Enzyme-instructed siRNA Release and Functional Self-assembly of Peptide-based Delivery System

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Cell-penetrating peptide (CPP)-based small interfering RNA (siRNA) delivery is one of the approaches with great potential to achieve RNA interference (RNAi) applied in gene therapy. CPP-based siRNA carriers hold many merits, including biodegradability, high transmembrane efficiency, and capability of endosomal escape [1-3]. Despite considerably high transfection efficiency achieved, there are still many challenges in further improving the CPP-based siRNA delivery systems. This proposal focuses on two of the challenges: (1) the dissociation of negatively charged siRNA from a positively charged peptide [4-7]; and (2) minimization of cytotoxicity of CPPs while maintaining capacity in endosomal escape. Herein, we propose to utilize enzyme-catalyzed phosphorylation to induce the transfer of negatively charged phosphate groups onto the cationic CPPs formulated with siRNA; the emergent phosphate groups can facilitate the dissociation of siRNAs from the complex due to electrostatic repulsion between the two negatively charged species, the phosphate groups on the peptide and the siRNA. The presence of phosphate groups also alters the balance between repulsive and attractive forces that govern the self-assembly of the peptide, resulting in conformational changes [8]. The physicochemical properties of CPP-siRNA are characterized using gel

electrophoresis, DLS, CDs, AFM and TEM; the cytotoxicity, cellular uptake, and the RNAi efficiency of the system are tested against cancer cells.

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Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Overview

1.1.1 RNA interference pathways

RNAi is a natural regulatory process of sequence-specific post-transcriptional silencing, first discovered in the 1990s by Andrew Fire and Craig Mello [9]. In 2006, Fire and Mello were awarded the Nobel Prize in Medicine or Physiology for their discovery of double-stranded RNA-mediated gene silencing by the process of RNA interference in 1998. Two decades later, RNAi has provided researchers with an unparalleled tool for investigations on genomics, diagnostics, and therapeutics. As discovered, the general process of RNAi consists of: a targeted gene sequence that is switched off by introducing specifically coded double-stranded RNA (dsRNA), usually 21-25 base pairs long, resulting in the reduction of the corresponding protein expression level [10]. This process has emerged as a valuable tool to recognize the crucial genes in various biological processes such as cell growth, tissue differentiation, and cell proliferation [11-13]. Furthermore, RNAi also shows great promise in therapeutic applications. Currently, synthetic siRNA has exhibited superior therapeutic efficacy in comparison to conventional treatments when treating severe diseases [14-16], such as HIV

[17], influenza [18], cancer [19] and CNS diseases [20]. Despite the outstanding performance, the practical application of siRNA therapeutics is limited due to their intrinsic properties of small size (typically 21-25 nucleotides), negative charges and vulnerability to RNase [21, 22]. Naked siRNAs are quickly degraded in human serum, excreted through renal filtration, and are not favorable to cell membrane penetration [22]. Therefore, efficient siRNA delivery systems that can protect and guide siRNA into targeted cells are crucial to the success of RNAi therapy. The criteria for an effective siRNA delivery system are; (a) the protection of siRNA from degradation during systemic delivery; (b) prolonged circulation time; (c) facilitating the cellular uptake of siRNA; (d) ability to escape from the endosomal compartment and release its siRNA cargo in the cytoplasm; (e) low toxicity and biocompatibility [10,22,23]. To fulfil these criteria mentioned, various cationic polymers have been developed to encapsulate siRNA via electrostatic interactions, forming nano-scaled particles [4,23]; the overall positive charges can stimulate cells to engulf the nanoparticles and “chemical triggers” are activated to allow the endosomal escape [1,3,4,24]. However, the attractive forces between the cationic polymers and negatively charged siRNAs hinder their dissociation, and the capacity of endosomal escape of cationic materials is always associated with toxicity [4,10,25].

1.1.2 CPP-based drug delivery system

Cell-penetrating peptides (CPPs) have received much attention in siRNA delivery due to their intrinsic ability to translocate through plasma membranes with low toxicity [4,26,27]. These peptides generally possess an overall positive charge, amphipathicity and consist of 5-30 amino acids. Arginine residues are usually employed to provide the positive charge, as arginine shows a stronger binding affinity to cell membranes than lysine residue, facilitating the cell membrane penetration of CPPs [28,29-31]. Some examples of CPP-based siRNA delivery include the CADY/siRNA system [32] and the C6 peptide series [27] developed by our group, exhibiting high efficiency in gene knockdown and low cytotoxicity. Conjugation of stearic acid to CPPs was also reported to significantly enhance the CPP-siRNA complex cellular internalization [26-28,32]. In order to promote the endosomal escape of the CPP-mediated delivery system, polyhistidine has been customarily utilized to induce the "proton sponge" effect [10,19,22,33]. The polyhistidine segment can be protonated at endosomal pH, resulting in a high influx of protons, chloride ions and water. Consequently, endosomal swelling and rupture occur. By combining the approaches of stearic acid and the "proton sponge" effect, our group has developed a CPP, NP1 (Stearyl- HHHHHHHHHHHHHHHHRRRRRRRR-NH2). The NP1

mediated siRNA delivery system has shown comparable knockdown efficiency to that of lipofectamine 2000 on several cell lines [34-37].

