bilayer of the cell membrane [9]. In addition, naked siRNA is susceptible to degradation from

nucleases in the plasma, and thus, its half-life in the bloodstream is generally less than an hour [8, 26]. Naked siRNA that is rapidly filtered by the glomerulus can also be readily excreted from the kidneys [11]. Consequently, the development of biologically safe and efficient siRNA delivery carriers have attracted significant attention from the research community [27, 28]. A variety of nanocarriers such as lipids, peptides, polymers, nanoparticles, and aptamers have been developed for siRNA delivery. Despite the promising siRNA delivery results, these newly developed nanocarriers are often limited by their high cytotoxicity and low transfection efficiency [7]. For example, cationic polymers such as poly(L-lysine), poly(vinyl pyridine) and polyethylenimine, have been frequently utilized as siRNA carriers. However, due to their high charge density and non-biodegradability, such cationic polymers generally exhibit high cytotoxicity, which limits their further clinical applications. As detailed in our previous work, biodegradable charged polyester-based vectors (BCPVs) based on a cationic polylactide have shown great promise as ideal siRNA nanocarriers, exhibiting high transfection efficiency, low toxicity, considerable hydrolytic degradability, and biocompatibility for delivering therapeutic siRNA into leukemia cells, a type of cancer cells well-known for being hard to transfect [264].

In this study, BCPVs were used as nanocarriers for the co-delivery of two kinds of siRNA (K-ras and Notch1 siRNA) in order to overcome the multiple gene mutations in pancreatic cancer cells, and to reduce drug resistance to GEM (**Fig 5.1**). In this study, BCPVs were used as a novel nanocarrier for co-delivering K-ras and Notch1 siRNA into MiaPaCa-2 (pancreatic cancer) cells at optimized conditions. The transfection efficiency of siRNA delivered by BCPVs was carefully assessed by flow cytometry, imaging, and real-time polymerase chain reaction (PCR). Moreover, the mechanism of siRNA release from the BCPV-siRNA nanocomplexes in the endosome was elucidated. BCPV-siRNA delivery led to significant downregulation of K-ras and Notch1 expression. Furthermore, K-ras and Notch1 knockdown in pancreatic cancer cells not only inhibited cell proliferation, migration, and invasion and induced cell apoptosis, but also enhanced the sensitivity of MiaPaCa-2 cells to GEM by reversing EMT. The results demonstrate that combinatorial therapy with gene knockdown and GEM treatment, exhibited more sustainable

antitumor activity than GEM or gene therapy alone. In conclusion, we showed that BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM combinatorial therapy shows enhanced synergistic anti-cancer effects, and provides a novel therapeutic strategy to treat pancreatic cancer effectively.

Figure 5.1 The roadmap in this chapter of the processed targeted/induced by the treatment suggested and detailed in this section, which includes BCPV-siRNA nanocomplex formation, uptake process and K-ras and Notch1 gene knockdown by siRNA transfection on pancreatic cancer cell.

5.2 Experimental Methods

5.2.1 Synthesis and characterization of BCPV-siRNA nanoplexes

The BCPV was synthesized by ring-opening polymerization (ROP) of allyl functionalized lactide (LA) and L-LA monomers followed by a thiol-ene modification, in accordance with 3.2.1 section.

5.2.3 Transfection

A custom-synthesized NC-siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCGGAGAATT-3') labeled with FAM or Cy3, and K-ras and Notch1 siRNA were purchased from Shanghai GenePharma Co., Ltd. The sequence of K-ras siRNA (sense: 5'-FAM-GUUGGAGCUUGUGGCGUAGUU-3'; Antisense: 5'-

CUACGCCACAAGCUCCAACUU-3'), and that of Notch1 siRNA (sense: 5'-AAGUGUCUGAGGCCAGCAAGAdTdT-3'; antisense: 5'-UCUUGCUGGCCUCAGACACUdTT-3') were determined as previously described [14, 265]. A BCPV (1 mg/ml) dispersion was mixed with siRNA at predetermined BCPV-siRNA weight ratios, followed by gentle vortexing and incubation for 30 min. The mixture was then added to cells, which were seeded onto 6-well plates (1×10^4 per well) in DMEM so as to achieve 30%–50% confluence at the time of transfection, and the cells were cultured for 4 h. In a parallel experiment, the same amount of free siRNA was used to transfect cells. The commercial DharmaFECT 1 Transfection Reagent (Dharmacon) coupled with siRNA^{FAM} and siRNA^{Cy3} was used as a positive control. Gene expression was monitored 48 h post-transfection. To determine the transfection efficiency, fluorescence imaging was performed 4 h post-transfection.

5.2.4 Transfection efficiency assessment

Four hours post-transfection, the cells were washed twice with PBS, fixed with 4% formaldehyde, and incubated with DAPI (Sigma) for nuclear counterstaining. The cells were then imaged *in vitro* with a fluorescence microscope (Eclipse-Ti, Nikon) with the filters set for DAPI (excitation: 405 nm; emission bandpass filter: 450/50 nm), FAM (excitation: 488 nm; emission bandpass filter: 525/50 nm) and Cy3 (excitation: 540 nm; emission bandpass filter: 605/50 nm) imaging. We further evaluated BCPV-siRNA complex uptake by the cells by staining cells that had been cultured with BCPV-siRNA complexes for different durations with LysoTracker Red. The intracellular distribution of siRNA^{FAM} and endosomes in MiaPaCa-2 cells was visualized with an inverted fluorescence microscope. For the flow cytometry experiments, the cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsin. Serving as a luminescence marker, the FAM and Cy3 (filter set for FITC was applied for FAM, and the filter set for PE was applied for Cy3) were used to determine the transfection efficiency in a quantitative manner. In detail, the operating parameters of the flow cytometry were set for FAM (excitation at 488nm and the emission was collected with a 530nm/30nm band pass filter), and Cy3 (excited with a 540nm

laser and the emission was collected with a 610/20nm band pass filter). The samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson, Mississauga, CA).

5.2.6 Gene expression analysis

The total RNA was extracted from MiaPaCa-2 cells 48 h post-transfection using an E.Z.N.A.™ Total RNA Kit (OMEGA) and its amount was quantified spectrophotometrically with a Nano-Drop ND-1000 as previously described [227]. Reverse transcription was conducted in a 20 µL reaction mixture and complementary DNA (cDNA) was synthesized from 2 µg total RNA using a reverse transcriptase kit (Promega) according to the manufacturer's instructions. The K-ras and Notch1 mRNA expression levels were determined by real time PCR using the SYBR green dye (Promega) in an ABI Prism 7500 Applied Biosystems system, and were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the most commonly used housekeeping genes for gene expression comparisons [42]. The primer sequences used for the different genes were as follows [14, 255]: K-ras forward: 5'-AGAGTGCCTTGACGATACAGC-3' and reverse: 5'-ACAAAGAAAGCCCTCCCCAGT-3'; Notch-1 forward 5'-CACTGTGGGCGGGTCC-3' and reverse 5'-GTTGTATTGGTTCGGCACCAT-3'; GAPDH, forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCT-GTTGCTGTA-3'.

5.2.7 Cell proliferation, invasion Assays, and cell apoptosis

The effect of BCPV-siRNA on MiaPaCa-2 cell proliferation was studied with an MTT assay. The invasive capacity of MiaPaCa-2 cells after siRNA transfection or after different treatments was tested with an 8.0-µm BD BioCoat Matrigel invasion Chamber Assay System (BD Biosciences) according to the manufacturer's protocol with minor modifications. Briefly, MiaPaCa-2 cells were transfected with BCPV-siRNA for 48 h as described in section 2.5. Subsequently, transfected MiaPaCa-2 cells (5×10^4) were seeded into the upper chamber of the system in FBS-free medium. The bottom chamber of the system was filled with medium containing 10% FBS as the chemoattractant. After 48 h, the cells in the upper chamber were removed with swabs, and the cells that had invaded through the Matrigel matrix membrane were

stained with 1% crystal violet in PBS for 30 min, and counted in 5 random fields of view under a microscope. The apoptosis assay was conducted using the FITC annexin V Apoptosis Detection Kit I (BD Pharmingen™) according to the manufacturer's instructions. Briefly, MiaPaCa-2 cells were seeded in a 6-well plate (approximately 1×10^6 per well), and were treated with BCPV-siRNA (weight ratio 8:1) for 48 h. The cells were then collected, washed twice with cold PBS, and suspended in Binding Buffer. Subsequently, the externalization of phosphatidylserine was measured. The cells were stained with FITC labeled annexinV and PI. The stained cells were analyzed by flow cytometry to determine the apoptotic cells.

5.2.8 Cell colony formation assay

MiaPaCa-2 cells were seeded in a 6-well plate (1×10^5 per well). After 24 h, the cells were treated with different formulations for 24 h, harvested by trypsinization, and 1,000 single viable cells were mixed with 0.3% agarose and 10% FBS, and plated in 35 mm Petri dishes that were pre-coated with 0.6% agarose. The cells were then incubated for two weeks to form colonies, which were stained with Giemsa-staining, and counted under an inverted microscope.

5.3 Results and discussion

In recent years, many attempts aiming at improving the survival rate of patients with pancreatic cancer have not yielded the expected results [1]. Therefore, the development of novel and effective treatment strategies to combat pancreatic cancer in the clinic has attracted significant attention [134, 156, 157, 266-268]. RNAi therapy has emerged as a new modality to treat cancer diseases by virtue of their specified targeting to mutated genes and low adverse effects [268-270]. However, naked siRNA, without a carrier, is difficult to cross the cell membrane and enter the cytosol, and this is a major hindrance in RNAi therapy. Therefore, we synthesized BCPVs as carriers for intracellular siRNA delivery, based on a biodegradable polymer, vial ring-opening polymerization and thiol-end click functionalization based on our previous study [14]. BCPVs had negligible cytotoxicity to various cell lines and can be used in large amounts as siRNA delivery carrier. Before as carrier of two kinds of siRNAs, the stability of BCPV-siRNA

nanocomplexes were firstly judged by monitoring the size change in a FBS solution via DLS (**Fig 5.2**). When comparing with the unchanged size of BCPV-siRNA nanocomplexes in deionized (DI) water at 3h, the size of the BCPV-siRNA nanocomplexes increased by 53 nm in 5% FBS solution, by 68 nm in 10% FBS solution, and by 84 nm in 50% FBS solution.

Figure 5.2 Stability of BCPV-siRNA nanocomplexes different solutions (DI water; 5%, 10%, and 50%FBS of PBS Buffer).

We speculate that the initial size changes of the BCPV-siRNA nanocomplexes is the result of the absorption of free ions and proteins in the FBS-PBS buffer solution. After 3 h, the size of BCPV-siRNA nanocomplexes remained constant in different solutions. No obvious changes were observed up to 48h.

As siRNA carrier, it has been suggested that BCPV-siRNA nanocomplexes may have additional functions that allow them to overcome several intracellular barriers affecting the success of RNAi treatment. These functions are related to the amount of siRNA the cells can uptake, the unpacking of siRNA, and the escape from the endosomal compartments to the cytosol, which is generally considered as one of the most challenging barriers for effective siRNA transfection. Accordingly, the cell uptake process and intracellular kinetics of BCPV-siRNA nanocomplexes were analyzed in MiaPaCa-2 cells. The cells were incubated with BCPV-

siRNA^{FAM} nanocomplexes at an 8:1 weight ratio for the specified times, and were then stained with LysoTracker Red. **Fig 5.3** shows the intracellular distribution of BCPV-siRNA^{FAM} nanocomplexes in MiaPaCa-2 cells observed with an inverted fluorescence microscope. After 0.5 h, siRNA^{FAM} (shown in green) was mostly located in the cell membrane, and was separated from intracellular endosomes (shown in red). After 1 h incubation of BCPV-siRNA^{FAM} with MiaPaCa-2 cells, a punctuated pattern of siRNA^{FAM} staining was observed in the cytoplasm, which completely correlated with the endosomes, displaying yellow fluorescence as a result of overlapping between the red and green fluorescence from the LysoTracker Red and siRNA^{FAM}

Figure 5.3 siRNA^{FAM} release from BCPV-siRNA^{FAM} nanocomplexes in the lysosome. Microscopic images (40×magnification) of MiaPaCa-2 cells treated with BCPV-siRNA^{FAM} for different time intervals. The weight ratio of BCPV and siRNA^{FAM} are fixed at 8:1, and the concentration of BCPV is 40 µg/ml. The lysosomes were stained with LysoTracker Red.

, respectively. After 4 h incubation, the green fluorescence that spread throughout the cell started to be distinctive from the red fluorescence (endosomes), as seen in the overlap image, indicating that part of the siRNA^{FAM} could escape from the endosomes to the cytosol, although the remaining siRNA was still observed in the endosomes. The separated green fluorescence could be observed for up to 12 h, indicating that the escape process of siRNA was maintained for a relatively long time. After 24 h incubation, no green fluorescence was detected in the cells, and only red fluorescence was observed, suggesting that the delivered siRNA has already performed its function. The imaging results show clearly the endosomal entrapment and endocytosis of BCPV-siRNA^{FAM} nanocomplexes. The release of siRNA^{FAM} from BCPV-siRNA^{FAM} nanocomplexes in the lysosome was also demonstrated, and we found that BCPV-siRNA^{FAM} nanocomplexes can effectively disassemble in the lysosomal environment. Moreover, the slow partial separation of green fluorescence from red fluorescence suggests that BCPVs are capable of effectively aiding siRNA release from endosomal compartments. This release could be attributed to the proton-sponge effect that renders cationic nanocomplexes capable of rupturing intracellular endosomes [55]. As a result of such siRNA escape mechanisms aided by carriers, most of the intracellular siRNA can be involved in RNAi instead of being degraded by hydrolases in the lysosomes.

Based on the above results, the capacity of BCPVs to deliver two kinds of siRNA with different fluorescence labels into MiaPaCa-2 cells was investigated. BCPVs (40 µg/mL) were mixed with FAM- and/or Cy3-labeled siRNA at an 8:1 weight ratio (BCPV-siRNA^{Cy3}, BCPV-siRNA^{FAM}, and BCPV-siRNA^{FAM}-siRNA^{Cy3} groups) or a 16:1 weight ratio (BCPV-semi-siRNA^{FAM}-siRNA^{Cy3} group) for 1 h, and were then incubated with MiaPaCa-2 cells for 4 h. Subsequently, the cells were observed under an inverted fluorescence microscope to assess the transfection efficiency. **Fig 5.4** shows the color-coded fluorescence images of MiaPaCa-2 cells treated with the different formulations. The commercialized transfection reagent DharmaFECT served as a positive control. Strong green (siRNA^{FAM}), red (siRNA^{Cy3}), yellow (overlay of siRNA^{FAM} and siRNA^{Cy3}), and blue (cell nuclei stained with DAPI) fluorescence signals were observed in all MiaPaCa-2 cells. Moreover, the bright green and red signals observed in cells

treated with the BCPV-siRNA^{FAM}-siRNA^{Cy3} formulation, reached levels similar to those of cells treated with DharmaFECT-siRNA^{FAM}-siRNA^{Cy3}. Thus, BCPVs showed a high capacity to simultaneously deliver two kinds of siRNA into cells. It should be noted that FBS was not deliberately removed from the medium before the addition of the BCPV-siRNA nanocomplexes. This revealed that the cells can be directly transfected with BCPV-siRNA nanocomplexes in the presence of FBS, thus indicating once more that BCPVs can protect siRNA from rapid degradation by RNases present in FBS. In contrast, most commercial transfection reagents do not perform satisfactorily when applied in the culture medium without first removing FBS. Thus, the use of BCPVs can greatly simplify the transfection protocol.

In order to quantify the transfection efficiency of BCPV-siRNA nanocomplexes on MiaPaCa-2 cells, flow cytometry was employed. The fluorescence intensities obtained by flow cytometry are shown in **Fig 5.5a**. Based on the intensity of the fluorescence signals, all gated events were classified into four populations: Q1, Q2, Q3, and Q4, where cells in Q3 show low autofluorescence signals, cells in Q1 show strong Cy3 fluorescence signals, cells in Q4 show strong FAM fluorescence signals, and cells in Q2 show a strong overlay between the FAM and Cy3 fluorescence signals. The statistical analysis from the results presented in **Fig. 5.5a** is summarized in **Figs. 5.5b** and **5.5c**. All BCPV-siRNA^{FAM}, BCPV-siRNA^{Cy3} and BCPV-siRNA^{FAM}-siRNA^{Cy3} groups exhibited a high transfection efficiency and strong fluorescence intensity resulting from FAM and/or Cy3, to levels comparable to those of the DharmaFEC-siRNA^{FAM}-siRNA^{Cy3} group. The BCPV-semi-siRNA^{FAM}-siRNA^{Cy3} group showed reduced FAM and Cy3 fluorescence intensities than the BCPV-siRNA^{FAM}-siRNA^{Cy3} group. This is due to the fact that the amount of siRNA in BCPV-siRNA^{FAM}-siRNA^{Cy3} was doubled than that in BCPV-semi-siRNA^{FAM}-siRNA^{Cy3}. The blank group (naked siRNA^{FAM} and siRNA^{Cy3}) showed negligible fluorescence intensity. These results indicate that BCPVs can effectively delivery siRNA into cells, and they are consistent with the results presented in **Fig 5.4**.

Figure 5.4 BCPVs as an effective transfection agent for pancreatic cancer line, MiaPaCa-2. MiaPaCa-2 cells were transfected by BCPV-siRNA^{FAM}-siRNA^{Cy3}. The observance of green (FAM), red (Cy3), Blue (DAPI) and yellow (overlay of FAM and Cy3) colors in the images obtained by an inverted fluorescence microscope (20×magnification) confirm the BCPV-assisted transfection of siRNA. The amount of siRNA^{FAM} and siRNA^{Cy3} in the BCPV-semi-siRNA^{FAM} - siRNA^{Cy3} group (2.5µg siRNA) is respectively equivalent to half of BCPV-siRNA^{FAM}-siRNA^{Cy3} group (5µg siRNA).

The aggressiveness of pancreatic cancer is partly due to its extrinsic and intrinsic drug resistance. Therefore, there is an urgent need for a novel therapeutic strategy to treat pancreatic cancer that can overcome drug resistance. K-ras and Notch1 expression is believed to be important for carcinogenesis [242, 252]. K-ras mutations are present in nearly every pancreatic cancer, and K-ras activation is an initiating event in pancreatic cancer. Although therapies targeting K-ras have so far failed to yield beneficial results in the clinic, identifying and targeting K-ras pathways is still of great interest for pancreatic cancer treatment [256]. Notch1, a downstream mediator of K-ras signaling, is responsible for maintaining the neoplastic phenotype of Ras mutant cells [242, 253]. Notch1 signaling acts synergistically with K-ras to promote pancreatic cancer initiation and progression. In particular, sustained activity of Notch1 is essential for maintaining the phenotype of Ras mutant cells [242, 252, 271, 272]. Hence, we aimed at delivering simultaneously Notch1

and K-ras siRNAs into pancreatic cells, and evaluating the applicability of this novel combination therapy for pancreatic cancer treatment.

Figure 5.6 BCPV-siRNA nanocomplexes mediated gene silencing in MiaPaCa-2 cells. The K-ras and Notch1 mRNA level were analyzed by Real-Time PCR. Graph was plotted after normalizing with the gene expression intensity of GAPDH.

Figure 5.7 Proliferation of the MiaPaCa-2 cells after treatment with different formulations. Cells were first treated with BCPVs alone, BCPV-siRNA^{K-ras}, BCPV-siRNA^{Notch1}, BCPV-semi-siRNA^{K-ras}-siRNA^{Notch1}, BCPV-siRNA^{K-ras}-siRNA^{Notch1}, and DharmaFECT-siRNA^{K-ras}-siRNA^{Notch1}, then continued to culture for 72 h. The cell viability was determined using MTT cell viability assay. The results are represented as mean $\pm$ SD, n=5.

Figure 5.8 Enhanced cell apoptosis as a consequence of high BCPV-siRNA transfection. (a) The cell groups were treated with different formulations and subjected to Annexin V: FITC Apoptosis assay, 72 h post treatment. In order to exclude the cell debris, the cell dots in the P1 region were select for evaluating the cell apoptosis. Cells presenting higher fluorescence signals for both FITC and PI staining in the flow cytometry micrographs were representative of the apoptotic cell count. (b) The BCPV-siRNA-treated cell group records the highest proportion of apoptotic cells. Blank cells were untreated. Results are represented as means $\pm$ SD, n = 3; **, P < 0.01.

Continuing from our previous results, we found that BCPVs could effectively deliver siRNA into cells, and such siRNA could be released from endosomes to the cytosol via the proton-sponge effect. To verify the performance of the released siRNA on regulating gene expression via RNAi, a series of gene-related experiments was conducted. **Fig 5.6** shows the gene knockdown efficiency of different BCPV-siRNA formulations on MiaPaCa-2 cell as assessed by real-time PCR. Both K-ras and Notch1 expression levels were significantly decreased after treating the cells with BCPV-siRNA^{K-ras}-siRNA^{Notch1}, BCPV-semi-siRNA^{K-ras}-siRNA^{Notch1}, and DharmaFECT-siRNA^{K-ras}-siRNA^{Notch1}. The latter two groups exhibited a similar knockdown efficiency for Notch1 and K-ras genes in MiaPaCa-2 cells, indicating that the transfection efficiency of BCPVs is comparable to that of commercial transfection agents for the same amount of siRNA. However, more effective downregulation of Notch1 and K-ras was observed with BCPV-siRNA^{K-ras}-siRNA^{Notch1} than with BCPV-semi-siRNA^{K-ras}-siRNA^{Notch1}, suggesting that the gene silencing efficiency is correlated with the siRNA amount. Moreover, the results indicate an inter-influence between K-ras and Notch1 was observed because BCPV-siRNA^{K-ras} not only inhibited K-ras gene expression, but also downregulated Notch1 gene expression. However, downregulation of Notch1 by BCPV-siRNA^{Notch1} did not influence K-ras gene expression. These results indicate that K-ras is upstream of the Notch1 signaling pathway. For the BCPV-only group, no obvious changes in K-ras and Notch1 expression were observed.