1.1.3 Enzyme-instructed drug release

The dynamic self-assembly of peptides or peptide amphiphiles (PAs) instructed by enzyme-catalyzed reactions have been a topic of interest in recent publications on self-assembling materials [8,38]. Among the enzyme-catalyzed reactions, the phosphorylation of peptides by protein kinase transfers a phosphate group from adenosine triphosphate (ATP) molecule to serine residue, inducing the changes in electrical properties of amino acid residues of specific peptides [39,40] (Figure 1.1). One example is the phosphorylation of peptides or proteins by protein kinase A (PKA) shown in Figure 1.1. However, serine alone cannot be recognized by PKA, and it requires a recognition motif including serine to be the substrate of PKA catalytic subunits. Such motif contains arginine, arbitrary amino acid, and serine, RRXS, where X is an arbitrary amino acid [50, 150]. This project proposes to utilize the phosphorylation of designed stearic CPP to trigger the dissociation between stearic CPP and siRNA. The phosphorylation of serine residue will also alter the molecular interactions among the stearic CPP molecules, leading to changes in surface properties and morphologies of the CPP self-assemblies [51]. Our recent work discovered that spherical arginine-rich PA self-assemblies are less toxic than

fibrous counterparts [147]. Therefore, except for siRNA release, phosphorylation-induced morphology change can be expected to affect the cytotoxicity. This project would provide a potential solution to the issues mentioned above in siRNA delivery.

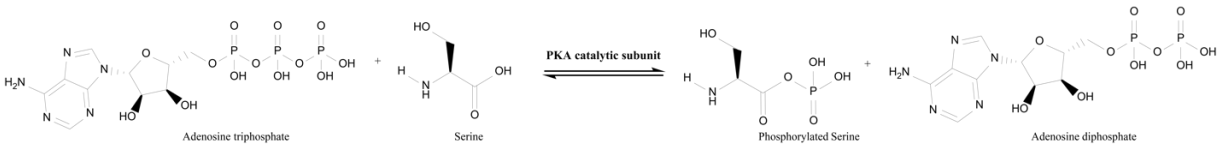

Figure 1.1 Serine phosphorylation by PKA, transferring the gamma phosphate group from ATP to serine.

1.1.4 Mechanism of CPP self-assembly, complex disassembly, and delivery of CPP-siRNA complex

The peptides designed in this research are NP1 (Styl-H16R8-NH2), NP1-SS (Styl-H16R3SR5S-NH2) and NP1-SS'(Styl-H16R3SR3S-NH2). NP1-SS is used in all the illustrations. There are four parts included in this peptide design: 1) stearic acid moiety functions to provide hydrophobic interaction and to assist the internalization of peptide, 2) oligohistidine serves as self-assembly/disassembly segment altered by pH, 3) cationic oligoarginine subsequence binds with siRNA and cell membrane, and also 4) forms a recognition motif with serine (RRRS) for the phosphorylation by PKA. The complete illustration for the composition of peptide is shown in Figure 1.2.

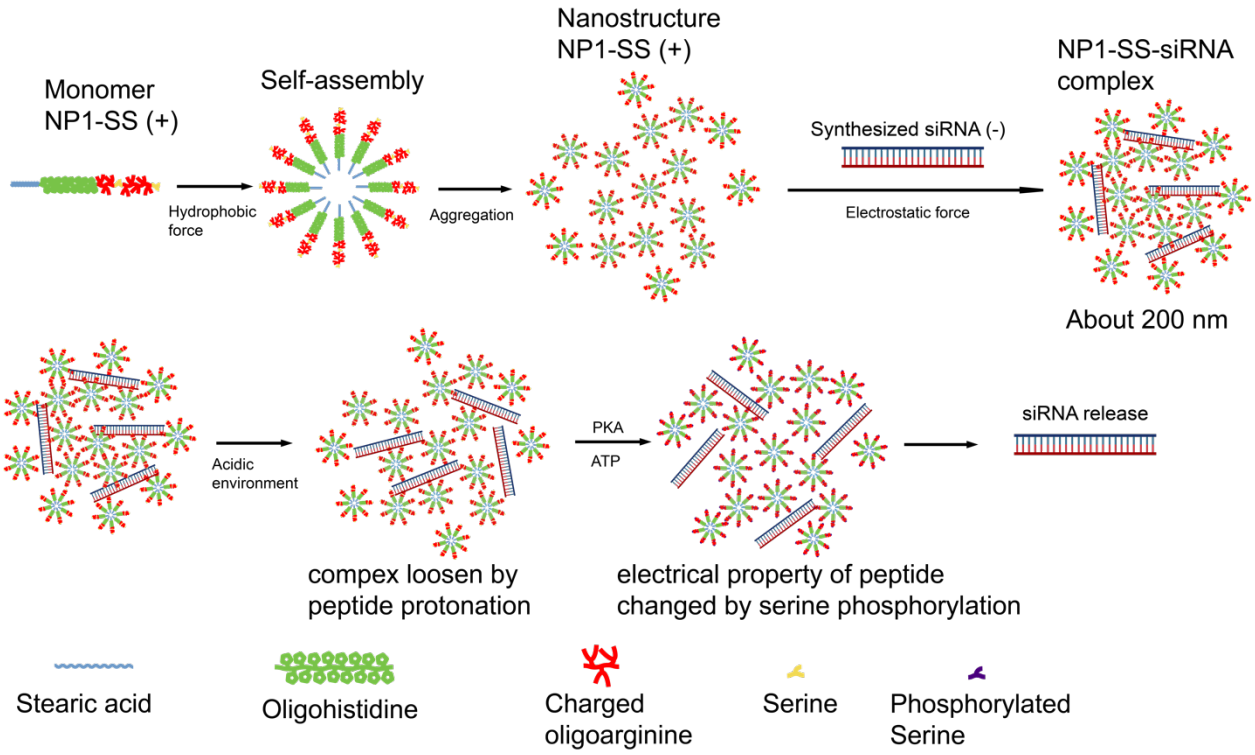

Figure 1.2 Mechanisms: NP1-SS self-assembly, formation of NP1-SS-siRNA complex, and disassembly of the complex triggered by pH and PKA, leading to siRNA release

The self-assembly of NP1-SS starts with dissolving peptide powder in high pH where the oligohistidine subsequence is deprotonated, and the stearic acid moiety experiences hydrophobic interaction in solution. It is pushed into the core while the hydrophilic oligoarginine head faces outwards. The self-assembled peptide then aggregates to form uneven-sized large nanostructures. When synthetic siRNAs are added to the system, peptides and siRNAs co-assemble via electrostatic attraction, stabilizing the structure and forming a

CPP-siRNA complex with sizes less than 200 nm in diameter. The CPP-siRNA complex is disassembled by adjusting the liquid environment to change oligohistidine to trigger the first stage of siRNA release. In such conditions, the oligohistidine subsequence is protonated, providing repulsive force between peptides, then loosening the complex structure, which mimics the process of intracellular proton sponge effect. The second stage of siRNA release is promoted through the enzyme-mediated phosphorylation of CPPs. As mentioned in 1.1.3, the phosphorylation reaction of peptides in this work is catalyzed by protein kinase A (PKA), which is an enzyme that exists in the cytoplasm involved in cellular regulation processes that work through a cyclic adenosine monophosphate (cAMP)-dependent signaling pathway [52]. In detail, PKA is a heterotetramer holoenzyme composed of four subunits. Two are regulatory subunits, and the other two are catalytic subunits [53]. cAMP is a second messenger derived from ATP and used for intracellular signal transduction [54]. PKA functions in response to the concentration of cAMP. As demonstrated in Figure 1.3, PKA is inactive in tetramer R2C2. When cAMP molecules bind to PKA regulatory subunits, the catalytic subunits are released and activated, and the R2C2 complexes dissociate [55]. The free catalytic subunits recognize and interact with NP1-SS to phosphorylate serine residues in the RRRS substrate section.

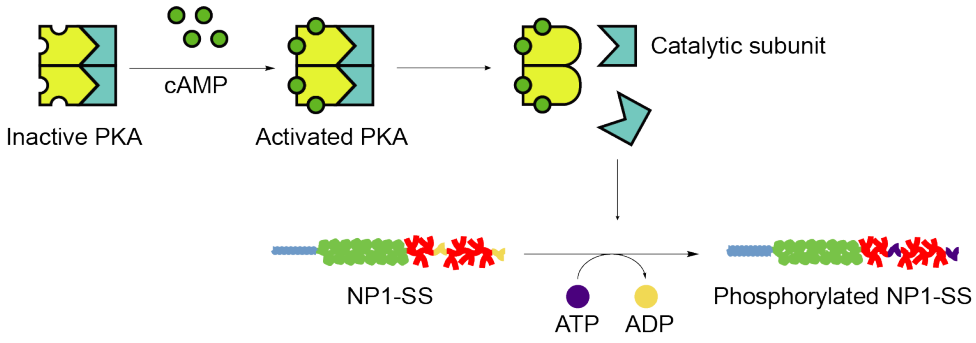

Figure 1.3 Example of NP1-SS phosphorylation by PKA catalytic subunits via cyclic AMP activation

The intracellular siRNA release and following gene silencing are presented in Figure 1.4. Cationic peptides can cross cell membrane via endocytosis, entering endosomes where oligohistidine can be protonated due to acidic conditions. In the meantime, the influx of hydrogen ions and chloride ions can cause the proton sponge effect, guiding the endosomal escape of loosened CPP-siRNA complexes during the first stage of siRNA release. After reaching the cytoplasm, CPP with RRRS can be phosphorylated by PKA catalytic subunits released via the combination of cAMP and PKA regulatory subunits. A phosphate group added to serine can weaken the electrostatic interaction between CPP and siRNA, triggering the second stage siRNA release, enhancing the dissociation of siRNA from CPP. The released siRNAs can form complexes with RISC, unwind and become active, and identify target mRNA.

Consequently, the combined mRNA will be cleaved and degraded, and gene knockdown can be attained.