According to previous reports, the K-ras and Notch1 genes are associated with cell growth, invasion, and apoptosis of pancreatic cancer cells [242]. However, the synergistic effect of these two genes on the growth of pancreatic cancer cells has never been investigated. Hence, the proliferation of MiaPaCa-2 cells after being treated with different BCPV-siRNA formulations was studied. **Fig 5.7** shows that the group treated solely with BCPVs did not show any differences from the blank group. In contrast, an obvious inhibition of cell proliferation was observed after treatment with the BCPV-siRNA^{K-ras} formulations. In particular, BCPV-siRNA^{K-ras}-siRNA^{Notch1} had a more pronounced effect in inhibiting pancreatic cancer cell proliferation than BCPV-siRNA^{K-ras}. The inhibition efficiencies of BCPV-siRNA^{K-ras}-siRNA^{Notch1} and DharmaFECT-

siRNA^{K-ras}-siRNA^{Notch1} were $65\% \pm 8.9\%$ and $62\% \pm 10.2\%$, respectively, indicating that BCPVs performed as well as DharmaFECT. In addition to the evaluation of cell proliferation evaluation, cell apoptosis and cell invasion were also investigated after treatment with the various formulations. As shown in **Fig 5.8** and **5.9**, the inhibition efficiency of BCPV-siRNA^{K-ras}-siRNA^{Notch1} for cell apoptosis and cell invasion was $23.5\% \pm 1.58\%$ and $77.2\% \pm 5.1\%$, respectively. These values are not significantly different from those observed with DharmaFECT-siRNA^{K-ras}-siRNA^{Notch1}, and are remarkably higher than those observed with the other formulations. These results indicate that co-delivery of K-ras and Notch1 siRNAs by BCPVs has a more pronounced effect on inhibiting cell growth, invasion, and inducing apoptosis.

Figure 5.9 Cell invasion assay based on a transwell chamber model of the MiaPaCa-2 cells after treatment with different formulations. (A) Bright field microscope images (20×magnification) of the cells that penetrated through the Matrigel-coated membrane in the transwell chamber. The cells were stained with Giemsa staining. (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 = 6.

Figure 5.10 Cell proliferation assay shows that repressing expression of K-ras and Notch1 together increased the sensitivity of MiaPaCa-2 cells to GEM.

Drug resistance associated with the treatment of pancreatic cancer was thought to be a major factor causing high mortality. GEM, one of the most commonly used chemotherapeutic drugs for pancreatic cancer treatment, has offered some relief for the patients over the past two decades [260, 262]. By inhibiting DNA replication and inactivating ribonucleotide reductase, GEM can inhibit cancer cell growth. However, pancreatic cancer treatment by the combination of GEM with other therapeutic agents has not been successful in terms of increasing overall patient survival [273]. After assessing the influence of BCPV-siRNA^{K-ras-siRNA^{Notch1}} on cell growth, we also investigated its therapeutic effect on reducing drug resistance of MiaPaCa-2 cells to GEM. MiaPaCa-2 cells were first treated with different BCPV-siRNA formulations, followed by the addition of various GEM concentrations for 96 h. The cell proliferation index was assessed by the MTT assay. As shown in **Fig 5.10**, MiaPaCa-2 cell sensitivity to GEM was clearly enhanced after treatment with BCPV-siRNA^{Notch1}, BCPV-siRNA^{K-ras-siRNA^{Notch1}}, and BCPV-siRNA^{K-ras-siRNA^{Notch1}} nanocomplexes. Particularly, cells co-incubated with BCPV-siRNA^{K-ras-siRNA^{Notch1}} and GEM at different concentrations (250, 500, 1,000, and 2,000 nM) had a higher inhibition efficiency ($71\% \pm 6.6\%$, $78\% \pm 8.2\%$, $85\% \pm 6.9\%$, and $92\% \pm 4.1\%$) on cell proliferation than treatment with GEM only ($12\% \pm 2.1\%$, $16\% \pm 1.9\%$, $23\% \pm 1.8\%$, and $28\% \pm 1.7\%$).

Moreover, treatment with BCPV-siRNA^{Notch1} and BCPV-semi-siRNA^{K-ras}-siRNA^{Notch1} also increased the sensitivity of cells to GEM. However, the efficacy was much lower than that observed with BCPV-siRNA^{K-ras}-siRNA^{Notch1} treatment. Accordingly, it was found that downregulation of K-ras and Notch1 expression enhanced the sensitivity of MiaPaCa-2 cells to GEM, thus leading to an improved efficiency in inhibiting the cell growth. To correlate the enhanced growth inhibition effect with the induction of apoptosis, the apoptosis of MiaPaCa-2 cells simultaneously treated with GEM and BCPV-siRNA^{K-ras}-siRNA^{Notch1} was measured with a FITC-labeled annexin V and PI staining kit. The results presented in **Fig 5.11** show that, compared to treatment with GEM only, BCPV-siRNA^{K-ras}-siRNA^{Notch1} induced a higher apoptosis level at all studied GEM concentrations. These data are consistent with the results shown in **Fig 5.10**.

Figure 5.11 FITC Annexin V and Propidium Iodide (PI) co-stained apoptosis assay on the MiaPaCa-2 cells treated with different concentration of GEM and/or BCPV-siRNA^{K-ras}-siRNA^{Notch1} formulations at 96h post-treatment. (A) Apoptotic cells stained with Annexin V/PI were measured by flow cytometry. (B) Apoptotic and dead cell counts in the quadrant (Q1+Q2+Q4) of different treatment groups. Error bars indicate the SD, *P < 0.01 compared between groups, n=5.

Figure 5.12 The morphology of MiaPaCa-2 cells changed from fibroblastoid to epithelial-like appearance after different formulations treatment were observed under phase contrast microscope (10×magnification). For each cell well, the GEM concentration was fixed at 500 nmol/ml, and BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes was fixed at 8:1 weight ratio with 40μg/ml BCPVs, 2.5μg/ml siRNA^{K-ras} and 2.5μg/ml siRNA^{Notch1}.

Figure 5.13 Real-time RT-PCR was used to quantify ZEB1 and E-cadherin mRNA expression in BLANK and BCPV-siRNA^{K-ras} -siRNA^{Notch1} treated MiaPaCa-2 cells. Error bars indicate the SD *, $P < 0.01$, compared with BLANK groups, $n=3$.

As was detailed in instruction, the presence of the EMT phenotype in pancreatic cancer cells accounts for the drug resistance displayed by most of the pancreatic cancer patients, and also contributes to the high mortality rate associated with pancreatic cancer. Therefore, reversing the

EMT induced phenotype can be a novel strategy to enhance the sensitivity of pancreatic cancer cells to anticancer drugs. Bin *et al.* reported that high Notch1 activation contributed to the acquisition of an EMT phenotype in pancreatic cancer cells. By activating mesenchymal cell markers, such as miR-200c, let-7a and let-7c, the resulting forced overexpression of Notch1 enhanced cell growth, migration, and invasion in various pancreatic cells lines [244]. The MiaPaCa-2 cell line showed typical EMT characteristics, including an elongated fibroblastoid shape, high invasion capacity, and resistance to GEM [244, 249]. Hence, the morphological changes of MiaPaCa-2 cells treated by BCPV-siRNA^{K-ras}-siRNA^{Notch1} were assessed microscopically. As shown in **Fig 5.12**, the 48 h post-transfection, the morphology of MiaPaCa-2 cells treated with BCPV-siRNA^{Notch1}, BCPV-semi-siRNA^{K-ras}-siRNA^{Notch1}, and BCPV-siRNA^{K-ras}-siRNA^{Notch1} was partially changed from an elongated fibroblastoid shape to an epithelial cobblestone-like appearance, and the growing cells appeared to be in close proximity with each other. However, upon treatment with BCPV-siRNA^{K-ras}, these phenotypic changes were not observed. The results indicate that downregulation of Notch1 gene expression is critical for the reversal of the EMT phenotype in MiaPaCa-2 cells, whereas the decreased expression of the K-ras gene plays a major role only in the inhibition of cell proliferation rather than the reversal of the EMT phenotype. Based on these results, we propose that the increased sensitivity of MiaPaCa-2 cells to GEM observed upon treatment with after BCPV-siRNA^{Notch1} and BCPV-siRNA^{K-ras}-siRNA^{Notch1} results from the EMT reversal.

To further confirm whether BCPV-siRNA^{K-ras}-siRNA^{Notch1}-treated MiaPaCa-2 cells underwent an EMT reversal process, we determined the expression of markers of epithelial and mesenchymal phenotypes by real-time PCR. The experimental method is as described in 5.2.6 section. Primers of ZEB1 and E-cadherin were designed with reference to previous report [274] (ZEB1: For, 5'-TTCAAACCCATAGTGGTTGCT-3', Reverse, 5'-TGGGAGATACCAAACCAACTG-3'; E-cadherin: Forward, 5'-CCCACCACGTACAAGGGTC-3', Reverse, 5'-CTGGGGTATTGGGGGCATC-3'). We found that significant reduction in ZEB1 expression was observed in BCPV-siRNA^{K-ras}-siRNA^{Notch1}

treated MiaPaCa-2 cells (**Fig 5.13**). Meanwhile, a significant increase in the transcription level of E-cadherin was observed in BCPV-siRNA^{K-ras}-siRNA^{Notch1} treated MiaPaCa-2 cells. The changes in the expression profile of these two factors suggest that BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes have indeed successfully induced EMT reversal in pancreatic cancer cells.

Figure 5.14 Wound healing assay in MiaPaCa-2 cell culture treated with different formulations. (A) Phase contrast microscope images (10×magnification) of the wound healing process monitored for 96 h post-treatment. (B) Quantitative evaluation on the percentage of the wound window closed after treatment, the values were normalized by the initial wound window width.

Figure 5.15 Cell invasion assay based on a transwell chamber model of the MiaPaCa-2 cells after treatment with different formulations. (A) Bright field microscope images (20×magnification) of the cells that penetrated through the Matrigel-coated membrane in the transwell chamber. For each cell well, the GEM concentration was fixed at 500 nmol/ml, and BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes was fixed at 8:1 mass ratio with 40 µg BCPVs, 2.5 µg siRNA^{K-ras} and 2.5 µg siRNA^{Notch1}. The cells were Giemsa-stained. (B) Quantitative cell counts of the cells that penetrated through the Matrigel-coated membrane. Each bar represents five random microscopic fields under 20 times magnification. Error bars indicate the SD, *P < 0.01 compared between the groups, n=5.

For pancreatic cancer patients, metastasis, a complex process that initiates the invasion of the primary cancer cells to the adjacent tissue, is a primary cause of death. Hence, MiaPaCa-2 cell migration and invasion following co-treatment with BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM were investigated. As shown in **Fig 5.14**, using a pipette tip, a scratch wound was created in a monolayer of MiaPaCa-2 cells treated with the different formulations. For the non-treated group, it was observed that $84\% \pm 6.6\%$ of the wounded area was healed after 72 h and the previously straight boundary lines would become unclear as a result of cells migrating to the open space between the wound margins. Following treatment with GEM only, a wound healing of $68 \pm 4.7\%$ was observed. On the contrary, co-delivery of K-ras and Notch1 siRNA by BCPVs significantly suppressed the healing process, and only $43\% \pm 6.7\%$ of the wound recovered. Noticeably, the slowest wound healing was observed upon simultaneous treatment with BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM, where only a wound healing of $12\% \pm 4.2\%$ was observed. This was a result of the downregulation of K-ras and Notch-1 gene expression, leading to an increased sensitivity of MiaPaCa-2 cells to GEM. These results suggest that the treatment with BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM all combined together could significantly inhibit MiaPaCa-2 cell migration. To evaluate the inhibitory effect of this combination treatment on MiaPaCa-2 cell invasion, the Matrigel invasion chamber assay was utilized to investigate the invasive potential of MiaPaCa-2 cells after being treated with different formulations. As shown in **Fig. 5.15**, cells co-treated with BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM showed a lower level of penetration through the Matrigel-coated chamber membrane than cells treated with the other formulations. Although the BCPV-siRNA^{K-ras}-siRNA^{Notch1} treatment yielded a $66 \pm 7.3\%$ inhibition efficiency on cell invasion, this value was still lower than that observed after co-treatment with BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM ($89\% \pm 4.1\%$). These results clearly indicate that our proposed combination treatment could synergistically inhibit cell invasion, and subsequently suppress the distal migration of pancreatic cancer cells.

Figure 5.16 Colony formation assay of MiaPaCa-2 cells were treated with different formulations, K-ras and Notch1 silencing enhance GEM's repression of the cancer cell colony formation. (a) Photomicrographs of Giemsa-stained colonies of MiaPaCa-2 cells growing in 35 mm cell culture dishes. (b) The number of cells per field under microscope (4×magnification) in each colony of MiaPaCa-2 cells was counted and normalized to that of the BLANK group. (c, d) The size of colonies as assessed under microscope.

In the following experiments, we also examined the synergistic effect of BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM co-treatment on MiaPaCa-2 cell clonogenicity. The four groups (BLANK, GEM only, BCPV-siRNA^{K-ras}-siRNA^{Notch1} and BCPV-siRNA^{K-ras}-siRNA^{Notch1}+GEM) were co-cultured with MiaPaCa-2 cells for two weeks to form colonies. As shown in **Fig. 5.16**, the number and size of MiaPaCa-2 cell colonies treated with BCPV-siRNA^{K-ras}-siRNA^{Notch1} and GEM were

clearly reduced compared with all the other groups. These results suggest that the downregulation of K-ras and Notch1 gene expression promotes the sensitivity of MiaPaCa-2 cells to GEM, thereby inhibiting cell colony formation. Based on the great improvements demonstrated in our study, we propose that the formulation developed here will serve as an effective candidate for pancreatic cancer therapy. Although there are several other issues that need to be addressed, particularly in the case of *in vivo* studies, such as the bio-distribution, immunotoxicity, and the metabolism of the nanoparticles *in vivo*, the *in vitro* findings in this study lay an important groundwork in providing meaningful information to guide future investigation in gene silencing-based pancreatic cancer therapy.

5.4. Conclusions

In this study, we developed BCPVs which encapsulate siRNA via electrostatic interactions and thus prevent its degradation in FBS-containing medium. The interaction between BCPVs and siRNA was the primary factor in the formation of stable extracellular nanocomplexes, while the BCPVs facilitated the siRNA release in the cytosol via the proton-sponge effect. We also showed that BCPVs can intracellularly deliver siRNA effectively. Learning on the excellent performance of BCPVs in siRNA delivery, we further designed and created BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes for overcoming drug resistance in pancreatic cancer. BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes effectively silenced the expression of the K-ras and Notch1 genes, induced cell apoptosis, and inhibited cell migration and proliferation. Most importantly, through the reversal of EMT in MiaPaCa-2 cells, BCPV-siRNA^{K-ras}-siRNA^{Notch1} enhanced the sensitivity of MiaPaCa-2 cells to GEM, and thus significantly improved the therapeutic effect of GEM. This combination therapy greatly inhibited proliferation, invasion, migration, and colony formation of MiaPaCa-2 cells, and enhanced cell apoptosis. In conclusion, we believe that based on the above results, this combination therapy through the use of BCPVs as siRNA carriers is a promising method for treating pancreatic cancer. To translate this technology to the clinic, future studies will have to focus on the use of BCPVs in *in vivo* gene silencing applications.

Chapter 6 Biodegradable polymer hybrid graphene quantum dots nanocomplex for synergetic chemo-gene therapy of pancreatic cancer

The K-ras mutation is one of the major mechanisms for pathogenesis, drug resistance and recurrence in pancreatic cancer. A new strategy to overcome pancreatic cancer is to use co-delivery of siRNA together with a chemotherapy drug to silence the mutation gene expression. In this study, nitrogen-doped graphene quantum dots (GQD) functionalized by biodegradable charged polyester vectors (BCPVs) were used as a platform for simultaneous Doxorubicin (DOX) and siRNA^{K-ras} delivery combined with NIR light-mediated biomolecules release. The functionalized-graphene quantum dots with BCPV allows co-loading DOX and siRNA by π - π interaction and electrostatic binding. After treatment with GQD/DOX/BCPV/siRNA nanocomplex, the proliferation, migration and invasion of pancreatic cancer cells were obviously suppressed, and the cell apoptosis was also promoted. The photothermal properties of the GQDs endow the enhanced intracellular release of DOX and siRNA from the nanocomplex under irradiation with near-infrared (NIR) laser light, thus enhanced the therapeutic effect. These results indicate the nitrogen-doped GQDs can be considered as a novel PTT agent, simultaneously allowing cell imaging and drug remotely control-release by NIR.

6.1 Introduction

Despite the huge effort and resources being channeled into pancreatic cancer research, the mortality rate of pancreatic cancer has not shown significant improvement, and it moved from the fourth to the third deadliest cancers in 2016, with yearly increased 53,070 new pancreatic cancer cases diagnosed and 41,780 deaths associated with pancreatic cancer in U.S.A alone [1, 275]. The high mortality rate is associated with K-ras gene mutation in over 90% of pancreatic cancer, resulting in rapid disease progression, high invasion, angiogenesis and resistance to chemotherapy[276]. DOX (doxorubicin) has been the first line drug for pancreatic cancer treatment, functions by disabling

Topoisomerase II, which is responsible for the synthesis of nucleic acids during the breaking and resealing of DNA strands during the replication process, leading to cell apoptosis. However, it is difficult to achieve the desired therapeutic effect through treatment with DOX only in most cases due to drug-resistance and systemic toxicity due to the toxic effects by the nonspecific distribution of anti-cancer drugs [3]. Hence, new treatment strategies for pancreatic cancer are imperatively and urgently needed. RNAi (RNA interference) is an effective method to inhibit K-ras oncogene expression by targeted cleavage of mRNA (messenger RNA) in pancreatic cancer cells [268]. However, the application of siRNAs faces a series of challenges. Naked siRNA is large molecular weight, negatively charged and degraded easily by RNase. Without a carrier, siRNA cannot penetrate the phospholipid bilayer of cell membrane by itself. A well-defined nano-carrier with low biological toxicity and highly biocompatible that is capable of effectively co-deliver DOX and K-ras siRNA into cells is a promising strategy for pancreatic cancer treatment. Graphene quantum dots (GQDs), which feature low cytotoxicity and high surface to volume ratio for easy conjugation with biomolecules, have great potential as a multifunctional nano-carrier [138, 277-279]. It has been reported that GQDs are excellent agents for loading hydrophobic drug molecules via π - π interaction between aromatic regions of the GQDs and drug molecule [280]. For constructing the multifunctional GQDs carriers, the selection of proper biocompatible molecules is important. PEI (polyethylenimine), which has a high positive charge, has been widely used to modify the charge properties of nanoparticle to form drug and gene co-loading layer by layer structure [281]. Nevertheless, PEI faces some serious drawbacks, such as long-term toxicity and the fact that it is non-degradable under normal biological aqueous conditions [282]. The possibility to reduce PEI toxicity by PEGylation is a mature and effective approach. However, PEGylation requires a dedicated study to find out and choose the best PEG structure and molecular weight, the number of chains to link per molecule and the coupling chemistry, which greatly increase the difficulty of manufacturing [46]. To combat these concerns, BCPVs have been reported in our previous study as an outstanding biodegradable siRNA carrier with

negligible cytotoxicity [283], but its potential in modifying nanoparticles for better cellular uptake that leads to efficient intracellular drug release and possible additional therapeutic approach has not been explored.

Light as an external stimulus offers a great opportunity to release a drug in a controllable manner at a desired time and in a desired area, which is crucial for boosting drug efficacy in cancer treatment while minimizing side effects [284, 285]. PTT (photothermal therapy) and PDT (photodynamic therapy) have attracted much interest in recent years as minimally invasive techniques to destroy cancerous cells without damaging surrounding healthy tissues [286, 287]. The photothermal properties of nanoparticles, such as Au nanorods (NRs), Au nanoparticles (NPs) and azobenzene have been used to trigger the release of biomolecular cargos of nanoparticles through near infrared (NIR)-induced hyperthermia [288-290]. These stimuli-responsive drug reservoirs can effectively respond to external triggers by changing their physical or chemical properties in order to regulate the movement of the pharmaceutical cargo [284]. NIR laser emitting in the 650 – 900 nm range provide an optimal affect as their wavelengths have deep tissue penetration with much less damage to the living tissues and biological specimens involved. The release of drugs encapsulated within the nanoparticles can be controlled and enhanced by the NIR energy. For example, Liu *et al.* reported the use of NIR to trigger the release of drug doxorubicin (DOX) from mesoporous silica-coated nanoparticles in a controllable manner [291]. Although MSNs-based drug delivery systems have been proven effective, MSNs don't have intrinsic fluorescent properties which limits their application in bio-imaging. Additionally, MSNs have no photothermal properties unless used in conjunction with other molecules and nanoparticles. Recently, Ge *et al.* demonstrated that GQDs can be used as PDT agents by produce O₂ via a multistate sensitization process, simultaneously allowing imaging and providing a highly efficient cancer therapy. However, the high O₂ generation yield of GQDs was achieved only in the visible light region [292]. To overcome this issue, it is highly

desirable to develop NIR remote-controllable GQDs-based system which can be used in cancer treatment, which has not been well addressed and remains a great challenge. Under 790 nm NIR irradiation, our fabricated nitrogen-doped GQDs can convert optical energy to heat and act as a photothermal therapy (PTT) agent. The nitrogen-doped fluorescent GQDs with photothermal property not only can serve as a nanocarrier and photothermal agent capable of converting optical energy to heat effectively under NIR irradiation allowing to trigger siRNA and DOX release to improve the therapeutic effect, but can also be used as bio-imaging agent in cellular microenvironments. Despite the wide use of NIR light as a considerable part of the advanced therapeutic strategies, the exploration of the use of NIR light based on GQD for synergistic chemo-gene-phototherapy has not yet been demonstrated to the best of our knowledge.