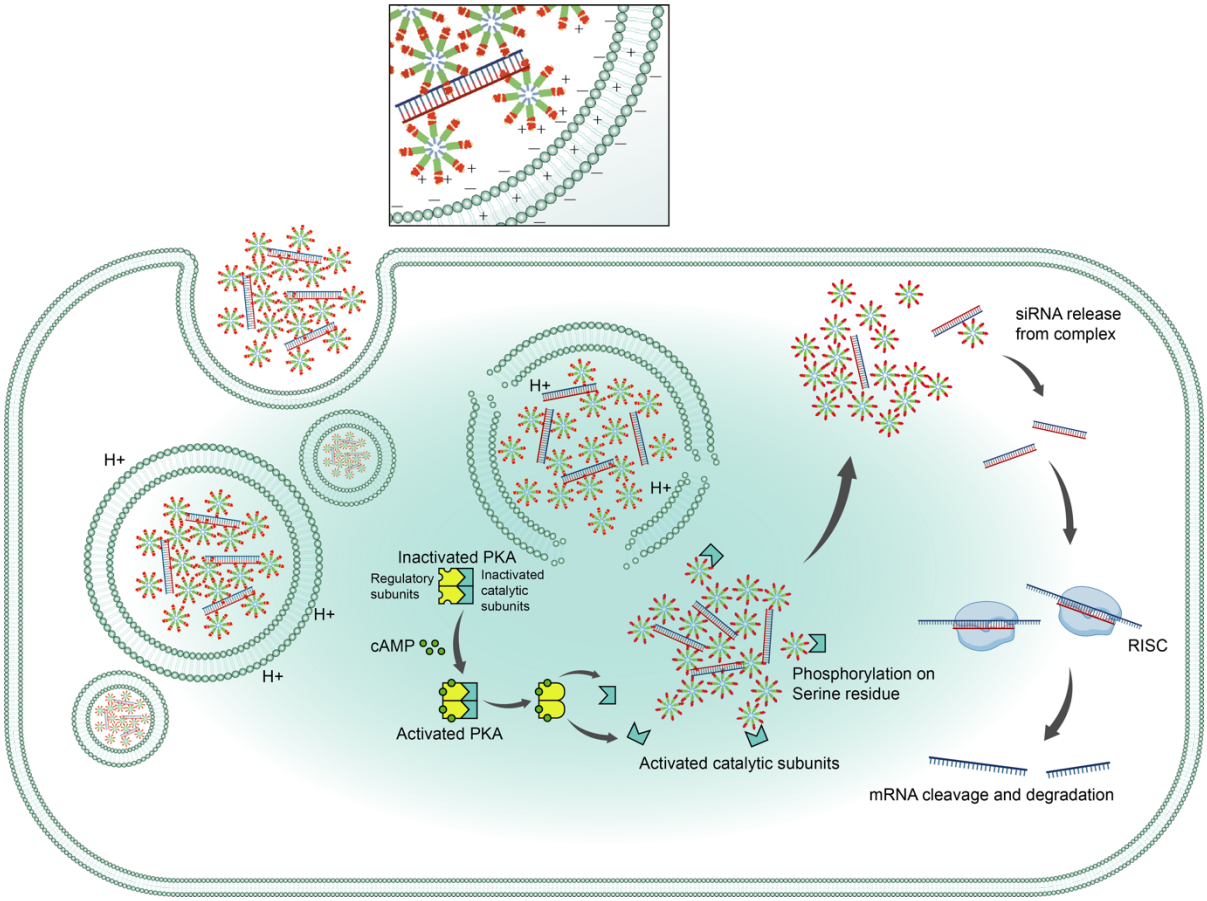

Figure 1.4 Endocytosis of CPP-siRNA complex, proton sponge effect induced endosome rupture, PKA regulation pathway and RNAi pathway of released siRNA.

1.2 Research Objectives

The general objective of this project is to design a self-assembled siRNA delivery system responsive to peptide phosphorylation to achieve enhanced release of siRNA.

The goal is to achieve:

- Modification of NP1 with serine for peptide phosphorylation.
- The formation of CPP-siRNA complexes and characterization of the physicochemical properties of CPPs and CPP-siRNA complexes.
- The self-assembly of CPPs and disassembly of CPP-siRNA complexes triggered by pH and PKA.
- Conducting *in vitro* tests of cell cytotoxicity, cellular uptake of CPP-siRNA complexes.
- Gene silencing and improving transfection efficiency.

1.3 Outline of the Thesis

There are five chapters included in this thesis.

Chapter 1 is the introduction of the project. It consists of four main ideas of this work: RNA interference, cell penetrating peptide, enzyme-mediated phosphorylation, and the mechanism

of the designed siRNA delivery system. The first three are the background behind this work, and the last part is the purpose of this project.

Chapter 2 provides the current reviews on RNAi, CPPs and phosphorylation mentioned in Chapter 1. RNAi is introduced with mechanism and applications. The CPP-based delivery system and their internalization mechanism are described. Phosphorylation-dependent dissociation is also discussed.

Chapter 3 describes the methodology in physicochemical characterizations of designed CPPs and CPP-siRNA complexes. Fluorescence spectroscopy, gel electrophoresis, DLS, CDs, TEM and AFM are used to characterize formulation of particle aggregation, CPP-siRNA complex, size and potential, secondary structure, and morphology, respectively. Result of each experiment is discussed accordingly, and an overall conclusion of the chapter is presented in the end.

Chapter 4 describes the methodology in siRNA release process *in vitro*. CCK8, FACS, Nanodrop, and qPCR are used to determine cytotoxicity, cellular uptake, released siRNA concentration, and gene knockdown efficacy for the performance of all three designed CPPs, respectively. Result of each experiment is discussed accordingly, and an overall conclusion of the chapter is presented in the end.

Chapter 5 summarizes the work done in current progress and recommends the future work that could be conducted to improve the result of this project and the future research direction that could be extended from this work.

Chapter 2

Literature Review

2.1 RNA interference as a competent tool

2.1.1 Mechanism of RNA interference in mammalian cells

RNA interference (RNAi), also known as post-transcriptional gene silencing (PTGS), is one of the gene regulatory mechanisms to achieve RNA silencing by limiting the transcript level. According to decades of research, RNAi is found to occur in almost all eukaryotic organisms, including plants [59], protozoans [56], insects [57], and mammals [58]. The processes and functions of RNAi serve as the protection of the genome against invasion caused by exogenous sources or endogenous lesions. The natural genetic mechanism of RNAi in eukaryotes occurs after DNA transcription (the synthesis of mRNA from DNA). Firstly, single-stranded RNAs transcribed by RNA polymerase II and III produce the primary miRNA, which is then cleaved by the DroshaDGCR8 complex into the precursor microRNA (pre-miRNA). The pre-miRNA migrates from the nucleus into the cytoplasm, and then an endoribonuclease named Dicer cleaves the pre-miRNA and produces a mature miRNA segment which is approximately 20 nucleotides in length, initiating the process of RNAi [60]. After the miRNA molecule is

produced, it will incorporate into an RNA-induced silencing complex (RISC) consisting of various proteins and a ribonuclease enzyme, forming a miRNA-RISC complex. Once incorporated, double-stranded RNA (dsRNA) is split into one passenger strand and one guide strand. The passenger strand will be released and degraded, while the guide strand will be catalyzed by the activated RISC [61]. Afterwards, the miRNA-RISC complex will bind to a subsequence domain of particular mRNA complementary to the guide strand sequence. Moreover, naturally occurring miRNA binds to the targeted sequence imperfectly, allowing miRNA to accept hundreds of mRNA sequences. As long as the binding with mRNA occurs, the ribonuclease enzyme contained in the miRNA-RISC complex would cleave targeted sites on the mRNA sequence, eventually leading to the inhibition of translating a specific gene into protein. As a result, the gene segment is silenced. The outcome, however, could be different when the condition of matching varies. With a precise match, the miRNA sequences can induce endonucleolytic cleavage, which results in mRNA degradation, whereas with an imperfect match, it could regulate translation, leading to inhibition of mRNA expression [62].

Thousands of years of evolution have armored our body with multitudinous fight or flight mechanisms, and RNAi is one of which can regulate genes and mediate cellular defence against infection by viruses, especially RNA viruses like influenza. Antiviral RNAi genes encoding

miRNAs with sequences complementary to viral sequences have been evolved in plenty of eukaryote organisms. Furthermore, RNAi contributes to the suppression of transposon activity in cells. Some transposons capable of self-duplication can be inserted into different segments of the genome, resulting in repetitive sequences of DNA, which could give rise to human diseases caused by fluctuant gene activity such as haemophilia [63], Duchenne muscular dystrophy [64] and certain types of cancer [65]. By regulating with RNAi, the possibility of irregular gene activity would be reduced, hence, lowering the rate of related diseases.

This discovery of the innate RNAi mechanism in eukaryotic cells revolutionizes the study of gene function by designing and introducing synthetic segments of double-stranded RNA (dsRNA) into cells in tissue culture. By imitating the function of miRNA during the process of natural RNAi, gene silencing can be achieved by synthetic dsRNA as well. Different types of synthetic dsRNAs have been developed over the years to manipulate gene functions. One of the universal dsRNAs is small interfering RNA or short interfering RNA (siRNA), which is about 20 to 25 nucleotides in length. Specifically, 21 nucleotides siRNAs with 3'-d(TT) or (UU) overhangs were the most effective and commonly studied [68]. According to early research conducted by Elbashir et al., siRNA can also bind to RISC and cleave targeted mRNA sequences like miRNA without Dicer pre-cut to exercise its activity, and it can suppress

mammalian gene expression in high efficiency [66, 67]. In detail, when RISC-siRNA is formed, Argonaute 2 (Ago-2), the catalytic core of RISC, will also bind to siRNA and then release one strand of the siRNA, activating the RISC-siRNA which further directs the recognition of complementary mRNA [69]. Since the synthesized siRNA cannot be produced in the cytoplasm, in order to induce RNAi, the designed siRNA should be transfected into the cells of choice. Therefore, transfection reagents with low cell cytotoxicity are required to achieve this step. Commonly used reagents are lipid-based and cationic for their overall net positive charge at physiological pH, ease of use and applicability to various cell types [70].