In this study, we report the synergistic treatment of pancreatic cancer with functionalized GQDs which serve as a multifunctional platform for simultaneous drug and siRNA delivery combined with NIR light-triggered release (**Fig 6.1**). Negatively charged nitrogen-doped GQDs were successfully synthesized through the hydrothermal method with citric acid as the precursor and urea as the nitrogen source. The well-defined BCPVs were synthesized by ring-open polymerization and thiol-ene functionalization. The tertiary amine groups grafted on biodegradable polylactide backbone endowed the BCPVs with positive charge. Then, the GQDs were functionalized in a step-by-step manner with DOX, BCPV, and siRNA to form a multifunctional co-delivery system. Firstly, the low molecular weight hydrophilic positively charged DOX was grafted onto the negatively-charged sheet-like GQDs by electrostatic and π - π stacking interaction. Secondly, a facile procedure was used to functionalize BCPVs onto the GQDs/DOX (negative charge) via electrostatic interaction. The introduction of BCPVs not only endowed GQDs with biocompatibility, stability and low cytotoxicity, but also rendered them with positive charge which favorably interacts with siRNA molecules for easily assimilation by cells. Finally, the siRNA was gently mixed with GQD/DOX/BCPV nanocomplexes (positively charged) to form multifunctional GQD/DOX/BCPV/siRNA

nanocomplexes. The delivery efficiency of DOX and siRNA by GQDs was evaluated on the pancreatic cancer cell line, MiaPaCa-2. Based on the NIR-enhanced DOX and siRNA release from GQDs nanocomplexes, the synergetic treatment effect of GQDs/DOX/BCPV/siRNA nanocomplexes on pancreatic cancer was investigated.

Figure 6.1 Schematic illustration of synthesis of GQD/DOX/BCPV/siRNA as NIR triggered gene and drug co-delivery system for efficient pancreatic cancer therapy.

6.2 Experimental Methods

6.2.1 Synthesis and characterization of nitrogen-doped GQD

The nitrogen-doped GQDs were synthesized using a hydrothermal method described earlier[293, 294], using citric acid as the precursor and urea as the nitrogen source. Briefly, 9 g of urea ((NH₂)₂CO, Sigma Aldrich) and 6 g citric acid (C₆H₈O₇, Sigma Aldrich) were dissolved in 100 ml ultrapure water at room temperature under stirring at 500 rpm for 5 minutes. The hydrothermal process was conducted in a stainless-steel autoclave with an inner Teflon liner for 4 h at a maintained temperature of 140°C. The pressure of the autoclave was maintained at about 1.5 to 2 MPa during the

whole duration of the hydrothermal process. To investigate the intrinsic crystal structure, transmission electron microscopy (JEM-3000F, JEOL, Tokyo, Japan) was used while the optical properties of the GQDs were analyzed using a Shimadzu UV-2450 spectrophotometer and Fluorolog-3 spectrofluorometer (Edison, NJ USA), obtaining absorption and PL spectra respectively. The zeta potential of the particles was measured using a Brookhaven 90Plus (Brookhaven Instruments Corporation).

6.2.2 Synthesis and characterization of biodegradable charged polyster-based vectors (BCPV)

To prepare BCPVs, a precursor of allyl functional polylactides (APLAs) needs to be first synthesized via ring-opening polymerization (ROP). Briefly, allyl functional lactide (ALA, 800 mg; 4.7 mmol; 35 eq), lactide (LA; 676 mg; 4.7 mmol; 35 eq), benzyl alcohol (BnOH; 14.5 mg; 0.134 mmol; 1 eq) and 4-dimethylaminopyridine (DMAP; 65.3 mg; 0.536 mmol; 4 eq) were introduced to a 10 mL polymerization flask, followed by adding 2 mL of dry dichloromethane (DCM). Sequentially, the temperature of the flask was controlled at 35°C using an oil bath. The polymerization reaction time was controlled for 7 days, at which the conversion of the ALA and LA monomers was higher than 90%. After the reaction, the resulting products were precipitated from cold methanol to remove the unreacted monomers and catalysts, followed by the analysis of $^1\text{H-NMR}$. The $^1\text{H-NMR}$ result indicated the APLA had a molecular structure of $\text{poly}(\text{ALA}_{0.43}\text{-co-LA}_{0.57})_{64}$. Subsequently, the APLA was converted to BCPVs through thiol-ene click functionalization. First, $\text{poly}(\text{ALA}_{0.43}\text{-co-LA}_{0.57})_{67}$ (100 mg; $[\text{ene}]_0 = 0.277$ mmol; 1 eq), 2-(diethylamino)ethanethiol hydrochloride (DEAET; 140 mg; $[\text{SH}]_0 = 0.831$ mmol; 3 eq) and 2,2'-Dimethoxy-2-phenylacetophenone (DMPA; 28.2 mg; 0.110 mmol; 0.4 eq) were introduced in a 10 mL reaction flask, followed by adding 3 mL of chloroform to dissolve all the reactant. Such reactant feeding amount led to the molar ratio of $[\text{ene}]_0:[\text{SH}]_0$ being equal to 1:3 in the reaction. Then, the flask was exposed to UV light (wavelength $\lambda_{\text{max}} = 365$ nm; 6 W; 0.12 A) for 30 min. After the UV irradiation, the resultants were dialyzed against acetone (MW cut-off: 3.5 kDa) to remove any unwanted impurities. After the dialysis, BCPVs were obtained and

characterized by $^1\text{H-NMR}$ to acquire their charge density. The result showed that the BCPV had a charge density of 43% relative to the PLA backbone.

6.2.3 Conjugation and characterization of GQD/DOX/BCPV/siRNA nanocomplexes

There are 3 stages of conjugation in the formation of the GQD/DOX/BCPV/siRNA nanocomplexes. In the first stage, GQDs are mixed with DOX for 24 hours. Next, the GQD/DOX nanocomplexes were dialyzed against ultrapure DI water for 48 hours. The loading efficiencies of the GQD/DOX were determined by measuring the absorbance of the GQD/DOX nanocomplexes followed by comparing the obtained absorbance spectra with the characteristic absorbance spectra of GQDs and DOX. In the second stage, the dialyzed GQD/DOX nanocomplex formulation was mixed with BCPV at different weight ratios for 1 hour. In the last stage of conjugation, different weight ratios of GQD/DOX/BCPV and siRNA were mixed for 30 minutes before being analyzed using agarose gel electrophoresis. The zeta potential was analyzed using a Brookhaven 90Plus (Brookhaven Instruments Corporation), while the particle size of the GQD/DOX/BCPV/siRNA nanocomplexes were observed using TEM.

6.2.4 Cell cytotoxicity assessment

Human pancreatic cancer cells MiaPaCa-2 (pancreatic cancer cell line) as well as Panc-1 (pancreatic cancer cell line), A549 (Lung cancer line), HepG2 (Hepatocellular carcinoma cell line) obtained from American Type Culture Collection were used in this study. The cells were maintained in culture with Dulbecco's Modified Eagle's Medium (DMEM, Hyclone), supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units/ml of penicillin (Gibco) and 100 $\mu\text{g/ml}$ of streptomycin (Gibco) with a 37°C , humidified atmosphere containing 5% carbon dioxide as described previously [268]. According to previous experience [13, 156], four different cell lines, namely A549, HepG2, PANC1 and MiaPaCa-2 cells, were seeded in 96-well plates with a cell count of 5×10^3 per well, leaving them to adhere for 24 hours. The cells were treated with different concentrations of GQDs and BCPVs for 48 hours. The cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) assay kit. The cell viability was obtained by normalizing the absorbance of the sample well against that from the BLANK well and was expressed in terms of percentage. The non-treated cells were assigned with 100% viability.

6.2.5 Transfection efficacy

One day before transfection, MiaPaCa-1 cells were seeded onto 6-well plates in DMEM medium with 10% FBS to give 30%-50% confluence at the time of transfection. K-ras siRNA (sense: 5'-FAM-GUUGGAGCUUGUGGCGUAGUU-3'; Antisense: 5'-CUACGCCACAAGCUCCAACUU-3')) was mixed with GQD/DOX/BCPV nanocomplexes with a gentle vortex and left to rest for 1 hour. Then, the above GQD/DOX/BCPV/siRNA nanocomplexes were then added into the 6-well plates and left for incubation for 6 hours. Then, some of the cells were imaged using an inverted fluorescence microscope (Nikon Eclipse-Ti). Naked GQDs, GQD/BCPV, DOX, GQD/DOX/BCPV, GQD/BCPV/siRNA^{FAM} were used in a separate parallel experiment at the same dosage level. The gene expression was monitored using quantitative real-time PCR at 48 hours post-transfection and western blot at 72 hours post-transfection. A Nikon Eclipse-Ti inverted microscope were used to capture in vitro fluorescence images. After four hours of incubation with GQD/DOX/BCPV/siRNA^{FAM} nanocomplexes, the transfected cells were washed thrice with PBS followed by fixation with 4% formaldehyde. Subsequently, the cells were incubated with DAPI (sigma) for nuclear counterstaining. The filter for the inverted microscope was set for GQDs (excitation at 405 nm and the emission was collected with a 450/50 nm band pass filter), FAM (excited with a 488 nm laser and the emission was collected with a 525/50nm band pass filter) and DOX (excited with 540 nm and emission was collected with a band pass filter 605/50 nm). The fluorescence intensity was determined using a FACScalibur flow cytometer (Becton Dickinson, Mississauga, CA). The cells were first washed with phosphate-buffered solution (PBS) trice before harvested by trypsinization. To quantify the fluorescence intensity, GQD, FAM and DOX served as

the luminescent markers with filter set respectively for Buv395, FITC and PE to collect fluorescence signals, respectively.

6.2.6 Gene expression analysis

48 hours after transfection, the total RNA was extracted from MiaPaCa-2 cells using TRIzol reagent (Invitrogen) and quantified using a micro-spectrophotometer (Nano-Drop ND-1000). 2 µg of total RNA was reverse transcribed to cDNA using reverse transcriptase kit from Promega in accordance with manufacturer's instructions. The mRNA levels of the targeted genes were measured by real time PCR normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used were as follows: K-Ras: 5'-AGAGTGCCTTGACGATACAGC-3' (sense), 5'-ACAAAGAAAGCCCTCCCCAGT-3' (antisense); GAPDH: 5'-ACCACAGTCCATGCCATCAC-3' (sense), GAPDH 5'-TCCACCACCCTGTTGCTGTA-3' (antisense). The PCR products were electrophoresed in a 1.2% GelRed stained agarose gel and observed under UV light illumination.

6.2.7 Anticancer effects *in vitro*

The effect of GQD/DOX/BCPV/siRNA nanocomplexes on proliferation of MiaPaCa-2 can be determined by the MTT assay as described above. The effect of cell migration was determined using the wound-healing assay. Briefly, 10⁴ of MiaPaCa-2 cells were seeded in 24-well plates followed by transfection with GQD/DOX/BCPV/siRNA nanocomplexes for 48 hours, in which the cells almost achieved confluence. Then, a scratch wound was created on the surface containing the cells using a sterile pipette tip. The cells were incubated in a complete culture medium and imaged using an inverted fluorescence microscope. The rate of wound healing was determined by evaluating the distance of migration in the scratch wound and was assessed at 0, and 48 hours (0 h is the time of scratching). NIS Elements Br Analysis 4.0 software was used to analyze the migration distance. The invasive capability of the MiaPaCa-2 cells transfected with GQD/DOX/BCPV/siRNA nanocomplexes was determine using an 8 µm BD BioCoat Matrigel invasion chamber assay system

(BD Biosciences) according to the manufacturer's protocol with minute modifications. Concisely, after 48 hours, GQD/DOX/BCPV/siRNA nanocomplexes-transfected MiaPaca-2 cells were seeded in the upper chamber of the system which contained FBS-free medium. The bottom chamber was filled with 10% FBS medium which serve as chemoattractant. The cells were incubated for a further 48 hours. Next, the cells in the upper chamber were removed with swabs followed by staining the cells that invaded the Matrigel matrix membrane with Giemsa staining in PBS for 5 minutes. The number of invaded cells were viewed and calculated under the microscope in five random views. The apoptosis rates of the transfected cells were determined using a FITC Annexin V Apoptosis Detection Kit I (BD PharmingenTM) according to manufacturer's protocol. Annexin V is widely used as an early apoptosis indicator as it has a strong affinity to the membrane phospholipid phosphatidylserine, which is widely available in the exterior of apoptotic cells. PI is a type of dye to identify the cells with a ruptured membrane and commonly used to classify dead and damaged cells. In short, approximately 10^6 MiaPaCa-2 cells were seeded in a six-well plate, followed by transfection with GQD/DOX/BCPV/siRNA nanocomplexes for 72 hours. The cells were then collected and washed twice with cold PBS and suspended in binding buffer. Then, flow cytometry analysis was used to determine the apoptotic cells.

6.2.8 NIR triggered DOX and siRNA release in cells from nanocomplexes

MiaPaCa-2 cells were seeded in a six-well plate with a density of 10^5 cells per well with DMEM culture medium, subsequently followed by transfection with GQD/DOX/BCPV/siRNA for four hours. Before illumination, the treated cells were rinsed thrice with PBS buffer to remove any free nanocomplexes. The multimode fiber coupled diode lasers with a 0.22 numerical aperture (NA) with a straight cleaved fiber end was used to illuminate the cells with NIR light. The output power was adjusted to achieve different power densities. The duration of illumination was fixed at 15 minutes. The fluorescence intensity from the cells was determined using flow cytometry.

6.2.9 Photothermal Effect of GQD/BCPV for Cancer Cells.

MiaPaCa-2 cells were seeded in a six-well plate with a density of 10^5 cells per well with DMEM culture medium, subsequently followed by transfection with GQD/BCPV for four hours. Next, the cells were rinsed thrice with PBS buffer to remove all free nanocomplexes before being illuminated from 2 W/cm^2 - 32 W/cm^2 with 780nm NIR laser for 15 minutes. The cells were being stained with PI (Propidium Iodide) to evaluate cell viability.

6.3 Results and Discussion

The nitrogen-doped GQDs were synthesized using the hydrothermal method described earlier (**Fig 6.2a**) [293]. In **Figure 6.2b**, the UV-VIS spectrum of GQDs exhibited a major absorption peak at 365 nm that assigned to the $n\text{-}\pi^*$ transition, as reported earlier[294]. The emitted blue luminescence can be viewed under UV lamp illumination, as shown in the inset of **Fig 6.2b**. HRTEM results showed that the bare GQDs had an average particle size of 28 ± 10 nm (**Fig 6.2c**). The synthesized nitrogen-doped GQDs exhibit an excitation-wavelength dependent photoluminescence (PL), and the most intense fluorescence PL peak at 440 nm upon 300 nm excitation (**Fig 6.2d**). As described in our previous report [294], the PL emission of the GQDs originates from the sp^2 clusters that are isolated within a sp^3 C-O matrix. Pyridinic-N and pyrrolic-N configurations contribute one or two electrons, respectively to π bond at GQD sp^2 as delocalized electron. These delocalized electrons are excited to π^* bonds with the existing π electrons and subsequently recombine with holes in the π bonds at GQDs sp^2 . On the other hand, the BCPV was synthesized according to previous report(**Fig 6.3a**) [283]. The resulting chemical structure of the BCPV was characterized using ^1H NMR. The ^1H NMR spectrum of BCPV was shown in **Fig 6.3b**. The average degree of polymerization (DP_n) of 64 was calculated using previously described methods [14]. The result showed that the BCPV had a charge density of 43% relative to the PLA backbone. In order to be able to perform easy coating on the GQDs, the molecular weight and positive charge of BPCV in this part is less than the previous chapters, which was achieved by controlling the reaction time in the synthetic process. Due to the presence of tertiary

amine-based cationic groups, BCPV has a high positive charge value of $+62 \pm 2.2$ mV. The intrinsic toxicity of GQDs and BCPV was evaluated via MTT assays on four different cell lines. The results showed that the cells still possess over 90% viability even at dosages of up to 4 mg/ml and 160 μ g/ml for GQDs and BCPV, respectively, after 48-hour post incubation (**Figure 6.4a**). This result clearly indicated that both GQDs and BCPV are highly biocompatible across a large dosage range.

Figure 6.2 (a) The nitrogen-doped GQDs were synthesized using a hydrothermal method. (b) The absorption spectra of the naked GQDs. The inset shows the naked GQDs imaged under white light and UV illumination. (c) TEM image of the naked GQDs. (d) Photoluminescence spectra of the GQDs, showing a PL emission peak at 440 nm, under 300 nm wavelength excitation.

For the efficient loading of biomolecules, it is important to optimize the weight ratios among GQDs, DOX, BCPV and siRNA as well as the sequence of conjugation order. Firstly, to determine the amount of conjugated DOX on the GQDs, two calibration curves with varying concentrations of GQDs and DOX were first prepared. GQDs were mixed with DOX for 24 hours before dialyzing it

against DI water for 48 hours. Subsequently, the absorbance spectra of the GQD/DOX nanocomplex was evaluated. It was observed that 1 mg GQD (2.5 mg/ml) was capable of loading 1.6 μ g DOX solution (4.1 μ g/ml). After conjugating with positive charge DOX, the zeta potential of GQDs/DOX nanocomplex was increased from -37 ± 0.9 mV of naked GQD to -16 ± 1.8 mV (**Fig 6.4b**). The negative surface charge of the GQD/DOX nanocomplex allows positively charged BCPV to be subsequently conjugated via electrostatic interaction. The GQD/DOX nanocomplex solution was mixed with BCPV at different weight ratios, and the resulting solution was left undisturbed for 4 h for conjugation. The BCPV conjugation efficiency of the resulting GQD/DOX/BCPV was evaluated by a 1.2% electrophoresis agarose gel. It was observed that the weight ratio of for GQD/DOX:BCPV at 1:0.12 is the optimized weight ratio as most of the GQD/DOX/BCPV (zeta potential: 43 ± 5.1 mV) migrated towards negative electrode (**Fig 6.4b and Fig 6.5a**). Similarly, agarose gel electrophoresis was also used to determine the optimum weight ratio of siRNA and GQD/DOX/BCPV nanocomplexes. In this process, GelRed, a type of siRNA stain, served as the indicator of the negative free siRNA running into agarose gel that would be attracted to the positive electrode. The optimized GQD/DOX/BCPV nanocomplex was mixed with siRNA at different weight ratios, ranging from 150:1 to 500:1. It can be observed that most of the siRNA has been fully retarded in the wells at weight ratio of 250:1 (**Fig 6.5b**). Hence, this optimized weight ratio was adopted in the subsequent experiments. The GQD/DOX/BCPV/siRNA nanocomplex (weight ratio at 250:1) had a particle size of 65 nm and zeta potential of 30 ± 3.4 mV (**Fig 6.4b**). To further investigate the photostability and chemical stability of the GQD/DOX/BCPV/siRNA nanocomplex, the nanocomplex was dispersed in different solution such as PBS, DMEM, DMEM+10% FBS, and 70% FBS for 48 hours. No significant fluorescence quenching and aggregation of the nanocomplex was observed, demonstrating the high colloidal stability of the nanocomplex in various biological solutions (**Fig 6.6**).

Figure 6.3 (a) Synthesis of BCPV via ROP (ring-open polymerization) and thiol-ene functionalization. (b) The chemical structure of the BCPV was confirmed from the NMR spectra (500 MHz ^1H NMR spectra of BCPV in CDCl_3).

Figure 6.4 (a) The cytotoxicity of GQDs and BCPV on four different cell lines. The cell viability was evaluated by MTT assay after 48h incubation with BCPV or GQDs. Blank was the negative control group. Results are represented as means $\pm$ SD, $n = 5$. (b) The zeta potential changes after step by step functionalization of naked GQDs with DOX, BCPV and siRNA. TEM image of the GQD/DOX/BCPV/siRNA nanocomplexes (inset), scale bar: 25nm.

Figure 6.5 (a) Agarose gel electrophoresis of BCPV:GQD/DOX at different weight ratio. (b) Agarose gel electrophoresis of GQD/DOX/BCPV:siRNA at different weight ratio.

Figure 6.6. GQD/DOX/BCPV/siRNA nanocomplexes in different solvents under UV illumination.

To demonstrate the transfection efficiency of the GQD/DOX/BCPV/siRNA^{FAM} nanocomplexes, MiaPaCa-2 cells, a pancreatic cancer cell line, were treated with the different formulations including naked GQDs, GQD/BCPV, DOX, GQD/DOX/BCPV, GQD/DOX/siRNA^{FAM} and GQD/DOX/BCPV/siRNA^{FAM}. **Figure 6.7** shows the color coded fluorescent signals from GQDs, DOX and siRNA^{FAM} in MiaPaCa-2 cells after they were treated with different formulations for 4 h. It was clearly observed that the uptake amount of GQDs was significantly enhanced by the BCPV coating. This is due to the change of the surface charge property of GQD/BCPV nanocomplex from negative (naked GQD) to positive, as positively charged nanocomplex would easily penetrate the negatively charged cell membrane. Meanwhile, the fluorescence intensity of DOX and siRNA inside the cells were also significantly increased when BCPV-modified GQDs were used as nanocarriers, proving a higher cellular uptake efficiency of GQD/DOX/BCPV, GQD/BCPV/siRNA^{FAM}, and GQD/DOX/BCPV/siRNA^{FAM} nanocomplexes. The DOX and siRNA fluorescence signals overlapped well with the fluorescence from GQDs which clearly suggests that GQDs can serve as a nanocarrier for co-delivery DOX and siRNA into cells. The fluorescence signals from the GQDs could render intracellular tracking capability and offers possibility for image-guided drug delivery and imaging tumour through enhanced permeability and retention effect. In order to further quantify the transfection efficiency of DOX and siRNA^{FAM}, flow cytometry was employed, and the results are shown in **Fig 6.8**. **Fig 6.8a** displays the representative fluorescence plots of MiaPaCa-2 cell treated with different formulations. The naked GQDs, GQD/BCPV and DOX alone are introduced as negative controls. As shown in **Fig 6.8b**, the cells treated with the GQD/DOX/BCPV/siRNA^{FAM} nanocomplexes present robust DOX and FAM fluorescence and their corresponding fluorescence signal intensity are estimated to be 3477 a.u (DOX) and 1806 a.u (FAM), respectively, which are much higher than these of the cells treated with free DOX and naked siRNA (not shown here) treated groups. Moreover, there are negligible differences of the fluorescence intensity of DOX between GQD/DOX/BCPV (3425 a.u) and GQD/DOX/BCPV/siRNA (3477 a.u) nanocomplexes-treated

tumour cells, as GQD/BCPV/siRNA^{FAM} and GQD/DOX/BCPV/siRNA^{FAM} nanocomplexes-treated cell groups, they exhibit similar fluorescent intensity of FAM. These flow cytometry results are consistent with those obtained from previous cell fluorescence imaging in **Fig 6.7**, and further demonstrated that GQD-based nanocomplexes can effectively co-deliver DOX and siRNA into pancreatic cancer cells.