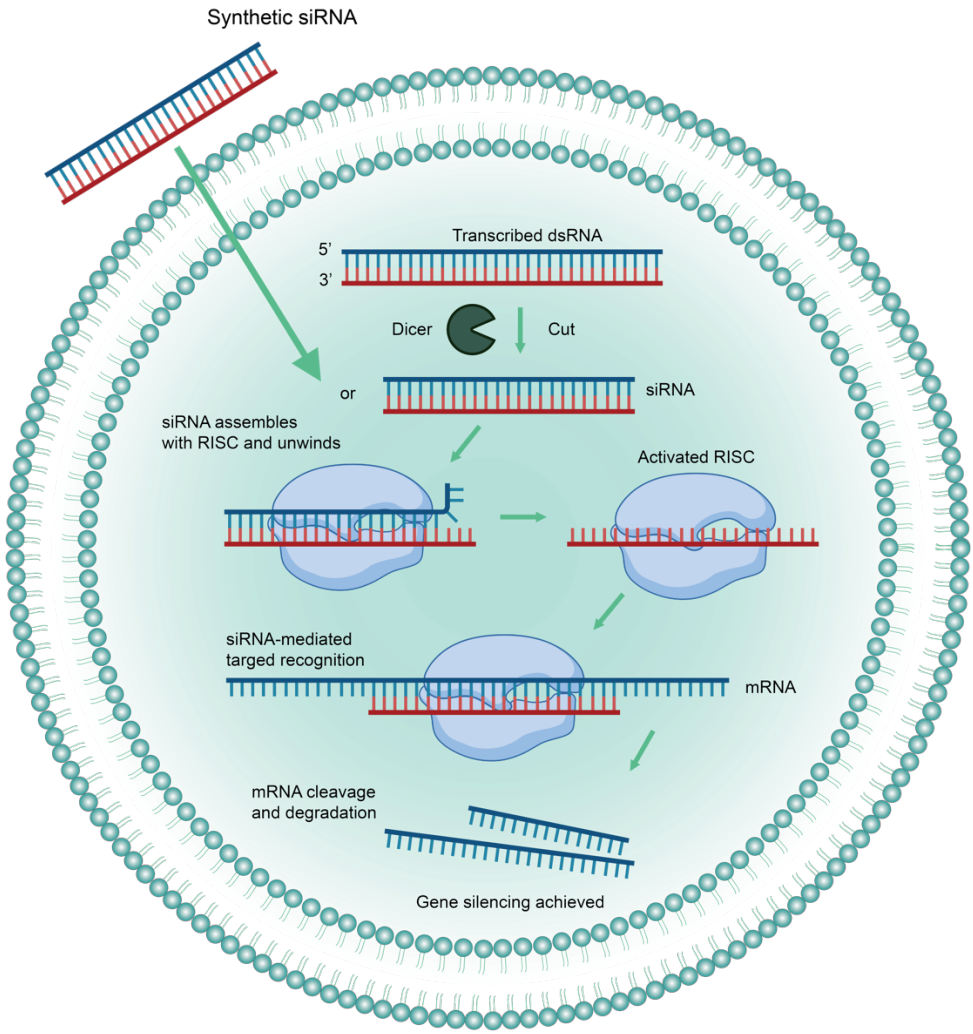

Figure 2.1 Intracellular RNAi pathway with endogenous or synthetic siRNA

The general purpose of RNAi is to investigate the activity of genes, understand their corresponding functions, and troubleshoot abnormally overactive genes that cause malignant mutations.

2.1.2 Applications of RNAi

RNAi is a universal intracellular process, and it participates in multiple cell regulations. Upon understanding the mechanism of RNAi, researchers can take advantage of the RNAi technique in genetic manipulation. RNAi has made remarkable progress in a wide range of fields such as functional genomics research, microbiology research, gene therapy, and signal transduction, which makes its application in medicine, including animal medicine, having broad prospects [71]. Comparing other therapeutic approaches such as small-molecule inhibitors that target proteins, RNAi overcomes challenges, including identifying specific agents to disrupt protein function and the relatively low specificity of the inhibitors [72]. Because the expression or function of specific genes is impaired in most pathologic circumstances, and miRNAs are unregulated in many diseases, RNAi might be used as a therapy to provide signaling molecules like transcription-suppressive factors to help restore gene expression of the impaired genes [159].

2.1.2.1 Useful tool for the discovery of gene function

In the era of post-genome, large-scale and high-throughput research on gene functions is in high demand. Due to the high degree of sequence specificity and effective interference activity of RNAi, it is capable of silencing targeted genes, disabling functions and reducing mutations,

which is suitable as a powerful tool for functional genomics [73]. Several laboratories have used RNAi technique to carry out large-scale genome screening. The basic principle is to firstly construct a dsRNA library for different parts of genome involving corresponding dsRNA, and then introduce them into different cells, and eventually gene screening and gene function could be determined by protein expression [74].

Studies have shown that RNAi can inhibit the expression of specific genes in mammals, creating a variety of phenotypes, and the time of inhibiting gene expression can be controlled at any stage of development, resulting in an effect similar to gene knockdown. Compared with traditional gene knockdown technology, this technique has the advantages of low investment, short cycle, simple operation, etc. [75]. Recently, there are more and more reports of successful use of RNAi to construct transgenic animal models, which indicates that RNAi will become indispensable tool for studying gene functions.

In genomics research, gene expression regulation is an important part of studying gene function, and gene knockdown strategy is a significant position in gene expression regulation. RNAi technique has considerable application prospects for establishing gene knockdown in animal models.

2.1.2.2 Therapeutic applications on viral diseases

Viruses- and bacteria-induced diseases have always been one essential cause of death worldwide. Some well-known representatives are HIV [78, 79], influenza [92] and Ebola [82]. Since synthetic siRNAs was found to limit viral infection based on directly silencing corresponding viral genes, it was considered as a potential alternative to traditional antiviral therapy. RNAi was able to inhibit the replication of viral agents. The duplication of virus depends on a limited set of viral genes, which makes RNAi easy and effective on treating viral infection [93].