Figure 6.7 Fluorescence microscopy images (20×magnification) of MiaPaCa-2 cells treated with different formulations. The blue signal originates from the PL of the GQDs while the red signal originates from DOX. The fluorescence signal from the FAM labeled siRNA is rendered in green. For the GQD/DOX/BCPV/siRNA treated group, the final concentration of DOX was fixed at 1.6 µg/ml and siRNA was fixed at 4 µg/ml, and these concentrations are all used in the following cell related experiments.

Figure 6.8 (a) Flow cytometry evaluation on the transfection efficiencies of MiaPaCa-2 cells transfected with GQDs, GQD/BCPV, DOX, GQD/DOX/BCPV, GQD/BCPV/siRNA^{FAM} and GQD/DOX/BCPV/siRNA^{FAM} with a non-treated BLANK group as reference. **(b)** Quantifying results of the flow cytometry analysis showing the fluorescence intensity of each group. The average fluorescent intensity is given in arbitrary units (a.u.).

In order to verify the knockdown efficiency of the GQD/DOX/BCPV/siRNA nanocomplexes, the transcription level of K-ras gene of treated MiaPaCa-2 cells was quantified through real-time PCR (polymerase chain reaction). By using GAPDH gene as the reference, the mRNA levels of K-ras gene in different groups were measured and normalized to the BLANK group. The results indicate that the cells treated with GQDs, BCPV, DOX and GQD/BCPV formulations showed no significant difference in the transcription level of the mutated K-ras gene as compared with the BLANK group.

On the other hand, the group treated with GQD/DOX/BCPV/siRNA nanocomplex exhibited higher K-ras transcription inhibition efficiency compared to the groups treated with other nanocomplexes (**Fig 6.9a**). The K-ras gene, encoding a membrane-bound guanosine triphosphate (GTP)-binding protein, is responsible in mediating various cellular functions such as the cell proliferation and cellular survival. K-ras mutation at codon 12 promotes cell proliferation, transformation and anti-apoptotic through multiple cell signaling pathways, eventually causing cell malignant transformation [241]. On the other hand, DOX functions by binding to DNA-associated enzymes, such as topoisomerase enzymes II, and inducing a range of cytotoxic effects that result in DNA damage. The apoptosis pathway is triggered when attempts to repair the DNA damage fails and the proliferation is inhibited at phase G₁ and G₂ [295]. In this study, the treatment effect of the GQD/DOX/BCPV/siRNA on pancreatic cancer were then systematically evaluated. In order to investigate the anti-proliferative effects of GQD/DOX/BCPV/siRNA nanocomplexes, the MTT assay was performed to analyze the ability of the nanocomplex to inhibit the growth of MiaPaCa-2 cells. **Fig 6.9b** shows that the co-delivery of DOX and siRNA into the cells via GQD/DOX/BCPV/siRNA remarkably reduced the cell proliferation by $67 \pm 8.2\%$ compared with the BLANK group. Moreover, the apoptosis of the treated MiaPaCa-2 cells was further evaluated by FITC labeled Annexin V and Propidium iodide (PI) staining. Annexin V has been widely used as an early apoptosis indicator depending on its high affinity to the membrane phospholipid phosphatidylserine, which is exposed to the external cellular environment in apoptotic cells. PI is a vital dye to label dead and damaged cells, and will be excluded by intact membrane of healthy cells. Our results indicated that the cell group treated with GQD/DOX/BCPV/siRNA nanocomplex have higher apoptotic cell population ($28 \pm 1.9\%$) as compared to the BLANK ($2.5 \pm 0.6\%$), GQD/BCPV ($2.3 \pm 0.5\%$) and DOX ($10.3 \pm 1.9\%$) groups, indicating that GQD/DOX/BCPV/siRNA nanocomplex treatment promoted more cells into apoptosis (**Fig 6.9c and 6.9d**).

Figure 6.9 (a) The cell groups were treated with different formulations, and the gene knockdown efficiency of the K-ras oncogene was evaluated by real time RT-PCR assay 48 h post-treatment. (b) Control of cell proliferation as a consequence of K-ras oncogene suppression. Results are represented as means $\pm$ SD, $n = 5$; *, $P < 0.01$. (c,d) Flow cytometry analysis showing apoptotic state of Annexin V and PI co-stained MiaPaCa-2 cells treated with different formulations, 72 hours post treatment. Cells presenting higher fluorescence signals for both FITC and PI staining in the flow cytometry micrographs were representative of the apoptotic cell count.

Figure 6.10 The cell migration and invasion inhibited by GQD-based nanocomplexes. (a) Wound healing assay in MiaPaCa-2 cell culture treated with different formulations. Phase contrast microscope images (10×magnification) of the wound healing process monitored for 48 hours post-treatment. (b) The cell invasion assay (below) based on a Transwell chamber model of the MiaPaCa-2 cells after treatment with different formulations. Bright field microscope images (20×magnification) of the cells that penetrated through the Matrigel-coated membrane in the transwell chamber. The cells were stained with trypan blue. (c) Quantitative evaluation on the percentage of the wound window closed after treatment, values were normalized by the initial wound window width. (d) Quantitative cell counts of the cells that penetrated through the Matrigel-coated membrane. Each bar represents five random microscopic fields under 20× magnification. Blank cells were untreated. Results are represented as means $\pm$ SD, $n = 6$; **, $P < 0.01$.

Metastatic is a primary cause of death in pancreatic cancer patients, and involves a complicated multi-step process which starts with the migration and invasion of the cancer cells to the adjacent tissues [296]. Hence, cancer treatments which are capable of reducing cell migration and invasion are very important for combating pancreatic cancer. By creating a wound on the monolayer of MiaPaCa-2 cells, the cell migration can be evaluated by monitoring the width of the wound after 48 hours of treatment with different formulations (**Fig 6.10a and 6.10c**). In the non-treated blank group, a decrease in the wound window width could be clearly observed after 24 hours. The cells started to migrate to the open space in-between the wound margins and the previously straight wound boundaries became cluttered. Similar phenomena were also observed for the GQD/BCPV and DOX treated groups. As comparison, an obvious suppressed wound healing was observed in the GQD/DOX/BCPV/siRNA treated group. On the other hand, the invasion capability of MiaPaCa-2 cells was assessed using a Matrigel invasion chamber assay. As shown in **Figure 6.10b and 6.10d**, the population of GQD/DOX/BCPV/siRNA-treated cells that managed to penetrate the invasion chamber was significantly lower than that of groups treated with other formulations. All these results suggest that the GQD/DOX/BCPV/siRNA combinational treatment have an enhanced synergistic anti-cancer performance on pancreatic cancer.

It has been reported that GQDs could also be used as photodynamic therapy agent, but the photothermal therapy using GQDs via NIR-triggered drug release for cancer treatment has not been reported [138]. Hence, after confirming the treatment effect of GQD/DOX/BCPV/siRNA nanocomplex, we next continued to evaluate the *in vitro* therapeutic effects of these nanocomplexes as photothermal agents and as drug carriers. In this study, the temperature of the GQD/BCPV solution (16 $\mu\text{g/ml}$ GQDs) can increase by 22°C upon 15 minutes of NIR laser irradiation with the beam size of 0.5 mm^2 (780 nm, 2 W/cm^2) (**Fig 6.11a**). In order to be consistent with the previous experiments, the final concentration of the GQD/BCPV (weight ratio: 0.12:1) was fixed at 1 mg/ml in the following experiments. The photothermal therapeutic effect of GQD/BCPV were investigated by exposing

transfected cells to NIR of different power densities for 15 minutes. Then, the cell viability of the groups treated with different power densities were evaluated by MTT assay. It was observed that the inhibition efficiency of cell viability was enhanced when the NIR power density was gradually increased. In comparison with the cells without treatment, the cell viability was negligibly reduced to be $97.7 \pm 10.21\%$ at 2 W/cm^2 NIR irradiation, and significantly reduced to $30.7 \pm 6.99\%$ at 32 W/cm^2 NIR irradiation (**Fig 6.11b**). We also directly observed this photothermal effect by staining the irradiated cells with PI, thus only dead cells will be stained. A distinct regions of cell death were observed as depicted in **Fig 6.11c**. In contrast, the MiaPaCa-2 cells without GQD/BCPV treatment were not affected by the NIR laser irradiation at 32 W/cm^2 . Nevertheless, the use of NIR irradiation with such high power density may not be practical for subsequent clinical translation as the used laser power is much higher than the maximum permissible exposure (MPE) of $1\text{-}2 \text{ W/cm}^2$ [297]. Hence, the GQDs need to be further engineered in a way that the absorption and photothermal conversion efficiency can be within the MPE limitation. However, we found that functionalization of GQDs with DOX, BCPV, or siRNA does not influence their photothermal conversion efficiency (**Fig 6.12a**). Meanwhile, after the GQD/DOX/BCPV/siRNA nanocomplex were dispersed in different biological solutions and irradiated with NIR light at 2 W/cm^2 for 15 minutes, similar temperature increased profile was observed (**Fig 6.12b**). This clearly demonstrated that the stable of photothermal property of the GQD/DOX/BCPV/siRNA nanocomplex in different biological solutions.

To further investigate the light-driven drug delivery profile of the nanocomplex system, MiaPaCa-2 cells were treated with GQD/DOX/BCPV/siRNA nanocomplexes for 6 hours (the optimized reaction time), then the treated cells were irradiated with 790 nm NIR light for 15 minutes. Next, the synergistic treatment effect on the MiaPaCa-2 cell was systematically studied. The MiaPaCa-2 cells treated with GQD/DOX/BCPV/siRNA nanocomplexes followed by NIR irradiation (15 minutes, 2 W/cm^2) presented higher fluorescence brightness originating from siRNA^{FAM} and DOX when compared to cells treated with GQD/DOX/BCPV/siRNA nanocomplexes (**Fig 6.13a**).

Figure 6.11 (a) The increased temperature of the GQD/BCPV nanocomplex formulation of various concentrations upon irradiation with NIR laser light (780nm, 2 W/cm², 15 minutes). (b) The summary of cell viability treated synergistically with GQD/BCPV under different power of NIR laser irradiation. (c) Microscopy images (4×magnification) of MiaPaCa-2 cells treated with GQD/BCPV subjected to NIR laser (780 nm, 32 W/cm², 15 minutes) illumination. The red fluorescence region indicates dead cells stained by propidium iodide.

Figure 6.12. (a) The increased temperature of the various GQD-based nanocomplexes formulation after being irradiated with 780 nm NIR light (2 W/cm²) up to 15 minutes. (b) The temperature increase of the GQD/DOX/BCPV/siRNA^{K-ras} nanocomplex formulation in different medium after being irradiated with NIR light (2 W/cm²) irradiation up to 15 minutes.

Figure 6.13 (a) Fluorescence images (20×magnification) of MiaPaCa-2 cells transfected by GQD/DOX/BCPV/siRNA^{K-ras} with/without NIR light irradiation. (b) Flow cytometry analysis indicating the release of siRNA^{K-ras} and DOX intracellularly in different treatment groups. (c) Quantitative summary indicating the fluorescence intensity of MiaPaCa-2 cells in various treatment groups. Results are represented as means $\pm$ SD, n = 5; *, P < 0.01.

Flow cytometry was further employed to quantify the increase in siRNA and DOX intracellular release from nanocomplex after 2 W/cm² NIR irradiation. The results indicate that, after irradiating the nanocomplex-treated cells, the fluorescence intensity of DOX had increased by 2.2 times while the fluorescence intensity of siRNA had increased by 1.8 times (Figs 6.13b and 6.13c). These results prove that NIR light irradiation can induce the photothermal effect of functionalized GQDs nanocarriers and this allows one to systematically control the release of their payloads. In the next set of experiments, we investigated the K-ras gene knock-down efficiency by PCR method after cells were treatment with nanocomplex and NIR light. It was observed that the NIR irradiation significantly enhanced the inhibition effectiveness of the K-ras gene expression on the nanocomplex-treated MiaPaCa-2 cells (Fig 6.14a). Furthermore, after NIR irradiation, the growth of GQD/DOX/BCPV/siRNA nanocomplex-treated cells was reduced by 82.6% (Fig 6.14b), which is

larger than that obtained for the cells treated with nanocomplexes only (72%). More importantly, the NIR irradiation also promoted more nanocomplex-treated cells to become apoptotic (**Figs 6.14c and 6.14d**). The improved treatment effect is primarily credited to the enhanced intracellular release of the DOX and siRNA from GQD/DOX/BCPV/siRNA nanocomplexes triggered by NIR irradiation.

Figure 6.14 (a) The K-ras expression of MiaPaCa-2 cells treated with different GQD-based formulations after NIR irradiation as detected by real time PCR assay. The inset at the top right corner shows NIR laser irradiation of cells in the culture well as seen using a NIR viewer. (b) After NIR irradiation, the proliferation of MiaPaCa-2 cells treated with different formulations were evaluated by MTT assay. (c) After NIR irradiation, the apoptotic cells in different formulations-treated MiaCaPa-2 cells was assessed by FITC Annexin V and PI co-stained. (d) Summary of the apoptotic rate of MiaPaCa-2 cells treated with different formulation after NIR condition. Results are represented as means $\pm$ SD, $n = 3$; *, $P < 0.01$.

These results clearly indicate that the combination of GQD/DOX/BCPV/siRNA nanocomplexes and NIR light irradiation can lead to a superior synergistic therapeutic effect for pancreatic cancer treatment. All these high biocompatible, low bio-toxicity, and light controllable features of the GQD/DOX/BCPV/siRNA nanocomplexes will allow to design personalized medicine treatment for pancreatic cancer with minimal collateral damage to normal tissues or cells. However, many more systematic anti-cancer studies, as well as some immuno-compatibility studies, still need to be performed *in vivo* before future clinical applications of GQD/DOX/BCPV/siRNA nanocomplex.

6.4 Conclusions

Overall, we have demonstrated that fluorescent GQDs can serve as a multifunctional delivery platform for the co-delivery of siRNA and DOX for pancreatic cancer therapeutic treatment. The expression of K-ras gene in pancreatic cancer cell was effectively suppressed and the cell viability was strongly inhibited. To further improve the therapeutic effect of the GQD-based nanocomplexes, NIR light was successfully employed to promote the intracellular release of DOX and siRNA via the photothermal effect. To the best of our knowledge, this is the first report on using biodegradable polymer-functionalized biocompatible graphene quantum dot-based nanocarrier for simultaneously delivering siRNA and DOX for treating pancreatic cancer *in vitro*. Moreover, the results in this study can be regarded as a breakthrough for treating pancreatic cancer using a combination therapy. The developed GQD/DOX/BCPV/siRNA nanocomplex co-delivery system that successfully integrate chemotherapy, gene therapy, bio-imaging guided and NIR light-mediated biomolecules release would be a promising approach for pancreatic cancer treatment in clinical.

Chapter 7 Conclusions, Ongoing, and Future Work

7.1 Conclusions

This Thesis investigates the synthesis and application of a novel biodegradable polymer, BCPV, as siRNA carrier for cancer treatment. The different treatment strategies of siRNA delivery targeting one or two mutation oncogenes by BCPV as nanocarrier were performed on both adherent and non-adherent growth cell lines. Meanwhile, the BCPV was further used to functionalize QDs to construct siRNA and chemotherapeutic drug multifunctional co-delivery system on the cancer treatment.

In recent years, RNAi has emerged as a promising strategy for the cancer treatment via regulation of the oncogene expression. However, the application of siRNA faces a series of challenges that need to be overcome before RNAi technology can be a feasible clinical practice. The siRNA's negative charge and high molecular weight restrict its entry through the cell membrane into the cytosol. Therefore, it is a crucial step to develop biologically safe and efficient siRNA carrier to achieve an effective targeted gene knock-down. In order to overcome these limitations, a novel biodegradable polymer BCPV, intended to be used as siRNA carrier, was designed and synthesized through ring-opening polymerization and thiol-ene click functionalization. In comparison with the traditional transfection agents, BCPV shows outstanding properties such as high biocompatibility, biodegradability, low cytotoxicity, and high transfection efficiency. Under optimized conditions, BCPV can effectively encapsulate siRNA to form a capsule-like structure nanocomplex, protecting siRNA from being degraded by RNase in the serum, and thus enabling to deliver siRNA into cytosol and assist its escape from endosome. Based on the BCPV's excellent performance in siRNA delivery, we have explored several therapeutic strategies on pancreatic cancer and leukemia cells. The expression of the K-ras mutation gene of pancreatic cancer cells was effectively inhibited after

treatment with BCPV-siRNA nanocomplexes, and the cells viability, proliferation, migration and invasion were clearly repressed.

Chronic Myeloid Leukemia (CML) is a hematopoietic malignant cancer characterized by reciprocal translocation of chromosomes 9 and 22 resulting in the formation of the BCR–ABL fusion oncoprotein. Leukemia cells are non-adhesive growth cells, and well known as difficult-to-transfect cell with the currently available siRNA transfection agents, resulting in reduced contact probability between nanoplexes and the suspended cells in the medium as compared to the adherent cells. The BCPV-siRNA successfully delivered BCR-ABL siRNA into the leukemia cell lines, and effectively inhibited the BCR-ABL gene expression, resulting in the reduction of cell viability and promotion of cell apoptosis. The BCPV provided a versatile platform to deliver siRNA to non-adherent leukemia cells with high transfection efficiency by effectively overcoming extra- and intra-cellular barriers.

Considering the carcinogenesis attribute to the multiple gene mutation, we delivered two kinds of siRNA by BCPV into pancreatic cancer cells for respective targeting of the K-ras and Notch1 genes, resulting in the EMT reversal of MiaPaCa-2 cells, thereby enhancing the sensitivity of MiaPaCa-2 cells to the gemcitabine anti-cancer drug. MiaPaCa-2 cell proliferation, migration, and invasion were effectively inhibited, and cell apoptosis was also significantly enhanced by the synergistic antitumor effect of BCPV-siRNA^{K-ras}-siRNA^{Notch1} nanocomplexes and GEM. These results suggest that this combination RNAi therapy can be used to improve cancer cell sensitivity to chemotherapeutic drugs.

In order to improve the therapeutic effect of RNAi therapy, BCPV was used to modify the GQDs to construct a siRNA and DOX co-loading hybrid-polymer nanoparticle system via π – π interaction and electrostatic binding. This hybrid material has the advantages of addressing multiple functions: siRNA-drug carrier, fluorescent imaging as well as synergistic photothermal therapy. After treatment with GQD/DOX/BCPV/siRNA nanocomplexes, the proliferation, migration and invasion of pancreatic cancer cells were strongly suppressed, and the cell apoptosis was also promoted. The photothermal properties of the GQDs also enable the enhanced intracellular release of DOX and

siRNA from the nanocomplex under irradiation with near-infrared (NIR) laser light, thus enhancing the therapeutic effect.

7.2 Ongoing and future works

The results presented in this Thesis successfully demonstrated that an outstanding anti-cancer effect was achieved via BCPV-siRNA nanocomplexes or BCPV hybrid nanoparticle treatment. However, there are still some factors that need to be addressed in future works before this siRNA delivery system can be used in clinical application.

7.2.1 *In vivo* assessment of therapeutic efficacy

The studies in this Thesis have been performed to investigate the cancer treatment effects of the proposed BCPV-based siRNA delivery systems *in vitro*. Although studies have been presented on how BCPV-siRNA can be utilized for specific inhibition of the oncogene expression, the treatment effect of BCPV-siRNA on the cancer have not yet been estimated *in vivo*. Obviously, siRNA delivery *in vivo* is much more difficult than that of *in vitro* setting due to the complication presented by physiological environments. In general, *in vivo* delivery of siRNA has more barriers compared *in vitro* delivery, as the former directly exposed BCPV-siRNA to enzymatic degradation by nucleases. *In vivo*, the positively charged polymer nanocarriers exhibit time-dependent aggregation as a result of adsorption of serum albumin and other blood proteins. On administration into the bloodstream, the siRNA carriers may be rapidly distributed to organs in the reticuloendothelial system (RES) and phagocytosed by the macrophage cells. Consequently, the nanoplexes tend to accumulate in RES organs (spleen and liver) before reaching any target tissues. After systemic administration, the BCPV-siRNA nanocomplexes need to extravagate from the blood vessels to reach tumor microenvironment region before they are internalized by cellular endocytosis, non-targeting aggregation and clearance. Hence, the evaluation of the siRNA delivery performance of BCPVs *in vivo* is of sheer importance for their clinical applicability and translation. In addition, it is required to elaborate on all of siRNA

delivery properties of BCPVs *in vivo*, including blood circulation time, intratumoral siRNA concentration, release process, pharmacokinetic profiles, and potential side adverse effects.

7.2.2 PEG modification and ligand targeting delivery

The efficacy of the BCPV-siRNA therapy is actually evaluated *in vitro* by directly exposing the cells to the BCPV-siRNA nanocomplex. To promote the potential BCPV application in clinical setting, the therapeutic effect needs to be assessed *in vivo*. Despite having promising transfection efficiency and low toxicity properties, preliminary results indicates that BCPV-siRNA nanocomplexes suffer from some obstacles in the form of low stability, slight aggregation and decreasing transfection efficiency in serum-containing medium. Thus, to overcome these limitations, grafting PEG on the BCPV chemical structure is an efficient approach. This strategy should allow it to avoid the undesirable non-specific interaction with blood negative proteins in the serum. PEGylation of BCPV could enhance the stability of BCPV-siRNA nanocomplex in the bloodstream. PEG modification could also cover the hydrophobic core to protect the nanoparticles from elimination by circulation system, and prolong their performance at the targeted site. In order to improve the amount of siRNA in the targeting tumor region, enhancing the interaction of polymer with tumor cells, prolonging the half-life of siRNA, and allowing cell uptake of siRNA via ligand-receptor regulated endocytosis, the development of ligand-coupled polymer is required. For active targeted siRNA delivery, BCPV could be modified with targeting molecules such as transferrin, folic acid, hyaluronic acid, aptamers and epidermal growth factor and other small molecules to enhance the selective transfection efficiency.

7.2.3 Photodynamic therapy

Photodynamic therapy (PDT) involves irradiation with a specific wavelength of light on photosensitizing agent that under irradiation produces reactive oxygen species (ROS), such as $^1\text{O}_2$ to elicit cancer cells death. PDT surpasses the traditional treatment methods due to its non-invasive

character, negligible drug resistance and low systemic toxicity. However, the current applications are often limited by the low cell uptake of these PDT agents. Hence, it should be meaningful and interesting to investigate BCPV's potential as functionalized polymer to modify photodynamic agents, such as porphyrin, phthalocyanines, graphene quantum dots (GQDs) and bacteriochlorin derivatives, to promote cell uptake, and enhance the PDT effect *in vivo*. This strategy can also be used to form multifunctional nanocomplexes consisting of a drug, siRNA, and a PDT agent for cancer combinational treatment.

References

- [1] R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer statistics, 2016," *CA: a cancer journal for clinicians*, vol. 66, pp. 7-30, 2016.
- [2] B. Stewart and C. P. Wild, "World cancer report 2014," *Health*, 2017.
- [3] C. Holohan, S. Van Schaeybroeck, D. B. Longley, and P. G. Johnston, "Cancer drug resistance: an evolving paradigm," *Nature Reviews Cancer*, vol. 13, pp. 714-726, 2013.
- [4] R. Misra, S. Acharya, and S. K. Sahoo, "Cancer nanotechnology: application of nanotechnology in cancer therapy," *Drug Discovery Today*, vol. 15, pp. 842-850, 2010.
- [5] K. Park, S. Lee, E. Kang, K. Kim, K. Choi, and I. C. Kwon, "New generation of multifunctional nanoparticles for cancer imaging and therapy," *Advanced functional materials*, vol. 19, pp. 1553-1566, 2009.
- [6] J. Shi, P. W. Kantoff, R. Wooster, and O. C. Farokhzad, "Cancer nanomedicine: progress, challenges and opportunities," *Nature Reviews Cancer*, vol. 17, pp. 20-37, 2017.
- [7] J. Conde, A. Ambrosone, Y. Hernandez, F. Tian, M. McCully, C. C. Berry, *et al.*, "15 years on siRNA delivery: Beyond the State-of-the-Art on inorganic nanoparticles for RNAi therapeutics," *Nano Today*, 2015.
- [8] R. Kanasty, J. R. Dorkin, A. Vegas, and D. Anderson, "Delivery materials for siRNA therapeutics," *Nature Materials*, vol. 12, pp. 967-977, Nov 2013.
- [9] J. Wang, Z. Lu, M. G. Wientjes, and J. L.-S. Au, "Delivery of siRNA therapeutics: barriers and carriers," *The AAPS journal*, vol. 12, pp. 492-503, 2010.
- [10] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello, "Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*," *nature*, vol. 391, pp. 806-811, 1998.
- [11] G. Ozcan, B. Ozpolat, R. L. Coleman, A. K. Sood, and G. Lopez-Berestein, "Preclinical and clinical development of siRNA-based therapeutics," *Advanced drug delivery reviews*, 2015.
- [12] G. Lin, R. Hu, W.-C. Law, C.-K. Chen, Y. Wang, H. Li Chin, *et al.*, "Biodegradable Nanocapsules as siRNA Carriers for Mutant K-Ras Gene Silencing of Human Pancreatic Carcinoma Cells," *Small*, vol. 9, pp. 2757-2763, 2013.
- [13] G. Lin, C. Yang, R. Hu, C.-K. Chen, W.-C. Law, T. Anderson, *et al.*, "Interleukin-8 gene silencing on pancreatic cancer cells using biodegradable polymer nanoplexes," *Biomaterials Science*, vol. 2, pp. 1007-1015, 2014.
- [14] C. Yang, R. Hu, T. Anderson, Y. Wang, G. Lin, W.-C. Law, *et al.*, "Biodegradable nanoparticle-mediated K-ras down regulation for pancreatic cancer gene therapy," *Journal of Materials Chemistry B*, vol. 3, pp. 2163-2172, 2015.

- [15] N. Zheng, Z. Song, Y. Liu, R. Zhang, R. Zhang, C. Yao, *et al.*, "Redox-responsive, reversibly-crosslinked thiolated cationic helical polypeptides for efficient siRNA encapsulation and delivery," *Journal of Controlled Release*, vol. 205, pp. 231-239, 2015.
- [16] Z. Wang, Y. Li, D. Kong, S. Banerjee, A. Ahmad, A. S. Azmi, *et al.*, "Acquisition of epithelial-mesenchymal transition phenotype of gemcitabine-resistant pancreatic cancer cells is linked with activation of the notch signaling pathway," *Cancer research*, vol. 69, pp. 2400-2407, 2009.
- [17] H. J. Kim, A. Kim, K. Miyata, and K. Kataoka, "Recent progress in development of siRNA delivery vehicles for cancer therapy," *Advanced drug delivery reviews*, vol. 104, pp. 61-77, 2016.
- [18] A. Birmingham, E. Anderson, K. Sullivan, A. Reynolds, Q. Boese, D. Leake, *et al.*, "A protocol for designing siRNAs with high functionality and specificity," *Nature protocols*, vol. 2, pp. 2068-2078, 2007.
- [19] G. J. Hannon, "RNA interference," *Nature*, vol. 418, pp. 244-251, Jul 2002.
- [20] E. Bernstein, A. A. Caudy, S. M. Hammond, and G. J. Hannon, "Role for a bidentate ribonuclease in the initiation step of RNA interference," *Nature*, vol. 409, pp. 363-366, Jan 2001.
- [21] C. Huang, M. Li, C. Y. Chen, and Q. Z. Yao, "Small interfering RNA therapy in cancer: mechanism, potential targets, and clinical applications," *Expert Opinion on Therapeutic Targets*, vol. 12, pp. 637-645, May 2008.
- [22] J. Wang, Z. Lu, M. G. Wientjes, and J. L. S. Au, "Delivery of siRNA Therapeutics: Barriers and Carriers," *Aaps Journal*, vol. 12, pp. 492-503, Dec 2010.
- [23] D. L. Lewis and J. A. Wolff, "Systemic siRNA delivery via hydrodynamic intravascular injection," *Advanced Drug Delivery Reviews*, vol. 59, pp. 115-123, Mar 2007.
- [24] F. Petrocca and J. Lieberman, "Promise and Challenge of RNA Interference-Based Therapy for Cancer," *Journal of Clinical Oncology*, vol. 29, pp. 747-754, Feb 2011.
- [25] S. L. Ameres and P. D. Zamore, "Diversifying microRNA sequence and function," *Nat Rev Mol Cell Biol*, vol. 14, pp. 475-488, 08//print 2013.
- [26] X. Zhao, F. Li, Y. Li, H. Wang, H. Ren, J. Chen, *et al.*, "Co-delivery of HIF1 α siRNA and gemcitabine via biocompatible lipid-polymer hybrid nanoparticles for effective treatment of pancreatic cancer," *Biomaterials*, vol. 46, pp. 13-25, 2015.
- [27] B. L. Davidson and P. B. McCray, "Current prospects for RNA interference-based therapies," *Nature Reviews Genetics*, vol. 12, pp. 329-340, 2011.
- [28] F. Petrocca and J. Lieberman, "Promise and challenge of RNA interference-based therapy for cancer," *Journal of Clinical Oncology*, vol. 29, pp. 747-754, 2011.
- [29] J. Conde, E. R. Edelman, and N. Artzi, "Target-responsive DNA/RNA nanomaterials for microRNA sensing and inhibition: The jack-of-all-trades in cancer nanotheranostics?," *Advanced Drug Delivery Reviews*, vol. 81, pp. 169-183, Jan 2015.
- [30] J. Conde, A. Ambrosone, Y. Hernandez, F. R. Tian, M. McCully, C. C. Berry, *et al.*, "15 years on siRNA delivery: Beyond the State-of-the-Art on inorganic nanoparticles for RNAi therapeutics," *Nano Today*, vol. 10, pp. 421-450, Aug 2015.
- [31] F. Perche, Y. Yi, L. Hespel, P. Mi, A. Dirisala, H. Cabral, *et al.*, "Hydroxychloroquine-conjugated gold nanoparticles for improved siRNA activity," *Biomaterials*, 2016.
- [32] Y. Wang, C. Yang, R. Hu, H. T. Toh, X. Liu, G. Lin, *et al.*, "Assembling Mn: ZnSe quantum dots-siRNA nanoplexes for gene silencing in tumor cells," *Biomaterials science*, vol. 3, pp. 192-202, 2015.
- [33] K. S. Siu, X. Zheng, Y. Liu, Y. Zhang, X. Zhang, D. Chen, *et al.*, "Single-walled carbon nanotubes noncovalently functionalized with lipid modified polyethylenimine for siRNA delivery in vitro and in vivo," *Bioconjugate chemistry*, vol. 25, pp. 1744-1751, 2014.
- [34] K. Moeller, K. Müller, H. Engelke, C. Brauchle, E. Wagner, and T. Bein, "Highly Efficient siRNA Delivery from Core-Shell Mesoporous Silica Nanoparticles with Multifunctional Polymer Caps," *Nanoscale*, 2015.
- [35] W. Ngamcherdtrakul, J. Morry, S. Gu, D. J. Castro, S. M. Goodyear, T. Sangvanich, *et al.*, "Cationic polymer modified mesoporous silica nanoparticles for targeted siRNA delivery to HER2+ breast cancer," *Advanced functional materials*, vol. 25, pp. 2646-2659, 2015.

- [36] P. Soriano, J. Dijkstra, A. Legrand, H. Spanjer, D. Londos-Gagliardi, F. Roerdink, *et al.*, "Targeted and nontargeted liposomes for in vivo transfer to rat liver cells of a plasmid containing the preproinsulin I gene," *Proceedings of the National Academy of Sciences*, vol. 80, pp. 7128-7131, 1983.
- [37] B. Dalby, S. Cates, A. Harris, E. C. Ohki, M. L. Tilkins, P. J. Price, *et al.*, "Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications," *Methods*, vol. 33, pp. 95-103, 2004.
- [38] M. Zhao, H. Yang, X. Jiang, W. Zhou, B. Zhu, Y. Zeng, *et al.*, "Lipofectamine RNAiMAX: an efficient siRNA transfection reagent in human embryonic stem cells," *Molecular biotechnology*, vol. 40, pp. 19-26, 2008.
- [39] A. D. Judge, V. Sood, J. R. Shaw, D. Fang, K. McClintock, and I. MacLachlan, "Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA," *Nature biotechnology*, vol. 23, pp. 457-462, 2005.
- [40] Z. Ma, J. Li, F. He, A. Wilson, B. Pitt, and S. Li, "Cationic lipids enhance siRNA-mediated interferon response in mice," *Biochemical and biophysical research communications*, vol. 330, pp. 755-759, 2005.
- [41] K. A. Howard, U. L. Rahbek, X. Liu, C. K. Damgaard, S. Z. Glud, M. Ø. Andersen, *et al.*, "RNA interference in vitro and in vivo using a chitosan/siRNA nanoparticle system," *Molecular Therapy*, vol. 14, pp. 476-484, 2006.
- [42] A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain, *et al.*, "A combinatorial library of lipid-like materials for delivery of RNAi therapeutics," *Nat Biotech*, vol. 26, pp. 561-569, 05//print 2008.
- [43] D. Palliser, D. Chowdhury, Q.-Y. Wang, S. J. Lee, R. T. Bronson, D. M. Knipe, *et al.*, "An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection," *Nature*, vol. 439, pp. 89-94, 2006.
- [44] J. E. Zuckerman and M. E. Davis, "Clinical experiences with systemically administered siRNA-based therapeutics in cancer," *Nature Reviews Drug Discovery*, vol. 14, pp. 843-856, 2015.
- [45] J.-M. Williford, J. Wu, Y. Ren, M. M. Archang, K. W. Leong, and H.-Q. Mao, "Recent advances in nanoparticle-mediated siRNA delivery," *Annual review of biomedical engineering*, vol. 16, pp. 347-370, 2014.
- [46] D. Pozzi, V. Colapicchioni, G. Caracciolo, S. Piovesana, A. L. Capriotti, S. Palchetti, *et al.*, "Effect of polyethyleneglycol (PEG) chain length on the bio-nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells," *Nanoscale*, vol. 6, pp. 2782-2792, 2014.
- [47] M. T. Abrams, M. L. Koser, J. Seitzer, S. C. Williams, M. A. DiPietro, W. Wang, *et al.*, "Evaluation of efficacy, biodistribution, and inflammation for a potent siRNA nanoparticle: effect of dexamethasone co-treatment," *Molecular Therapy*, vol. 18, pp. 171-180, 2010.
- [48] S. Warashina, T. Nakamura, Y. Sato, Y. Fujiwara, M. Hyodo, H. Hatakeyama, *et al.*, "A lipid nanoparticle for the efficient delivery of siRNA to dendritic cells," *Journal of Controlled Release*, vol. 225, pp. 183-191, 3/10/ 2016.
- [49] B. Yu, S.-H. Hsu, C. Zhou, X. Wang, M. C. Terp, Y. Wu, *et al.*, "Lipid nanoparticles for hepatic delivery of small interfering RNA," *Biomaterials*, vol. 33, pp. 5924-5934, 2012.
- [50] W. Tao, J. P. Davide, M. Cai, G.-J. Zhang, V. J. South, A. Matter, *et al.*, "Noninvasive Imaging of Lipid Nanoparticle-Mediated Systemic Delivery of Small-Interfering RNA to the Liver," *Molecular Therapy*, vol. 18, pp. 1657-1666, 2010.
- [51] M. Jayaraman, S. M. Ansell, B. L. Mui, Y. K. Tam, J. Chen, X. Du, *et al.*, "Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo," *Angewandte Chemie International Edition*, vol. 51, pp. 8529-8533, 2012.
- [52] C. A. Alabi, K. T. Love, G. Sahay, H. Yin, K. M. Luly, R. Langer, *et al.*, "Multiparametric approach for the evaluation of lipid nanoparticles for siRNA delivery," *Proceedings of the National Academy of Sciences*, vol. 110, pp. 12881-12886, 2013.

- [53] S. C. De Smedt, J. Demeester, and W. E. Hennink, "Cationic polymer based gene delivery systems," *Pharmaceutical research*, vol. 17, pp. 113-126, 2000.
- [54] L. D. Shea, E. Smiley, J. Bonadio, and D. J. Mooney, "DNA delivery from polymer matrices for tissue engineering," *Nature biotechnology*, vol. 17, pp. 551-554, 1999.
- [55] A. Aied, U. Greiser, A. Pandit, and W. Wang, "Polymer gene delivery: overcoming the obstacles," *Drug discovery today*, vol. 18, pp. 1090-1098, 2013.
- [56] J.-P. Nam and J.-W. Nah, "Target gene delivery from targeting ligand conjugated chitosan-PEI copolymer for cancer therapy," *Carbohydrate polymers*, vol. 135, pp. 153-161, 2016.
- [57] S. H. Kim, J. H. Jeong, S. H. Lee, S. W. Kim, and T. G. Park, "Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer," *Journal of controlled release*, vol. 129, pp. 107-116, 2008.
- [58] B. Urban-Klein, S. Werth, S. Abuharbeid, F. Czubayko, and A. Aigner, "RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo," *Gene therapy*, vol. 12, p. 461, 2005.
- [59] C. Zhu, S. Jung, S. Luo, F. Meng, X. Zhu, T. G. Park, *et al.*, "Co-delivery of siRNA and paclitaxel into cancer cells by biodegradable cationic micelles based on PDMAEMA-PCL-PDMAEMA triblock copolymers," *Biomaterials*, vol. 31, pp. 2408-2416, 2010.
- [60] A. J. Convertine, D. S. Benoit, C. L. Duvall, A. S. Hoffman, and P. S. Stayton, "Development of a novel endosomolytic diblock copolymer for siRNA delivery," *Journal of Controlled Release*, vol. 133, pp. 221-229, 2009.
- [61] H. Y. Cho, A. Srinivasan, J. Hong, E. Hsu, S. Liu, A. Shrivats, *et al.*, "Synthesis of biocompatible PEG-based star polymers with cationic and degradable core for siRNA delivery," *Biomacromolecules*, vol. 12, pp. 3478-3486, 2011.
- [62] X. x. Liu, P. Rocchi, F. q. Qu, S. q. Zheng, Z. c. Liang, M. Gleave, *et al.*, "PAMAM dendrimers mediate siRNA delivery to target Hsp27 and produce potent antiproliferative effects on prostate cancer cells," *ChemMedChem*, vol. 4, pp. 1302-1310, 2009.
- [63] M. L. Patil, M. Zhang, and T. Minko, "Multifunctional triblock nanocarrier (PAMAM-PEG-PLL) for the efficient intracellular siRNA delivery and gene silencing," *ACS nano*, vol. 5, pp. 1877-1887, 2011.
- [64] C. L. Waite and C. M. Roth, "PAMAM-RGD conjugates enhance siRNA delivery through a multicellular spheroid model of malignant glioma," *Bioconjugate chemistry*, vol. 20, pp. 1908-1916, 2009.
- [65] Y. Kodama, H. Kuramoto, Y. Mieda, T. Muro, H. Nakagawa, T. Kurosaki, *et al.*, "Application of biodegradable dendrigraft poly-L-lysine to a small interfering RNA delivery system," *Journal of drug targeting*, vol. 25, pp. 49-57, 2017.
- [66] R.-g. Qi, S.-h. Wu, Y. Wang, J. Chen, Z.-g. Xie, Y.-b. Huang, *et al.*, "Contribution of cholesterol moieties attached on MPEG-b-PCL-b-PLL to the cell uptake, endosomal escape and gene knockdown of the micelleplexes of siRNA," *Chinese Journal of Polymer Science*, vol. 31, pp. 912-923, 2013.
- [67] C. Zheng, M. Zheng, P. Gong, J. Deng, H. Yi, P. Zhang, *et al.*, "Polypeptide cationic micelles mediated co-delivery of docetaxel and siRNA for synergistic tumor therapy," *Biomaterials*, vol. 34, pp. 3431-3438, 2013.
- [68] S. M. Noh, M. O. Park, G. Shim, S. E. Han, H. Y. Lee, J. H. Huh, *et al.*, "Pegylated poly-L-arginine derivatives of chitosan for effective delivery of siRNA," *Journal of Controlled Release*, vol. 145, pp. 159-164, 2010.
- [69] P. Sun, W. Huang, M. Jin, Q. Wang, B. Fan, L. Kang, *et al.*, "Chitosan-based nanoparticles for survivin targeted siRNA delivery in breast tumor therapy and preventing its metastasis," *International Journal of Nanomedicine*, vol. 11, p. 4931, 2016.
- [70] Y. Hu, J. Xie, Y. W. Tong, and C.-H. Wang, "Effect of PEG conformation and particle size on the cellular uptake efficiency of nanoparticles with the HepG2 cells," *Journal of Controlled Release*, vol. 118, pp. 7-17, 2007.
- [71] J. Y. Yhee, S. Song, S. J. Lee, S.-G. Park, K.-S. Kim, M. G. Kim, *et al.*, "Cancer-targeted MDR-1 siRNA delivery using self-cross-linked glycol chitosan nanoparticles to overcome drug resistance," *Journal of Controlled Release*, vol. 198, pp. 1-9, 2015.

- [72] S. Hu-Lieskovan, J. D. Heidel, D. W. Bartlett, M. E. Davis, and T. J. Triche, "Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma," *Cancer research*, vol. 65, pp. 8984-8992, 2005.
- [73] J. D. Heidel, Z. Yu, J. Y.-C. Liu, S. M. Rele, Y. Liang, R. K. Zeidan, *et al.*, "Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA," *Proceedings of the National Academy of Sciences*, vol. 104, pp. 5715-5721, 2007.
- [74] T. Liu, W. Xue, B. Ke, M.-Q. Xie, and D. Ma, "Star-shaped cyclodextrin-poly (l-lysine) derivative co-delivering docetaxel and MMP-9 siRNA plasmid in cancer therapy," *Biomaterials*, vol. 35, pp. 3865-3872, 2014.
- [75] J.-M. Li, Y.-Y. Wang, W. Zhang, H. Su, L.-N. Ji, and Z.-W. Mao, "Low-weight polyethylenimine cross-linked 2-hydroxypopyl- β -cyclodextrin and folic acid as an efficient and nontoxic siRNA carrier for gene silencing and tumor inhibition by VEGF siRNA," *Int J Nanomedicine*, vol. 8, pp. 2101-2117, 2013.
- [76] S. J. Lee, J. Y. Yhee, S. H. Kim, I. C. Kwon, and K. Kim, "Biocompatible gelatin nanoparticles for tumor-targeted delivery of polymerized siRNA in tumor-bearing mice," *Journal of Controlled Release*, vol. 172, pp. 358-366, 2013.
- [77] S. R. Youngren, R. K. Tekade, B. Gustilo, P. R. Hoffmann, and M. B. Chougule, "STAT6 siRNA matrix-loaded gelatin nanocarriers: formulation, characterization, and ex vivo proof of concept using adenocarcinoma cells," *BioMed research international*, vol. 2013, 2013.
- [78] J. M. Freire, I. R. de Figueiredo, J. Valle, A. S. Veiga, D. Andreu, F. J. Enguita, *et al.*, "siRNA-cell-penetrating peptides complexes as a combinatorial therapy against chronic myeloid leukemia using BV173 cell line as model," *Journal of Controlled Release*, vol. 245, pp. 127-136, 2017.
- [79] B. Fang, L. Jiang, M. Zhang, and F. Z. Ren, "A novel cell-penetrating peptide TAT-A1 delivers siRNA into tumor cells selectively," *Biochimie*, vol. 95, pp. 251-257, 2013.
- [80] C. Ganas, A. Weiß, M. Nazarenus, S. Rösler, T. Kissel, P. Rivera_Gil, *et al.*, "Biodegradable capsules as non-viral vectors for in vitro delivery of PEI/siRNA polyplexes for efficient gene silencing," *Journal of Controlled Release*, vol. 196, pp. 132-138, 2014.
- [81] K. A. Whitehead, R. Langer, and D. G. Anderson, "Knocking down barriers: advances in siRNA delivery," *Nature reviews Drug discovery*, vol. 8, pp. 129-138, 2009.
- [82] A. C. R. Grayson, A. M. Doody, and D. Putnam, "Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro," *Pharmaceutical research*, vol. 23, pp. 1868-1876, 2006.
- [83] O. M. Merkel, R. Urbanics, P. Bedőcs, Z. Rozsnyay, L. Rosivall, M. Toth, *et al.*, "In vitro and in vivo complement activation and related anaphylactic effects associated with polyethylenimine and polyethylenimine-graft-poly (ethylene glycol) block copolymers," *Biomaterials*, vol. 32, pp. 4936-4942, 2011.
- [84] M.-E. Bonnet, P. Erbacher, and A.-L. Bolcato-Bellemin, "Systemic delivery of DNA or siRNA mediated by linear polyethylenimine (L-PEI) does not induce an inflammatory response," *Pharmaceutical research*, vol. 25, pp. 2972-2982, 2008.
- [85] T. Nomoto, Y. Matsumoto, K. Miyata, M. Oba, S. Fukushima, N. Nishiyama, *et al.*, "In situ quantitative monitoring of polyplexes and polyplex micelles in the blood circulation using intravital real-time confocal laser scanning microscopy," *Journal of controlled release*, vol. 151, pp. 104-109, 2011.
- [86] O. M. Merkel, A. Beyerle, D. Librizzi, A. Pfestroff, T. M. Behr, B. Sproat, *et al.*, "Nonviral siRNA delivery to the lung: Investigation of PEG- PEI polyplexes and their in vivo performance," *Molecular pharmaceutics*, vol. 6, pp. 1246-1260, 2009.
- [87] A. Malek, F. Czubayko, and A. Aigner, "PEG grafting of polyethylenimine (PEI) exerts different effects on DNA transfection and siRNA-induced gene targeting efficacy," *Journal of drug targeting*, vol. 16, pp. 124-139, 2008.
- [88] Y.-K. Oh and T. G. Park, "siRNA delivery systems for cancer treatment," *Advanced drug delivery reviews*, vol. 61, pp. 850-862, 2009.