Chronic HBV (CHB) infection is one case of viral diseases that can generate severe problem in immune system [94]. It is a long-standing health problem with unmet medical needs. DNA of HBV reproduces through a genomic RNA intermediate and makes use of an encoded viral reverse transcriptase during its replication. It is found that HBV proteins conjugate with pre-genomic RNA, supply the virus, and interact with the host immune system. By applying RNAi, viral mRNA cleavage prevents the production of viral proteins and replication products. Many progresses have been made to confront this disease. In recent research conducted by Wooddell's team (2018), RNAi therapeutic ARO-HBV can combine with S and X open

reading frames triggering RNAi, which is designed to inhibit all HBV transcripts, including from host-integrated HBV DNA. Hence, the coverage of genotype can be broadened, and resistance development can be avoided. Mice was hydrodynamically injected with an HBV plasmid that can express HBV RNA. ARO-HBV was administered to mice by hypodermic injection to treat the infectious mice. Consequently, ARO-HBV exhibited efficient reductions in viral antigens and DNA of HBV. Moreover, the result also showed low toxicity in mice. According to the progress mentioned in Wooddell's work, ARO-HBV is proceeding to clinical trials at phase I/II in patients with CHB [90].

Respiratory syncytial virus (RSV) infection is a deadly disease that can easily affect children in early age, produce annual pestilence of respiratory sickness, and cause lower respiratory tract disease, bronchiolitis, and otitis media. Not friendly to adults and elderlies either, RSV can produce upper respiratory illness like pneumonia [95]. RSV propagates nearly exclusively in the respiratory epithelium cells, especially in the outermost layer. The proteolytic enzymes of the infected cells cut F protein on cell membrane, and the virus can attach to membrane and enter the cell cytoplasm where they can reproduce. The RNA virus is transcribed into several distinct proteins, including 2 non-structural proteins, NS1 and NS2, as essential factors in reproduction. During the past decade, RNAi therapeutic for RSV has progressed into clinical

trials. ALN-RSV01 is an siRNA targeting the mRNA encoding the N protein of RSV. It has been extensively tested and has shown great antiviral effect in vitro and in vivo prior to clinical trials. RSV replication in animal models of infection can be reduced by the delivery of ALN-RSV01into cells of the respiratory tract. This siRNA is the first antiviral siRNA aiming for microbial pathogen to be administered into clinical trials, delivery to the human respiratory tract. ALN-RSV01 displays specific antiviral activity against RSV in animal experiments, which also shows a solid safety profile. DeVincenzo' team conducted the preclinical trails and evaluated the safety and tolerance of this siRNA delivered intranasally as a spray to the upper respiratory tract with positive results [81, 91]. It appears to be a potential candidate for further development.

Ebola virus disease (EVD) or Ebola hemorrhagic fever is a killing disease for human firstly appeared in central Africa in 1976. The largest outbreak of EVD was during 2014-2016 in West Africa [96]. EVD is transmitted to and spread in human populations through physical contact with bodily fluids. The pathophysiological characteristics of sever Ebola virus diseases are vascular leakage, shock, coagulopathy, and multiple organ damage [97]. Therefore, antiviral agents are in serious demand for the drug development of EVD. One advanced preclinical therapy evaluated by WHO was TKM-100802 (Tekmira Pharmaceuticals, British

Columbia, Canada) which is a lipid nanoparticle (LNP) formulated with siRNAs targeting genes encoding L polymerase and Viral Protein-35. The two proteins are involved in propagation of Zaire ebolavirus and suppression of the host immune response, respectively [98, 151].

A new formulation of TKM-100802 is named TKM-130803, in which the siRNA component has been adapted and substituted nucleotides in both L polymerase and Viral Protein-35 siRNAs to ensure specificity to the West African Makona variant of *Zaire ebolavirus*. The siRNA drug component of TKM-130803 is termed siEbola-3 and share the same LNP composition. However, the survival in those receiving TKM-130803 did not improve the historic survival rates. The trial failed to identify the effectiveness and harmlessness ang has been terminated. In short, even the administration of TKM-130803 with certain dosage was proved well tolerated in the trial, further investigation is required on the potential influence of drug formulation and improvement of survival [82].

2.1.2.3 Therapeutic applications on hereditary diseases

Genetic disorders are generally caused by the mutation of gene subsequence. Since there is no cure or treatment available to eliminate the disease, only a few to alleviate the symptoms, therapies that can target mutant genes selectively are crucial for the effective treatment of

genetic disorders [99]. Thus, RNAi and RNA targeting drugs like antisense are considered promising due to their specific selectivity on targeting genes with single nucleotide polymorphism mutations.