- [89] A. Beyerle, A. Braun, A. Banerjee, N. Ercal, O. Eickelberg, T. H. Kissel, *et al.*, "Inflammatory responses to pulmonary application of PEI-based siRNA nanocarriers in mice," *Biomaterials*, vol. 32, pp. 8694-8701, 2011.
- [90] G. Cavallaro, C. Sardo, and E. F. Craparo, "Polymeric nanoparticles for siRNA delivery: production and applications," *International Journal of Pharmaceutics*, 2017.
- [91] D. S. Benoit, S. M. Henry, A. D. Shubin, A. S. Hoffman, and P. S. Stayton, "pH-responsive polymeric sirna carriers sensitize multidrug resistant ovarian cancer cells to doxorubicin via knockdown of polo-like kinase 1," *Molecular pharmaceutics*, vol. 7, pp. 442-455, 2010.
- [92] C. E. Nelson, J. R. Kintzing, A. Hanna, J. M. Shannon, M. K. Gupta, and C. L. Duvall, "Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time, and bioactivity in vivo," *ACS nano*, vol. 7, pp. 8870-8880, 2013.
- [93] K. Chaturvedi, K. Ganguly, A. R. Kulkarni, V. H. Kulkarni, M. N. Nadagouda, W. E. Rudzinski, *et al.*, "Cyclodextrin-based siRNA delivery nanocarriers: a state-of-the-art review," *Expert opinion on drug delivery*, vol. 8, pp. 1455-1468, 2011.
- [94] H. Ragelle, G. Vandermeulen, and V. Préat, "Chitosan-based siRNA delivery systems," *Journal of Controlled Release*, vol. 172, pp. 207-218, 2013.
- [95] D. Jere, H.-L. Jiang, Y.-K. Kim, R. Arote, Y.-J. Choi, C.-H. Yun, *et al.*, "Chitosan-graft-polyethylenimine for Akt1 siRNA delivery to lung cancer cells," *International journal of pharmaceutics*, vol. 378, pp. 194-200, 2009.
- [96] K. Sharma, S. Somavarapu, A. Colombani, N. Govind, and K. M. Taylor, "Nebulised siRNA encapsulated crosslinked chitosan nanoparticles for pulmonary delivery," *International journal of pharmaceutics*, vol. 455, pp. 241-247, 2013.
- [97] S. Mao, W. Sun, and T. Kissel, "Chitosan-based formulations for delivery of DNA and siRNA," *Advanced drug delivery reviews*, vol. 62, pp. 12-27, 2010.
- [98] S. Tosatti, S. De Paul, A. Askendal, S. VandeVondele, J. Hubbell, P. Tengvall, *et al.*, "Peptide functionalized poly (L-lysine)-g-poly (ethylene glycol) on titanium: resistance to protein adsorption in full heparinized human blood plasma," *Biomaterials*, vol. 24, pp. 4949-4958, 2003.
- [99] J. N. Zheng, H. G. Xie, W. T. Yu, X. D. Liu, W. Y. Xie, J. Zhu, *et al.*, "Chitosan-g-MPEG-modified alginate/chitosan hydrogel microcapsules: a quantitative study of the effect of polymer architecture on the resistance to protein adsorption," *Langmuir*, vol. 26, pp. 17156-17164, 2010.
- [100] E. Logashenko, A. Vladimirova, M. Repkova, A. Venyaminova, E. Chernolovskaya, and V. Vlassov, "Silencing of MDR 1 gene in cancer cells by siRNA," *Nucleosides, Nucleotides and Nucleic Acids*, vol. 23, pp. 861-866, 2004.
- [101] C.-g. Zhang, W.-j. Zhu, Y. Liu, Z.-q. Yuan, S.-d. Yang, W.-l. Chen, *et al.*, "Novel polymer micelle mediated co-delivery of doxorubicin and P-glycoprotein siRNA for reversal of multidrug resistance and synergistic tumor therapy," *Scientific reports*, vol. 6, 2016.
- [102] M. E. Davis, "The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic," *Molecular pharmaceutics*, vol. 6, pp. 659-668, 2009.
- [103] S. H. Pun, N. C. Bellocq, A. Liu, G. Jensen, T. Machemer, E. Quijano, *et al.*, "Cyclodextrin-modified polyethylenimine polymers for gene delivery," *Bioconjugate chemistry*, vol. 15, pp. 831-840, 2004.
- [104] A. O. Elzoghby, "Gelatin-based nanoparticles as drug and gene delivery systems: reviewing three decades of research," *Journal of Controlled Release*, vol. 172, pp. 1075-1091, 2013.
- [105] I.-D. Kim, E. Sawicki, H.-K. Lee, E.-H. Lee, H. J. Park, P.-L. Han, *et al.*, "Robust neuroprotective effects of intranasally delivered iNOS siRNA encapsulated in gelatin nanoparticles in the postischemic brain," *Nanomedicine: Nanotechnology, Biology and Medicine*, vol. 12, pp. 1219-1229, 2016.
- [106] Y. Obata, T. Nishino, T. Kushibiki, R. Tomoshige, Z. Xia, M. Miyazaki, *et al.*, "HSP47 siRNA conjugated with cationized gelatin microspheres suppresses peritoneal fibrosis in mice," *Acta biomaterialia*, vol. 8, pp. 2688-2696, 2012.
- [107] W. B. Tan, S. Jiang, and Y. Zhang, "Quantum-dot based nanoparticles for targeted silencing of HER2/neu gene via RNA interference," *Biomaterials*, vol. 28, pp. 1565-1571, 2007.

- [108] H. Zhu, J. Zhu, A. Xie, Y. Lin, B. Zhang, Y. Wang, *et al.*, "Visible quantum-dot-based targeted siRNA delivery for HIF-1 α gene silencing," *Journal of Drug Delivery Science and Technology*, vol. 24, pp. 445-451, 2014.
- [109] G. Chen, J. Y. Zhu, Z. L. Zhang, W. Zhang, J. G. Ren, M. Wu, *et al.*, "Transformation of Cell - Derived Microparticles into Quantum - Dot - Labeled Nanovectors for Antitumor siRNA Delivery," *Angewandte Chemie International Edition*, vol. 54, pp. 1036-1040, 2015.
- [110] Z. Medarova, W. Pham, C. Farrar, V. Petkova, and A. Moore, "In vivo imaging of siRNA delivery and silencing in tumors," *Nature medicine*, vol. 13, pp. 372-377, 2007.
- [111] A. M. Derfus, A. A. Chen, D.-H. Min, E. Ruoslahti, and S. N. Bhatia, "Targeted quantum dot conjugates for siRNA delivery," *Bioconjugate chemistry*, vol. 18, pp. 1391-1396, 2007.
- [112] M. V. Yezhelyev, L. Qi, R. M. O'Regan, S. Nie, and X. Gao, "Proton-sponge coated quantum dots for siRNA delivery and intracellular imaging," *Journal of the American Chemical Society*, vol. 130, pp. 9006-9012, 2008.
- [113] H. Zhu, S. Zhang, Y. Ling, G. Meng, Y. Yang, and W. Zhang, "pH-responsive hybrid quantum dots for targeting hypoxic tumor siRNA delivery," *Journal of Controlled Release*, vol. 220, Part A, pp. 529-544, 12/28/ 2015.
- [114] Y. Wang, B. Wu, C. Yang, M. Liu, T. C. Sum, and K. T. Yong, "Synthesis and characterization of Mn: ZnSe/ZnS/ZnMnS sandwiched QDs for multimodal imaging and theranostic applications," *small*, vol. 12, pp. 534-546, 2016.
- [115] J. Selvaraj, A. Mahesh, V. Asokan, V. Baskaralingam, A. Dhayalan, and T. Paramasivam, "Phosphine-Free, Highly Emissive, Water-Soluble Mn: ZnSe/ZnS Core-Shell Nanorods: Synthesis, Characterization, and in Vitro Bioimaging of HEK293 and HeLa Cells," *ACS Applied Nano Materials*, 2017.
- [116] P. Subramaniam, S. J. Lee, S. Shah, S. Patel, V. Starovoytov, and K. B. Lee, "Generation of a Library of Non - Toxic Quantum Dots for Cellular Imaging and siRNA Delivery," *Advanced Materials*, vol. 24, pp. 4014-4019, 2012.
- [117] P. G. Luo, S. Sahu, S.-T. Yang, S. K. Sonkar, J. Wang, H. Wang, *et al.*, "Carbon "quantum" dots for optical bioimaging," *Journal of Materials Chemistry B*, vol. 1, pp. 2116-2127, 2013.
- [118] L. Ye, K.-T. Yong, L. Liu, I. Roy, R. Hu, J. Zhu, *et al.*, "A pilot study in non-human primates shows no adverse response to intravenous injection of quantum dots," *Nat Nano*, vol. 7, pp. 453-458, 07//print 2012.
- [119] T. Xia, M. Kovochich, M. Liong, H. Meng, S. Kabehie, S. George, *et al.*, "Polyethyleneimine coating enhances the cellular uptake of mesoporous silica nanoparticles and allows safe delivery of siRNA and DNA constructs," *ACS nano*, vol. 3, pp. 3273-3286, 2009.
- [120] X. Li, Y. Chen, M. Wang, Y. Ma, W. Xia, and H. Gu, "A mesoporous silica nanoparticle-PEI-fusogenic peptide system for siRNA delivery in cancer therapy," *Biomaterials*, vol. 34, pp. 1391-1401, 2013.
- [121] J. Shen, H.-C. Kim, H. Su, F. Wang, J. Wolfram, D. Kirui, *et al.*, "Cyclodextrin and polyethylenimine functionalized mesoporous silica nanoparticles for delivery of siRNA cancer therapeutics," *Theranostics*, vol. 4, pp. 487-497, 2014.
- [122] S. R. Bhattarai, E. Muthuswamy, A. Wani, M. Brichacek, A. L. Castañeda, S. L. Brock, *et al.*, "Enhanced gene and siRNA delivery by polycation-modified mesoporous silica nanoparticles loaded with chloroquine," *Pharmaceutical research*, vol. 27, pp. 2556-2568, 2010.
- [123] H. Meng, M. Liong, T. Xia, Z. Li, Z. Ji, J. I. Zink, *et al.*, "Engineered design of mesoporous silica nanoparticles to deliver doxorubicin and Pgp siRNA to overcome drug resistance in a cancer cell line," *ACS nano*, vol. 4, p. 4539, 2010.
- [124] H. K. Na, M. H. Kim, K. Park, S. R. Ryoo, K. E. Lee, H. Jeon, *et al.*, "Efficient functional delivery of siRNA using mesoporous silica nanoparticles with ultralarge pores," *Small*, vol. 8, pp. 1752-1761, 2012.
- [125] S. A. Mackowiak, A. Schmidt, V. Weiss, C. Argyo, C. von Schirnding, T. Bein, *et al.*, "Targeted drug delivery in cancer cells with red-light photoactivated mesoporous silica nanoparticles," *Nano letters*, vol. 13, pp. 2576-2583, 2013.
- [126] C. Hom, J. Lu, M. Liong, H. Luo, Z. Li, J. I. Zink, *et al.*, "Mesoporous silica nanoparticles facilitate delivery of siRNA to shutdown signaling pathways in mammalian cells," *Small*, vol. 6, pp. 1185-1190, 2010.

- [127] H. Meng, W. X. Mai, H. Zhang, M. Xue, T. Xia, S. Lin, *et al.*, "Codelivery of an optimal drug/siRNA combination using mesoporous silica nanoparticles to overcome drug resistance in breast cancer in vitro and in vivo," *ACS nano*, vol. 7, pp. 994-1005, 2013.
- [128] S. Kim, H.-K. Na, C. Won, and D.-H. Min, "In-Depth Study on Gene Silencing Capability of Silica Nanoparticles with Different Pore Sizes: Degree and Duration of RNA Interference," *RSC Advances*, 2016.
- [129] C. Park, H. Youn, H. Kim, T. Noh, Y. H. Kook, E. T. Oh, *et al.*, "Cyclodextrin-covered gold nanoparticles for targeted delivery of an anti-cancer drug," *Journal of Materials Chemistry*, vol. 19, pp. 2310-2315, 2009.
- [130] C. Peng, J. Qin, B. Zhou, Q. Chen, M. Shen, M. Zhu, *et al.*, "Targeted tumor CT imaging using folic acid-modified PEGylated dendrimer-entrapped gold nanoparticles," *Polymer Chemistry*, vol. 4, pp. 4412-4424, 2013.
- [131] J.-S. Lee, J. J. Green, K. T. Love, J. Sunshine, R. Langer, and D. G. Anderson, "Gold, poly (β -amino ester) nanoparticles for small interfering RNA delivery," *Nano letters*, vol. 9, p. 2402, 2009.
- [132] C. J. Bishop, S. Y. Tzeng, and J. J. Green, "Degradable polymer-coated gold nanoparticles for co-delivery of DNA and siRNA," *Acta biomaterialia*, vol. 11, pp. 393-403, 2015.
- [133] Y. Yi, H. J. Kim, P. Mi, M. Zheng, H. Takemoto, K. Toh, *et al.*, "Targeted systemic delivery of siRNA to cervical cancer model using cyclic RGD-installed unimer polyion complex-assembled gold nanoparticles," *Journal of Controlled Release*, vol. 244, pp. 247-256, 2016.
- [134] F. Yin, C. Yang, Q. Wang, S. Zeng, R. Hu, G. Lin, *et al.*, "A Light-Driven Therapy of Pancreatic Adenocarcinoma Using Gold Nanorods-Based Nanocarriers for Co-Delivery of Doxorubicin and siRNA," *Theranostics*, vol. 5, p. 818, 2015.
- [135] C. Liu, P. Zhang, X. Zhai, F. Tian, W. Li, J. Yang, *et al.*, "Nano-carrier for gene delivery and bioimaging based on carbon dots with PEI-passivation enhanced fluorescence," *Biomaterials*, vol. 33, pp. 3604-3613, 2012.
- [136] Y.-F. Wu, H.-C. Wu, C.-H. Kuan, C.-J. Lin, L.-W. Wang, C.-W. Chang, *et al.*, "Multi-functionalized carbon dots as theranostic nanoagent for gene delivery in lung cancer therapy," *Scientific reports*, vol. 6, 2016.
- [137] T. Feng, X. Ai, G. An, P. Yang, and Y. Zhao, "Charge-convertible carbon dots for imaging-guided drug delivery with enhanced in vivo cancer therapeutic efficiency," *ACS nano*, vol. 10, pp. 4410-4420, 2016.
- [138] X. T. Zheng, A. Ananthanarayanan, K. Q. Luo, and P. Chen, "Glowing graphene quantum dots and carbon dots: properties, syntheses, and biological applications," *Small*, vol. 11, pp. 1620-1636, 2015.
- [139] H. Dong, W. Dai, H. Ju, H. Lu, S. Wang, L. Xu, *et al.*, "Multifunctional poly (l-lactide)-polyethylene glycol-grafted graphene quantum dots for intracellular microRNA imaging and combined specific-gene-targeting agents delivery for improved therapeutics," *ACS applied materials & interfaces*, vol. 7, pp. 11015-11023, 2015.
- [140] J. H. Lee, K. Lee, S. H. Moon, Y. Lee, T. G. Park, and J. Cheon, "All - in - One Target - Cell - Specific Magnetic Nanoparticles for Simultaneous Molecular Imaging and siRNA Delivery," *Angewandte Chemie*, vol. 121, pp. 4238-4243, 2009.
- [141] X. Wang, L. Zhu, X. Hou, L. Wang, and S. Yin, "Polyethylenimine mediated magnetic nanoparticles for combined intracellular imaging, siRNA delivery and anti-tumor therapy," *RSC Advances*, vol. 5, pp. 101569-101581, 2015.
- [142] J. Y. Lee, C. Crake, B. Teo, D. Carugo, M. de Saint Victor, A. Seth, *et al.*, "Ultrasound - Enhanced siRNA Delivery Using Magnetic Nanoparticle - Loaded Chitosan - Deoxycholic Acid Nanodroplets," *Advanced Healthcare Materials*, 2017.
- [143] Y. Shin, J. H. Chang, J. Liu, R. Williford, Y.-K. Shin, and G. J. Exarhos, "Hybrid nanogels for sustainable positive thermosensitive drug release," *Journal of controlled release*, vol. 73, pp. 1-6, 2001.
- [144] S. V. Vinogradov, "Colloidal microgels in drug delivery applications," *Current pharmaceutical design*, vol. 12, pp. 4703-4712, 2006.
- [145] H. Lee, H. Mok, S. Lee, Y.-K. Oh, and T. G. Park, "Target-specific intracellular delivery of siRNA using degradable hyaluronic acid nanogels," *Journal of Controlled Release*, vol. 119, pp. 245-252, 2007.

- [146] W. H. Blackburn, E. B. Dickerson, M. H. Smith, J. F. McDonald, and L. A. Lyon, "Peptide-functionalized nanogels for targeted siRNA delivery," *Bioconjugate chemistry*, vol. 20, p. 960, 2009.
- [147] E. B. Dickerson, W. H. Blackburn, M. H. Smith, L. B. Kapa, L. A. Lyon, and J. F. McDonald, "Chemosensitization of cancer cells by siRNA using targeted nanogel delivery," *Bmc Cancer*, vol. 10, p. 10, 2010.
- [148] K. Raemdonck, B. Naeye, K. Buyens, R. E. Vandenbroucke, A. Høgset, J. Demeester, *et al.*, "Biodegradable dextran nanogels for RNA interference: focusing on endosomal escape and intracellular siRNA delivery," *Advanced Functional Materials*, vol. 19, pp. 1406-1415, 2009.
- [149] R.-Q. Li, W. Wu, H.-Q. Song, Y. Ren, M. Yang, J. Li, *et al.*, "Well-defined reducible cationic nanogels based on functionalized low-molecular-weight PGMA for effective pDNA and siRNA delivery," *Acta biomaterialia*, vol. 41, pp. 282-292, 2016.
- [150] D. Li, C. F. van Nostrum, E. Mastrobattista, T. Vermonden, and W. E. Hennink, "Nanogels for intracellular delivery of biotherapeutics," *Journal of Controlled Release*, 2016.
- [151] S. Z. Khaled, A. Cevenini, I. K. Yazdi, A. Parodi, M. Evangelopoulos, C. Corbo, *et al.*, "One-pot synthesis of pH-responsive hybrid nanogel particles for the intracellular delivery of small interfering RNA," *Biomaterials*, vol. 87, pp. 57-68, 2016.
- [152] G. Aguirre, J. Ramos, and J. Forcada, "Advanced design of t and pH dual - responsive PDEAEMA - PVCL core - shell nanogels for siRNA delivery," *Journal of Polymer Science Part A: Polymer Chemistry*, vol. 54, pp. 3203-3217, 2016.
- [153] V. Bitko, A. Musiyenko, O. Shulyayeva, and S. Barik, "Inhibition of respiratory viruses by nasally administered siRNA," *Nature medicine*, vol. 11, pp. 50-55, 2005.
- [154] Y. Y. Huang, J. M. Hong, S. Q. Zheng, Y. Ding, S. T. Guo, H. Y. Zhang, *et al.*, "Elimination Pathways of Systemically Delivered siRNA," *Molecular Therapy*, vol. 19, pp. 381-385, Feb 2011.
- [155] A. Cardoso, S. Simoes, L. De Almeida, J. Pelisek, C. Culmsee, E. Wagner, *et al.*, "siRNA delivery by a transferrin - associated lipid - based vector: a non - viral strategy to mediate gene silencing," *The journal of gene medicine*, vol. 9, pp. 170-183, 2007.
- [156] N. Panwar, C. Yang, F. Yin, H. S. Yoon, T. S. Chuan, and K.-T. Yong, "RNAi-based therapeutic nanostrategy: IL-8 gene silencing in pancreatic cancer cells using gold nanorods delivery vehicles," *Nanotechnology*, vol. 26, p. 365101, 2015.
- [157] T. Anderson, R. Hu, C. Yang, H. S. Yoon, and K.-T. Yong, "Pancreatic cancer gene therapy using an siRNA-functionalized single walled carbon nanotubes (SWNTs) nanoplex," *Biomaterials Science*, vol. 2, pp. 1244-1253, 2014.
- [158] B.-K. Wang, X.-F. Yu, J.-H. Wang, Z.-B. Li, P.-H. Li, H. Wang, *et al.*, "Gold-nanorods-siRNA nanoplex for improved photothermal therapy by gene silencing," *Biomaterials*, vol. 78, pp. 27-39, 2016.
- [159] A. Elbakry, A. Zaky, R. Liebl, R. Rachel, A. Goepferich, and M. Breunig, "Layer-by-layer assembled gold nanoparticles for siRNA delivery," *Nano letters*, vol. 9, pp. 2059-2064, 2009.
- [160] C. G. Chen and Z. T. Yu, "siRNA Targeting HIF-1 α Induces Apoptosis of Pancreatic Cancer Cells through NF- κ B-independent and -dependent Pathways under Hypoxic Conditions," *Anticancer Research*, vol. 29, pp. 1367-1372, Apr 2009.
- [161] Y. H. Zhi, M. M. Song, P. L. Wang, T. Zhang, and Z. Y. Yin, "Suppression of matrix metalloproteinase-2 via RNA interference inhibits pancreatic carcinoma cell invasiveness and adhesion," *World Journal of Gastroenterology*, vol. 15, pp. 1072-1078, Mar 2009.
- [162] J. Guillermet-Guibert, L. Davenne, D. Pchejetski, N. Saint-Laurent, L. Brizuela, C. Guilbeau-Frugier, *et al.*, "Targeting the sphingolipid metabolism to defeat pancreatic cancer cell resistance to the chemotherapeutic gemcitabine drug," *Molecular Cancer Therapeutics*, vol. 8, pp. 809-820, Apr 2009.
- [163] D. Peer and J. Lieberman, "Special delivery: targeted therapy with small RNAs," *Gene Therapy*, vol. 18, pp. 1127-1133, Dec 2011.
- [164] K. A. Whitehead, R. Langer, and D. G. Anderson, "Knocking down barriers: advances in siRNA delivery," *Nat Rev Drug Discov*, vol. 8, pp. 129-138, 02//print 2009.
- [165] A. El-Aneed, "An overview of current delivery systems in cancer gene therapy," *Journal of Controlled Release*, vol. 94, pp. 1-14, Jan 2004.

- [166] S. C. Semple, A. Akinc, J. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, *et al.*, "Rational design of cationic lipids for siRNA delivery," *Nat Biotech*, vol. 28, pp. 172-176, 02//print 2010.
- [167] J. P. Dassie, X.-y. Liu, G. S. Thomas, R. M. Whitaker, K. W. Thiel, K. R. Stockdale, *et al.*, "Systemic administration of optimized aptamer-siRNA chimeras promotes regression of PSMA-expressing tumors," *Nat Biotech*, vol. 27, pp. 839-846, 09//print 2009.
- [168] K. Lappalainen, I. Jaaskelainen, K. Syrjanen, A. Urtti, and S. Syrjanen, "COMPARISON OF CELL-PROLIFERATION AND TOXICITY ASSAYS USING 2 CATIONIC LIPOSOMES," *Pharmaceutical Research*, vol. 11, pp. 1127-1131, Aug 1994.
- [169] M. A. Collins, J. C. Brisset, Y. Q. Zhang, F. Bednar, J. Pierre, K. A. Heist, *et al.*, "Metastatic Pancreatic Cancer Is Dependent on Oncogenic Kras in Mice," *Plos One*, vol. 7, Dec 2012.
- [170] R. Siegel, D. Naishadham, and A. Jemal, "Cancer Statistics, 2012," *Ca-a Cancer Journal for Clinicians*, vol. 62, pp. 10-29, Jan-Feb 2012.
- [171] Y. Li, T. G. VandenBoom, Z. Wang, D. Kong, S. Ali, P. A. Philip, *et al.*, "miR-146a suppresses invasion of pancreatic cancer cells," *Cancer research*, vol. 70, pp. 1486-1495, 2010.
- [172] A. Arlt, S. S. Muerkoster, and H. Schafer, "Targeting apoptosis pathways in pancreatic cancer," *Cancer Letters*, vol. 332, pp. 346-358, May 2013.
- [173] M. Tada, M. Ohashi, Y. Shiratori, T. Okudaira, Y. Komatsu, T. Kawabe, *et al.*, "Analysis of K-ras gene mutation in hyperplastic duct cells of the pancreas without pancreatic disease," *Gastroenterology*, vol. 110, pp. 227-231, Jan 1996.
- [174] T. Tsuchida, H. Kijima, S. Hori, Y. Oshika, T. Tokunaga, K. Kawai, *et al.*, "Adenovirus-mediated anti-K-ras ribozyme induces apoptosis and growth suppression of human pancreatic carcinoma," *Cancer Gene Therapy*, vol. 7, pp. 373-383, Mar 2000.
- [175] J. B. Fleming, G. L. Shen, S. E. Holloway, M. Davis, and R. A. Brekken, "Molecular consequences of silencing mutant K-ras in pancreatic cancer cells: Justification for K-ras-directed therapy," *Molecular Cancer Research*, vol. 3, pp. 413-423, Jul 2005.
- [176] C. K. Chen, W. C. Law, R. Aalink, B. Nair, A. Kopwitthaya, S. D. Mahajan, *et al.*, "Well-Defined Degradable Cationic Polylactide as Nanocarrier for the Delivery of siRNA to Silence Angiogenesis in Prostate Cancer," *Advanced Healthcare Materials*, vol. 1, pp. 751-761, Nov 2012.
- [177] J. C. Stockert, A. Blázquez-Castro, M. Cañete, R. W. Horobin, and Á. Villanueva, "MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets," *Acta histochemica*, vol. 114, pp. 785-796, 2012.
- [178] C. B. Yang, X. C. Mo, J. Z. Lv, X. Y. Liu, M. C. Yuan, M. Dong, *et al.*, "Lipopolysaccharide enhances Fc epsilon RI-mediated mast cell degranulation by increasing Ca²⁺ entry through store-operated Ca²⁺ channels: implications for lipopolysaccharide exacerbating allergic asthma," *Experimental Physiology*, vol. 97, pp. 1315-1327, Dec 2012.
- [179] C. Xu, D.-m. Hu, and Q. Zhu, "eEF1A2 promotes cell migration, invasion and metastasis in pancreatic cancer by upregulating MMP-9 expression through Akt activation," *Clinical & Experimental Metastasis*, vol. 30, pp. 933-944, Oct 2013.
- [180] K. Yoshikawa, E. Tanabe, A. Shibata, S. Inoue, M. Kitayoshi, S. Okimoto, *et al.*, "Involvement of oncogenic K-ras on cell migration stimulated by lysophosphatidic acid receptor-2 in pancreatic cancer cells," *Experimental Cell Research*, vol. 319, pp. 105-112, Feb 1 2013.
- [181] B. B. Friday and A. A. Adjei, "K-ras as a target for cancer therapy," *Biochimica Et Biophysica Acta-Reviews on Cancer*, vol. 1756, pp. 127-144, Nov 2005.
- [182] D. Lowy and B. Willumsen, "Function and regulation of ras," *Annual review of biochemistry*, vol. 62, pp. 851-891, 1993.
- [183] M. Trahey and F. McCormick, "A cytoplasmic protein stimulates normal N-ras p21 GTPase, but does not affect oncogenic mutants," *Science*, vol. 238, pp. 542-545, 1987.
- [184] A. Takashima and D. V. Faller, "Targeting the RAS oncogene," *Expert Opinion on Therapeutic Targets*, vol. 17, pp. 507-531, May 2013.

- [185] P. Jasinski, P. Zwolak, K. Terai, and A. Z. Dudek, "Novel Ras pathway inhibitor induces apoptosis and growth inhibition of K-ras-mutated cancer cells in vitro and in vivo," *Transl Res*, vol. 152, pp. 203-12, Nov 2008.
- [186] Y. Miura, S. Ohnami, K. Yoshida, M. Ohashi, M. Nakano, S. Ohnami, *et al.*, "Intraperitoneal injection of adenovirus expressing antisense K-ras RNA suppresses peritoneal dissemination of hamster syngeneic pancreatic cancer without systemic toxicity," *Cancer Letters*, vol. 218, pp. 53-62, Jan 31 2005.
- [187] T. R. Brummelkamp, R. Bernards, and R. Agami, "Stable suppression of tumorigenicity by virus-mediated RNA interference," *Cancer Cell*, vol. 2, pp. 243-247, Sep 2002.
- [188] B. L. Coomber and A. I. Gotlieb, "In vitro endothelial wound repair. Interaction of cell migration and proliferation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 10, pp. 215-222, 1990.
- [189] J.-M. Zahm, H. Kaplan, A.-L. Hérard, F. Doriot, D. Pierrot, P. Somelette, *et al.*, "Cell migration and proliferation during the in vitro wound repair of the respiratory epithelium," *Cell motility and the cytoskeleton*, vol. 37, pp. 33-43, 1997.
- [190] M. G. Lampugnani, "Cell migration into a wounded area in vitro," in *Adhesion Protein Protocols*, ed: Springer, 1999, pp. 177-182.
- [191] J. C. Yarrow, Z. E. Perlman, N. J. Westwood, and T. J. Mitchison, "A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods," *BMC biotechnology*, vol. 4, p. 21, 2004.
- [192] H. A. Chapman, "Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration," *Current Opinion in Cell Biology*, vol. 9, pp. 714-724, 10// 1997.
- [193] M. Björklund and E. Koivunen, "Gelatinase-mediated migration and invasion of cancer cells," *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, vol. 1755, pp. 37-69, 5/25/ 2005.
- [194] I. J. Fidler, "The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited," *Nat Rev Cancer*, vol. 3, pp. 453-458, 06//print 2003.
- [195] L. A. Liotta, "Tumor Invasion and Metastases—Role of the Extracellular Matrix: Rhoads Memorial Award Lecture," *Cancer Research*, vol. 46, pp. 1-7, January 1, 1986 1986.
- [196] J. D. Hood and D. A. Cheresh, "Role of integrins in cell invasion and migration," *Nat Rev Cancer*, vol. 2, pp. 91-100, 02//print 2002.
- [197] P. Friedl and K. Wolf, "Tumour-cell invasion and migration: diversity and escape mechanisms," *Nat Rev Cancer*, vol. 3, pp. 362-374, 05//print 2003.
- [198] A. Valster, N. L. Tran, M. Nakada, M. E. Berens, A. Y. Chan, and M. Symons, "Cell migration and invasion assays," *Methods*, vol. 37, pp. 208-215, 10// 2005.
- [199] A. Lal, C. A. Glazer, H. M. Martinson, H. S. Friedman, G. E. Archer, J. H. Sampson, *et al.*, "Mutant Epidermal Growth Factor Receptor Up-Regulates Molecular Effectors of Tumor Invasion," *Cancer Research*, vol. 62, pp. 3335-3339, June 15, 2002 2002.
- [200] H. K. Kleinman, M. L. McGarvey, J. R. Hassell, V. L. Star, F. B. Cannon, G. W. Laurie, *et al.*, "Basement membrane complexes with biological activity," *Biochemistry*, vol. 25, pp. 312-318, 1986/01/01 1986.
- [201] H. Kobayashi, H. Ohi, M. Sugimura, H. Shinohara, T. Fujii, and T. Terao, "Inhibition of in Vitro Ovarian Cancer Cell Invasion by Modulation of Urokinase-type Plasminogen Activator and Cathepsin B," *Cancer Research*, vol. 52, pp. 3610-3614, July 1, 1992 1992.
- [202] R. Ren, "Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia," *Nat Rev Cancer*, vol. 5, pp. 172-83, Mar 2005.
- [203] A. McGahon, R. Bissonnette, M. Schmitt, K. M. Cotter, D. R. Green, and T. G. Cotter, "BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death," *Blood*, vol. 83, pp. 1179-87, Mar 1 1994.
- [204] J. R. McWHIRTER and J. Wang, "Activation of tyrosinase kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion proteins," *Molecular and Cellular Biology*, vol. 11, pp. 1553-1565, 1991.

- [205] S. Salesse and C. M. Verfaillie, "BCR/ABL-mediated Increased Expression of Multiple Known and Novel Genes That May Contribute to the Pathogenesis of Chronic Myelogenous Leukemia1," *Molecular cancer therapeutics*, vol. 2, pp. 173-182, 2003.
- [206] P. Ramaraj, H. Singh, N. Niu, S. Chu, M. Holtz, J. K. Yee, *et al.*, "Effect of mutational inactivation of tyrosine kinase activity on BCR/ABL-induced abnormalities in cell growth and adhesion in human hematopoietic progenitors," *Cancer Res*, vol. 64, pp. 5322-31, Aug 1 2004.
- [207] R. C. Zhao, Y. Jiang, and C. M. Verfaillie, "A model of human p210(bcr/ABL)-mediated chronic myelogenous leukemia by transduction of primary normal human CD34(+) cells with a BCR/ABL-containing retroviral vector," *Blood*, vol. 97, pp. 2406-12, Apr 15 2001.
- [208] C. Barthe, P. Cony-Makhoul, J. V. Melo, and J. R. F.-X. Mahon, "Roots of Clinical Resistance to STI-571 Cancer Therapy," *Science*, vol. 293, p. 2163, September 21, 2001 2001.
- [209] S. Branford, Z. Rudzki, S. Walsh, A. Grigg, C. Arthur, K. Taylor, *et al.*, "High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance," *Blood*, vol. 99, pp. 3472-3475, 2002.
- [210] N. von Bubnoff, F. Schneller, C. Peschel, and J. Duyster, "BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study," *The Lancet*, vol. 359, pp. 487-491.
- [211] M. Azam, R. R. Latek, and G. Q. Daley, "Mechanisms of Autoinhibition and STI-571/Imatinib Resistance Revealed by Mutagenesis of BCR-ABL," *Cell*, vol. 112, pp. 831-843.
- [212] A. Hochhaus, S. Kreil, A. S. Corbin, P. La Rosee, M. C. Muller, T. Lahaye, *et al.*, "Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy," *Leukemia*, vol. 16, pp. 2190-6, Nov 2002.
- [213] S. I. Pai, Y. Y. Lin, B. Macaes, A. Meneshian, C. F. Hung, and T. C. Wu, "Prospects of RNA interference therapy for cancer," *Gene Ther*, vol. 13, pp. 464-477, 12/08/online 2005.
- [214] N. Panwar, C. Yang, F. Yin, H. S. Yoon, T. S. Chuan, and K. T. Yong, "RNAi-based therapeutic nanostrategy: IL-8 gene silencing in pancreatic cancer cells using gold nanorods delivery vehicles," *Nanotechnology*, vol. 26, p. 365101, Sep 11 2015.
- [215] J. M. Withey, S. B. Marley, J. Kaeda, A. J. Harvey, M. R. Crompton, and M. Y. Gordon, "Targeting primary human leukaemia cells with RNA interference: Bcr - Abl targeting inhibits myeloid progenitor self - renewal in chronic myeloid leukaemia cells," *British journal of haematology*, vol. 129, pp. 377-380, 2005.
- [216] X. Guo and L. Huang, "Recent advances in nonviral vectors for gene delivery," *Accounts of chemical research*, vol. 45, pp. 971-979, 2011.
- [217] A. H. Elmaagacli, M. Koldehoff, R. Peceny, L. Klein-Hitpass, H. Ottinger, D. W. Beelen, *et al.*, "WT1 and BCR-ABL specific small interfering RNA have additive effects in the induction of apoptosis in leukemic cells," *Haematologica*, vol. 90, pp. 326-34, Mar 2005.
- [218] W. Glienke, L. Maute, U. Koehl, R. Esser, E. Milz, and L. Bergmann, "Effective treatment of leukemic cell lines with wt1 siRNA," *Leukemia*, vol. 21, pp. 2164-70, Oct 2007.
- [219] M. A. Mintzer and E. E. Simanek, "Nonviral Vectors for Gene Delivery," *Chemical Reviews*, vol. 109, pp. 259-302, 2009/02/11 2009.
- [220] S. Zhang, B. Zhao, H. Jiang, B. Wang, and B. Ma, "Cationic lipids and polymers mediated vectors for delivery of siRNA," *J Control Release*, vol. 123, pp. 1-10, 2007.
- [221] A. C. Rinkenauer, A. Schallon, U. Günther, M. Wagner, E. Betthausen, U. S. Schubert, *et al.*, "A Paradigm Change: Efficient Transfection of Human Leukemia Cells by Stimuli-Responsive Multicompartment Micelles," *ACS Nano*, vol. 7, pp. 9621-9631, 2013/11/26 2013.
- [222] C. KeeáLai, "Biodegradable cationic polymeric nanocapsules for overcoming multidrug resistance and enabling drug-gene co-delivery to cancer cells," *Nanoscale*, vol. 6, pp. 1567-1572, 2014.
- [223] J. Valencia-Serna, H. Gul-Uludağ, P. Mahdipoor, X. Jiang, and H. Uludağ, "Investigating siRNA delivery to chronic myeloid leukemia K562 cells with lipophilic polymers for therapeutic BCR-ABL down-regulation," *Journal of controlled release*, vol. 172, pp. 495-503, 2013.

- [224] F. Wang, Y. Wang, X. Zhang, W. Zhang, S. Guo, and F. Jin, "Recent progress of cell-penetrating peptides as new carriers for intracellular cargo delivery," *Journal of Controlled Release*, vol. 174, pp. 126-136, 2014.
- [225] Y. Arthanari, A. Pluen, R. Rajendran, H. Aojula, and C. Demonacos, "Delivery of therapeutic shRNA and siRNA by Tat fusion peptide targeting bcr–abl fusion gene in chronic myeloid leukemia cells," *Journal of Controlled Release*, vol. 145, pp. 272-280, 2010.
- [226] M. Wilda, U. Fuchs, W. Wössmann, and A. Borkhardt, "Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi)," *Oncogene*, vol. 21, pp. 5716-5724, 2002.
- [227] C. Yang, X. Mo, J. Lv, X. Liu, M. Yuan, M. Dong, *et al.*, "Lipopolysaccharide enhances FcεRI - mediated mast cell degranulation by increasing Ca²⁺ entry through store - operated Ca²⁺ channels: implications for lipopolysaccharide exacerbating allergic asthma," *Experimental physiology*, vol. 97, pp. 1315-1327, 2012.
- [228] Y. Arthanari, A. Pluen, R. Rajendran, H. Aojula, and C. Demonacos, "Delivery of therapeutic shRNA and siRNA by Tat fusion peptide targeting BCR-ABL fusion gene in Chronic Myeloid Leukemia cells," *J Control Release*, vol. 145, pp. 272-80, Aug 3 2010.
- [229] M. Merkerova, H. Klamova, R. Brdicka, and H. Bruchova, "Targeting of gene expression by siRNA in CML primary cells," *Mol Biol Rep*, vol. 34, pp. 27-33, Mar 2007.
- [230] H. Ohba, Z. Zhelev, R. Bakalova, A. Ewis, T. Omori, M. Ishikawa, *et al.*, "Inhibition of bcr-abl and/or c-abl gene expression by small interfering, double-stranded RNAs," *Cancer*, vol. 101, pp. 1390-1403, 2004.
- [231] J.-P. Behr, "Synthetic gene transfer vectors II: back to the future," *Accounts of chemical research*, vol. 45, pp. 980-984, 2012.
- [232] C. K. Chen, W. C. Law, R. Aalinkeel, B. Nair, A. Kopwiththaya, S. D. Mahajan, *et al.*, "Well-defined degradable cationic polylactide as nanocarrier for the delivery of siRNA to silence angiogenesis in prostate cancer," *Adv Healthc Mater*, vol. 1, pp. 751-61, Nov 2012.
- [233] J. D. Rowley, "A New Consistent Chromosomal Abnormality in Chronic Myelogenous Leukaemia identified by Quinacrine Fluorescence and Giemsa Staining," *Nature*, vol. 243, pp. 290-293, 06/01/print 1973.
- [234] A. Eguchi, B. R. Meade, Y. C. Chang, C. T. Fredrickson, K. Willert, N. Puri, *et al.*, "Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein," *Nat Biotechnol*, vol. 27, pp. 567-71, Jun 2009.
- [235] A. K. Shalek, J. T. Gaubomme, L. Wang, N. Yosef, N. Chevrier, M. S. Andersen, *et al.*, "Nanowire-Mediated Delivery Enables Functional Interrogation of Primary Immune Cells: Application to the Analysis of Chronic Lymphocytic Leukemia," *Nano Letters*, vol. 12, pp. 6498-6504, 2012/12/12 2012.
- [236] L. Wohlbold, H. van der Kuip, A. Moehring, G. Granot, M. Oren, H. Vornlocher, *et al.*, "All common p210 and p190 Bcr-abl variants can be targeted by RNA interference," *Leukemia*, vol. 19, pp. 290-292, 2005.
- [237] M. Wilda, U. Fuchs, W. Wossmann, and A. Borkhardt, "Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi)," *Oncogene*, vol. 21, pp. 5716-24, Aug 22 2002.
- [238] J. Rangatia and D. Bonnet, "Transient or long-term silencing of BCR-ABL alone induces cell cycle and proliferation arrest, apoptosis and differentiation," *Leukemia*, vol. 20, pp. 68-76, 11/10/online 2005.
- [239] D. Cortez, L. Kadlec, and A. M. Pendergast, "Structural and signaling requirements for BCR-ABL-mediated transformation and inhibition of apoptosis," *Molecular and Cellular Biology*, vol. 15, pp. 5531-41, October 1, 1995 1995.
- [240] N. Waddell, M. Pajic, A.-M. Patch, D. K. Chang, K. S. Kassahn, P. Bailey, *et al.*, "Whole genomes redefine the mutational landscape of pancreatic cancer," *Nature*, vol. 518, pp. 495-501, 2015.
- [241] J. B. Fleming, G.-L. Shen, S. E. Holloway, M. Davis, and R. A. Brekken, "Molecular consequences of silencing mutant K-ras in pancreatic cancer cells: justification for K-ras–directed therapy," *Molecular cancer research*, vol. 3, pp. 413-423, 2005.

- [242] S. Weijzen, P. Rizzo, M. Braid, R. Vaishnav, S. M. Jonkheer, A. Zlobin, *et al.*, "Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells," *Nature medicine*, vol. 8, pp. 979-986, 2002.
- [243] Y. Miyamoto, A. Maitra, B. Ghosh, U. Zechner, P. Argani, C. A. Iacobuzio-Donahue, *et al.*, "Notch mediates TGF α -induced changes in epithelial differentiation during pancreatic tumorigenesis," *Cancer cell*, vol. 3, pp. 565-576, 2003.
- [244] B. Bao, Z. Wang, S. Ali, D. Kong, Y. Li, A. Ahmad, *et al.*, "Notch-1 induces epithelial–mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells," *Cancer letters*, vol. 307, pp. 26-36, 2011.
- [245] G. Z. Cheng, J. Chan, Q. Wang, W. Zhang, C. D. Sun, and L.-H. Wang, "Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel," *Cancer research*, vol. 67, pp. 1979-1987, 2007.
- [246] B. C. Fuchs, T. Fujii, J. D. Dorfman, J. M. Goodwin, A. X. Zhu, M. Lanuti, *et al.*, "Epithelial-to-mesenchymal transition and integrin-linked kinase mediate sensitivity to epidermal growth factor receptor inhibition in human hepatoma cells," *Cancer research*, vol. 68, pp. 2391-2399, 2008.
- [247] J. P. Thiery, "Epithelial–mesenchymal transitions in tumour progression," *Nature Reviews Cancer*, vol. 2, pp. 442-454, 2002.
- [248] T. Brabletz, A. Jung, S. Reu, M. Porzner, F. Hlubek, L. A. Kunz-Schughart, *et al.*, "Variable β -catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment," *Proceedings of the National Academy of Sciences*, vol. 98, pp. 10356-10361, 2001.
- [249] Y. Li, T. G. VandenBoom, D. Kong, Z. Wang, S. Ali, P. A. Philip, *et al.*, "Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells," *Cancer research*, vol. 69, pp. 6704-6712, 2009.
- [250] M. Korpai, E. S. Lee, G. Hu, and Y. Kang, "The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2," *Journal of Biological Chemistry*, vol. 283, pp. 14910-14914, 2008.
- [251] J. L. Avila and J. L. Kissil, "Notch signaling in pancreatic cancer: oncogene or tumor suppressor?," *Trends in molecular medicine*, vol. 19, pp. 320-327, 2013.
- [252] J.-P. De La O, L. L. Emerson, J. L. Goodman, S. C. Froebe, B. E. Illum, A. B. Curtis, *et al.*, "Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia," *Proceedings of the National Academy of Sciences*, vol. 105, pp. 18907-18912, 2008.
- [253] S. Yabuuchi, S. G. Pai, N. R. Campbell, R. F. de Wilde, E. De Oliveira, P. Korangath, *et al.*, "Notch signaling pathway targeted therapy suppresses tumor progression and metastatic spread in pancreatic cancer," *Cancer letters*, vol. 335, pp. 41-51, 2013.
- [254] E. Vakiani and D. B. Solit, "KRAS and BRAF: drug targets and predictive biomarkers," *The Journal of pathology*, vol. 223, pp. 220-230, 2011.
- [255] Z. Wang, S. Banerjee, Y. Li, K. W. Rahman, Y. Zhang, and F. H. Sarkar, "Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor- κ B, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells," *Cancer research*, vol. 66, pp. 2778-2784, 2006.
- [256] Z. Ji, F. C. Mei, J. Xie, and X. Cheng, "Oncogenic KRAS activates hedgehog signaling pathway in pancreatic cancer cells," *Journal of Biological Chemistry*, vol. 282, pp. 14048-14055, 2007.
- [257] M. A. Collins, J.-C. Brisset, Y. Zhang, F. Bednar, J. Pierre, K. A. Heist, *et al.*, "Metastatic pancreatic cancer is dependent on oncogenic Kras in mice," *PLoS One*, vol. 7, p. e49707, 2012.
- [258] H. r. Burris, M. J. Moore, J. Andersen, M. R. Green, M. L. Rothenberg, M. R. Modiano, *et al.*, "Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial," *Journal of clinical oncology*, vol. 15, pp. 2403-2413, 1997.
- [259] T. Conroy, F. Desseigne, M. Ychou, O. Bouché, R. Guimbaud, Y. Bécouarn, *et al.*, "FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer," *New England Journal of Medicine*, vol. 364, pp. 1817-1825, 2011.

- [260] A. Mittal, D. Chitkara, S. W. Behrman, and R. I. Mahato, "Efficacy of gemcitabine conjugated and miRNA-205 complexed micelles for treatment of advanced pancreatic cancer," *Biomaterials*, vol. 35, pp. 7077-7087, 2014.
- [261] C. M. S. R. Lima, D. Savarese, H. Bruckner, A. Dudek, J. Eckardt, J. Hainsworth, *et al.*, "Irinotecan plus gemcitabine induces both radiographic and CA 19-9 tumor marker responses in patients with previously untreated advanced pancreatic cancer," *Journal of clinical oncology*, vol. 20, pp. 1182-1191, 2002.
- [262] J. D. Berlin, S. Adak, D. J. Vaughn, D. Flinker, L. Blaszczewski, J. E. Harris, *et al.*, "A phase II study of gemcitabine and 5-fluorouracil in metastatic pancreatic cancer: an Eastern Cooperative Oncology Group Study (E3296)," *Oncology*, vol. 58, pp. 215-218, 2000.
- [263] C. Liu, G. Zhao, J. Liu, N. Ma, P. Chivukula, L. Perelman, *et al.*, "Novel biodegradable lipid nano complex for siRNA delivery significantly improving the chemosensitivity of human colon cancer stem cells to paclitaxel," *Journal of controlled release*, vol. 140, pp. 277-283, 2009.
- [264] C. Yang, N. Panwar, Y. Wang, B. Zhang, M. Liu, H. T. Toh, *et al.*, "Biodegradable charged polyester-based vector (BCPVs) as an efficient non-viral transfection nanoagent for gene knockdown of BCR-ABL hybrid oncogene in human chronic myeloid leukemia cell line," *Nanoscale*, 2016.
- [265] L. Song, Y. Peng, J. Yun, P. Rizzo, V. Chaturvedi, S. Weijzen, *et al.*, "Notch-1 associates with IKK α and regulates IKK activity in cervical cancer cells," *Oncogene*, vol. 27, pp. 5833-5844, 2008.
- [266] R. Siegel, J. Ma, Z. Zou, and A. Jemal, "Cancer statistics, 2014," *CA: a cancer journal for clinicians*, vol. 64, pp. 9-29, 2014.
- [267] A. M. Lennon, C. L. Wolfgang, M. I. Canto, A. P. Klein, J. M. Herman, M. Goggins, *et al.*, "The early detection of pancreatic cancer: what will it take to diagnose and treat curable pancreatic neoplasia?," *Cancer research*, vol. 74, pp. 3381-3389, 2014.
- [268] R. Hu, C. Yang, Y. Wang, G. Lin, W. Qin, Q. Ouyan, *et al.*, "Aggregation-induced emission (AIE) dye loaded polymer nanoparticles for gene silencing in pancreatic cancer and their in vitro and in vivo biocompatibility evaluation," *Nano Research*, vol. 8, pp. 1563-1576, 2015.
- [269] L. Li, W. Gu, J. Liu, S. Yan, and Z. P. Xu, "Amine-functionalized SiO₂ nanodot-coated layered double hydroxide nanocomposites for enhanced gene delivery," *Nano Research*, vol. 8, pp. 682-694, 2015.
- [270] M. McCully, Y. Hernandez, J. Conde, P. V. Baptista, M. Jesus, A. Hursthouse, *et al.*, "Significance of the balance between intracellular glutathione and polyethylene glycol for successful release of small interfering RNA from gold nanoparticles," *Nano Research*, vol. 8, pp. 3281-3292, 2015.
- [271] D. La O, J. Paul, and C. L. Murtaugh, "Notch and Kras in pancreatic cancer," *Cell Cycle*, vol. 8, pp. 1860-1864, 2009.
- [272] M. V. Sundaram, "The love-hate relationship between Ras and Notch," *Genes & development*, vol. 19, pp. 1825-1839, 2005.
- [273] S. Ali, A. Ahmad, S. Banerjee, S. Padhye, K. Dominiak, J. M. Schaffert, *et al.*, "Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF," *Cancer research*, vol. 70, pp. 3606-3617, 2010.
- [274] P. A. Gregory, A. G. Bert, E. L. Paterson, S. C. Barry, A. Tsykin, G. Farshid, *et al.*, "The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1," *Nature cell biology*, vol. 10, pp. 593-601, 2008.
- [275] A. Khanal, C. Ullum, C. W. Kimbrough, N. C. Garbett, J. A. Burlison, M. W. McNally, *et al.*, "Tumor targeted mesoporous silica-coated gold nanorods facilitate detection of pancreatic tumors using Multispectral optoacoustic tomography," *Nano Research*, vol. 8, pp. 3864-3877, 2015.
- [276] D. Yadav and A. B. Lowenfels, "The epidemiology of pancreatitis and pancreatic cancer," *Gastroenterology*, vol. 144, pp. 1252-1261, 2013.
- [277] I. Al-Ogaidi, H. Gou, Z. P. Aguilar, S. Guo, A. K. Melconian, A. K. A. Al-Kazaz, *et al.*, "Detection of the ovarian cancer biomarker CA-125 using chemiluminescence resonance energy transfer to graphene quantum dots," *Chemical Communications*, vol. 50, pp. 1344-1346, 2014.
- [278] M. Nurunnabi, Z. Khatun, K. M. Huh, S. Y. Park, D. Y. Lee, K. J. Cho, *et al.*, "In vivo biodistribution and toxicology of carboxylated graphene quantum dots," *ACS nano*, vol. 7, pp. 6858-6867, 2013.

- [279] S. Zhu, Y. Song, X. Zhao, J. Shao, J. Zhang, and B. Yang, "The photoluminescence mechanism in carbon dots (graphene quantum dots, carbon nanodots, and polymer dots): current state and future perspective," *Nano Research*, vol. 8, pp. 355-381, 2015.
- [280] J.-E. Lee, I. In, H. Lee, K. D. Lee, J. H. Jeong, and S. Y. Park, "Target delivery and cell imaging using hyaluronic acid-functionalized graphene quantum dots," *Molecular pharmaceuticals*, vol. 10, pp. 3736-3744, 2013.
- [281] X. Du, B. Shi, J. Liang, J. Bi, S. Dai, and S. Z. Qiao, "Developing Functionalized Dendrimer - Like Silica Nanoparticles with Hierarchical Pores as Advanced Delivery Nanocarriers," *Advanced materials*, vol. 25, pp. 5981-5985, 2013.
- [282] C. H. Jones, C.-K. Chen, M. Jiang, L. Fang, C. Cheng, and B. A. Pfeifer, "Synthesis of cationic polylactides with tunable charge densities as nanocarriers for effective gene delivery," *Molecular pharmaceuticals*, vol. 10, pp. 1138-1145, 2013.
- [283] C. Yang, N. Panwar, Y. Wang, B. Zhang, M. Liu, H. Toh, *et al.*, "Biodegradable charged polyester-based vectors (BCPVs) as an efficient non-viral transfection nanoagent for gene knockdown of the BCR-ABL hybrid oncogene in a human chronic myeloid leukemia cell line," *Nanoscale*, vol. 8, pp. 9405-9416, 2016.
- [284] Y. T. Chang, P. Y. Liao, H. S. Sheu, Y. J. Tseng, F. Y. Cheng, and C. S. Yeh, "Near - Infrared Light - Responsive Intracellular Drug and siRNA Release Using Au Nanoensembles with Oligonucleotide - Capped Silica Shell," *Advanced Materials*, vol. 24, pp. 3309-3314, 2012.
- [285] G. Luo, W. Chen, H. Jia, Y. Sun, H. Cheng, R. Zhuo, *et al.*, "An indicator-guided photo-controlled drug delivery system based on mesoporous silica/gold nanocomposites," *Nano Research*, vol. 8, p. 1893, 2015.
- [286] X. Song, Q. Chen, and Z. Liu, "Recent advances in the development of organic photothermal nano-agents," *Nano Research*, vol. 8, pp. 340-354, 2015.
- [287] T. Liu, Y. Chao, M. Gao, C. Liang, Q. Chen, G. Song, *et al.*, "Ultra-small MoS₂ nanodots with rapid body clearance for photothermal cancer therapy," *Nano Research*, vol. 9, pp. 3003-3017, 2016.
- [288] J. Su, H. Sun, Q. Meng, Q. Yin, P. Zhang, Z. Zhang, *et al.*, "Bioinspired Nanoparticles with NIR - Controlled Drug Release for Synergetic Chemophotothermal Therapy of Metastatic Breast Cancer," *Advanced Functional Materials*, vol. 26, pp. 7495-7506, 2016.
- [289] Y. Yang, B. Velmurugan, X. Liu, and B. Xing, "NIR photoresponsive crosslinked upconverting nanocarriers toward selective intracellular drug release," *Small*, vol. 9, pp. 2937-2944, 2013.
- [290] N. Wang, Z. Zhao, Y. Lv, H. Fan, H. Bai, H. Meng, *et al.*, "Gold nanorod-photosensitizer conjugate with extracellular pH-driven tumor targeting ability for photothermal/photodynamic therapy," *Nano Research*, vol. 7, pp. 1291-1301, 2014.
- [291] J. Liu, W. Bu, L. Pan, and J. Shi, "NIR - Triggered Anticancer Drug Delivery by Upconverting Nanoparticles with Integrated Azobenzene - Modified Mesoporous Silica," *Angewandte Chemie International Edition*, vol. 52, pp. 4375-4379, 2013.
- [292] J. Ge, M. Lan, B. Zhou, W. Liu, L. Guo, H. Wang, *et al.*, "A graphene quantum dot photodynamic therapy agent with high singlet oxygen generation," *Nature communications*, vol. 5, 2014.
- [293] T. Ogi, H. Iwasaki, K. Aishima, F. Iskandar, W.-N. Wang, K. Takimiya, *et al.*, "Transient nature of graphene quantum dot formation via a hydrothermal reaction," *Rsc Advances*, vol. 4, pp. 55709-55715, 2014.
- [294] F. A. Permatasari, A. H. Aimon, F. Iskandar, T. Ogi, and K. Okuyama, "Role of C-N configurations in the photoluminescence of graphene quantum dots synthesized by a hydrothermal route," *Scientific reports*, vol. 6, 2016.
- [295] I. Judson, J. Verweij, H. Gelderblom, J. T. Hartmann, P. Schöffski, J.-Y. Blay, *et al.*, "Doxorubicin alone versus intensified doxorubicin plus ifosfamide for first-line treatment of advanced or metastatic soft-tissue sarcoma: a randomised controlled phase 3 trial," *The lancet oncology*, vol. 15, pp. 415-423, 2014.

- [296] C. Yang, R. Hu, T. Anderson, Y. Wang, G. Lin, W.-C. Law, *et al.*, "Biodegradable nanoparticle-mediated K-ras down regulation for pancreatic cancer gene therapy," *Journal of Materials Chemistry B*, vol. 3, pp. 2163-2172, 2015.
- [297] F. C. Delori, R. H. Webb, and D. H. Sliney, "Maximum permissible exposures for ocular safety (ANSI 2000), with emphasis on ophthalmic devices," *JOSA A*, vol. 24, pp. 1250-1265, 2007.

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Publication list

1. **Chengbin Yang**, Kok Ken Chan, Wen-Jen Lin, Muhammad Danang Birowosuto, Shuwen Zeng, Liying Hong, Fitri Aulia Permatasari, Ferry Iskandar, Takashi Ogi, Kikuo Okuyama, Ho Sup Yoon, Chih-Kuang Chen and Ken-Tye Yong. Near-infrared light-triggered drug and gene molecules release graphene quantum dots nanocomplex for synergetic chemo-gene-photothermal therapy of pancreatic cancer.2017. (Under Major revision)
2. **Chengbin Yang**, Kok Ken Chan, Wen-Jen Lin, Alana Mauluidy Soehartono, Guimiao Lin, Huiting Toh, Ho Sup Yoon, Chih-Kuang Chen, and Ken-Tye Yong. Biodegradable nanocarrier for siRNA co-delivery strategy increasing the chemosensitivity of pancreatic cancer cells to Gemcitabine. *Nano Research*, 2017, 10(9), 3049–3067.
3. **Chengbin Yang**, Nishtha Panwar, Yucheng Wang, Butian Zhang, Maixian Liu, Huiting Toh, Ho Sup Yoon, Swee Chuan Tjin, Peter Han Joo Chong, Wing-Cheung Law, Chih-Kuang

- Chen and Ken-Tye Yong. Biodegradable charged polyester-based vectors (BCPVs) as an efficient non-viral transfection nanoagent for gene knockdown of the BCR–ABL hybrid oncogene in a human chronic myeloid leukemia cell line. *Nanoscale*, 2016, 8, 9405-9416.
4. **Chengbin Yang**, Rui Hu, Tommy Anderson, Yucheng Wang, Guimiao Lin, Wing-Cheung Law, Wei-Jen Lin, Quoc Toan Nguyen, Hui Ting Toh, Ho Sup Yoon, Chih-Kuang Chen, Ken-Tye Yong. Biodegradable nanoparticle-mediated K-ras down regulation for pancreatic cancer gene therapy. *Journal of Materials Chemistry B*, 2015, 3, 2163-2172.
 5. Nishtha Panwar, **Chengbin Yang**, Feng Yin, Ho Sup Yoon, Tjin Swee Chuan, Ken-Tye Yong. RNAi-based therapeutic nanostrategy: IL-8 gene silencing in pancreatic cancer cells using gold nanorods delivery vehicles. *Nanotechnology*, 2015, 26, 365101. **(Co-first author)**
 6. Feng Yin, **Chengbin Yang**, Qianqian Wang, Shuwen Zeng, Rui Hu, Guimiao Lin, Jinglin Tian, Siyi Hu, Rong Feng Lan, Ho Sup Yoon, Fei Lu, Kuan Wang, Ken-Tye Yong. A light-driven therapy of pancreatic adenocarcinoma using gold nanorods-based nanocarriers for co-delivery of doxorubicin and siRNA. *Theranostics*, 2015, 5(8), 818–833. **(Co-first author)**
 7. Guimiao Lin, **Chengbin Yang**, Rui Hu, Chih-Kuang Chen, Wing-Cheung Law, Tommy Anderson, Butian Zhang, Quoc Toan Nguyen, Hui Ting Toh, Ho Sup Yoon, Chong Cheng, Ken-Tye Yong. Interleukin-8 gene silencing on pancreatic cancer cells using biodegradable polymer nanoplexes. *Biomaterials Science*, 2014, 2 (7), 1007-1015. **(Co-first author)**.
 8. Xiaomin Liu, **Chengbin Yang**, Jing Liu, Jianwei Liu, Rui Hu, Ken-Tye Yong, Ling Ye. In Vitro evaluation and monitoring of the expression level and localization of aldose reductase using functionalized quantum dots and EGFP. *Biotechnology and Bioprocess Engineering*, 2015, 20 (4), 800-806. **(Co-first author)**
 9. Butian Zhang, **Chengbin Yang**, Yuan Gao, Yue Wang, Chengfei Bu, Siyi Hu, Liwei Liu, Hilmi Volkan Demir, Junle Qu, Ken-Tye Yong. Engineering quantum dots with different emission wavelengths and specific fluorescence lifetimes for spectrally and temporally multiplexed imaging of cells. *Nanotheranostics*, 2017, 1(1), 131-140.
 10. Jianwei Liu, **Chengbin Yang**, Jing Liu, Rui Hu, Yazhuo Hu, Hongyan Chen, Wing-Cheung Law, Mark T. Swihart, Ling Ye, Kuan Wang, Ken-Tye Yong. Effects of Cd-based Quantum Dot Exposure on the Reproduction and Offspring of Kunming Mice over Multiple Generations. *Nanotheranostics*, 2017, 1(1), 23-37.
 11. Guimiao Lin, Chih-Kuang Chen, Feng Yin, **Chengbin Yang**, Jinglin Tian, Ting Chen, Gaixia Xu, Chunxiao He, Marie Chia-Mi Lin, Jie Wang, Fei Lu, Xiaomei Wang and Ken-Tye Yong. Biodegradable nanoparticles as siRNA carriers for in vivo gene silencing and pancreatic cancer therapy. *Journal of Materials Chemistry B*, 2017, **5**, 3327-3337.

12. Yucheng Wang, Bo Wu, **Chengbin Yang**, Maixian Liu, Tze Chien Sum, Ken-Tye Yong. Synthesis and Characterization of Mn: ZnSe/ZnS/ZnMnS Sandwiched QDs for Multimodal Imaging and Theranostic Applications. *Small*, 2016, 12(4), 534-546.
13. Tai-Lok Cheung, Liying Hong, Nanxi Rao, **Chengbin Yang**, Libo Wang, Wenn Jing Lai, Peter Han Joo Chong, Wing-Cheung Law, Ken-Tye Yong. The non-aqueous synthesis of shape controllable Cu₂-xS plasmonic nanostructures in a continuous-flow millifluidic chip for the generation of photo-induced heating. *Nanoscale*, 2016, 8(12), 6609-6622.
14. Rui Hu, **Chengbin Yang**, Yucheng Wang, Guimiao Lin, Wei Qin, Qingling Ouyang, Wing-Cheung Law, Quoc Toan Nguyen, Ho Sup Yoon, Xiaomei Wang, Ken-Tye Yong, Ben Zhong Tang. Aggregation-induced emission (AIE) dye loaded polymer nanoparticles for gene silencing in pancreatic cancer and their in vitro and in vivo biocompatibility evaluation. *Nano Research*, 2015, 8(5), 1563-1576.
15. Yucheng Wang, **Chengbin Yang**, Rui Hu, Hui Ting Toh, Xin Liu, Guimiao Lin, Feng Yin, Ho Sup Yoon, Ken-Tye Yong. Assembling Mn: ZnSe quantum dots-siRNA nanoplexes for gene silencing in tumor cells. *Biomaterials Science*, 2015, 3 (1), 192-202.
16. Butian Zhang, Yucheng Wang, **Chengbin Yang**, Siyi Hu, Yuan Gao, Yiping Zhang, Yue Wang, Hilmi Volkan Demir, Liwei Liu, Ken-Tye Yong. Composition effect on the optical properties of aqueous synthesized Cu-In-S and Zn-Cu-In-S quantum dot nanocrystals. *Physical Chemistry Chemical Physics*, 2015, 17, 25133-25141.
17. Danny Jian Hang Tng, Peiyi Song, Rui Hu, **Chengbin Yang**, Cher Heng Tan, Ken-Tye Yong. Standalone Lab-on-a-Chip Systems toward the Evaluation of Therapeutic Biomaterials in Individualized Disease Treatment. *ACS Biomaterials Science & Engineering*, 2015, 1 (11), 1055–1066.
18. Danny Jian Hang Tng, Peiyi Song, Guimiao Lin, Alana Mauluidy Soehartono, Guang Yang, **Chengbin Yang**, Feng Yin, Cher Heng Tan, Ken-Tye Yong. Synthesis and characterization of multifunctional hybrid-polymeric nanoparticles for drug delivery and multimodal imaging of cancer. *International Journal of Nanomedicine*, 2015; 10: 5771–5786.