Hereditary transthyretin amyloidosis is a progressive and fatal genetic disease caused by transthyretin (TTR) gene mutations which are produced primarily in liver. The transcribed TTR protein, either mutant or wild type, can deposit in various tissues and organs as amyloid, leading to polyneuropathy and cardiomyopathy [100]. Current available treatments for hereditary transthyretin amyloidosis are liver transplantation and TTR stabilizers, yet still cannot thoroughly and effectively inhibit disease progression [101]. Prominently, there is an FDA approved RNAi therapeutic agent named Patisiran (sold under ONPATTRO) is currently in use for the treatment of hereditary TTR-mediated amyloidosis. It is the first siRNA-based drug approved to treat this disease and is considered to be a first-in-class medication, and currently it is marketed by Alnylam. Patisiran was designed to decrease the production of both wild-type and mutant TTR, which is a formulation of TTR targeting siRNA in lipid nanoparticles (LNPs) that delivery siRNA to TTR production site. Patisiran siRNA targets a subsequence in the untranslated domain of TTR mRNA where wild-type and all TTR variants are encoded. Therefore, it suppresses the translation of both wild-type and pathogenic proteins.

The release of patisiran siRNA into the cytoplasm is achieved by subsequent endocytosis which assists the fusion of LNP with the endosomal membrane. Patisiran is well tolerated and has demonstrated a manageable safety profile in patients with hereditary transthyretin amyloidosis. Moreover, the potential for future management of hereditary transthyretin amyloidosis suggests the combination of patisiran therapy with TTR stabilizers or antibodies to provide multiple modes of action [76, 88].

Hypercholesterolemia or familial hypercholesterolemia impedes the regular process of cholesterol, leading to high risk of cardiac arrest and acceleration the occurrence of heart attack. It is caused by inherited gene, and symptoms can be presented at early age [102]. The mutant gene inhibits the elimination of low-density lipoprotein (LDL) which can narrow and harden arteries, increasing the risk of cardiac-related malfunctions. Medications and healthy diet can ameliorate the morbidity, yet still, it is easier for people carrying this gene defect to acquire elevated LDL cholesterol level [103]. Therefore, additional treatment options are expected for decreasing LDL cholesterol lever, moreover, decreasing risk of cardiovascular diseases. Proprotein convertase subtilisin-kexin type 9 (PCSK9) is a serine protease that is mainly responsible for increasing LDL cholesterol levels by intracellular and extracellular binding to LDL receptors, initiating the receptor degradation in liver. The administration of siRNA

molecules can decrease PCSK9 levels effectively. A formulation of LNP and siRNA, ALN-PCS, has been proved to be capable of inhibition of PCSK9 synthesis and reduction of LDL cholesterol in adult. Inclisiran (ALN-PCSsc) is a conjugate of synthetic PCSK9 targeting siRNA and triantennary N-acetyl galactosamine carbohydrates which can bind to hepatocytes membrane and guide inclisiran uptake into hepatocytes. The clinical result observed significant reductions in PCSK9 and LDL cholesterol levels. And for long-term persistence, result suggested that it is highly possible for inclisiran to provide effective treatment of hypercholesterolemia with semiannual or quadrennial administration which is currently in phase III trial [83].

2.1.2.4 Therapeutic applications on cancers

Cancer is a sever and life-threatening illness which occurs during abnormal cellular reproduction process. The defective DNA of cells is the cause of malfunctional cell growth and division. Tumors are the result of gene network regulation by the interaction of multiple genes, which are formed by uncontrollable cancer cell replication. Uncontrolled metastasis of the tumor is the cause of its malignancy. The current cancer statistics stated that many patients are still inaccessible to efficient and harmless treatment. Patients with cancer can ameliorate symptoms by receiving traditional surgery, radiotherapy, and chemotherapy at the expense of

financial and mental burden [104]. The discovery of RNAi therapies has inaugurated a new direction for the treatment of cancer. The blockade of a single oncogene induced by traditional techniques cannot completely inhibit or reverse the growth of tumors, while RNAi can make use of the homology of multiple genes in the same gene family with the characteristic of highly conserved sequence. Then, a dsRNA molecule targeting this segment sequence could be designed and this kind of dsRNA can be injected to eliminate the performance of multiple genes at once. Meanwhile, it is also possible to inject multiple dsRNAs simultaneously to eliminate multiple unrelated genes at the same time [105].

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy accounts for most (over 90%) of pancreatic cancer cases. Only 7% patients with pancreatic cancer can survive over five years. It exists and develops in the lining of the ducts in the pancreas and from cells that produce pancreatic enzymes [106]. A siRNA delivery system, Atu027, was developed to target the expression of protein kinase N3 (PKN3) gene which produce proteins mediating metastatic cell growth, and currently is under development for the treatment of advanced solid cancer. Atu027 is a liposomal siRNA formulation based on cationic lipids, which impedes the function of PKN3 such as the formation on extracellular matrix and cell migration. Systemic Atu027 administration resulted in therapeutic efficacy in the inhibition of invasive cell growth, and the

observed efficacy was not related to toxic side effects including unspecific stimulation of the innate immune system [77, 87].

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer, which occurs in population with chronic liver diseases or infection like hepatitis and cirrhosis [107]. Polo-like kinase 1 (PLK1) is over expressed in multiple tumors including liver cancer and is associated with poor prognoses in cancers. PLK1 plays multiple roles during cell cycle including controlling mitotic entry, regulating chromosome segregation, facilitating DNA replication, etc. Studies have discovered that the inhibition of PLK1 expression can interfere with multiple stages of mitosis, leading to cancer cell apoptosis. TKM-080301 is a LNP formulation of a siRNA against PLK1. In preclinical studies, TKM-080301 silenced PLK1 expression and induced strong antiproliferative activity in various cancer cell lines. The single administration of TKM-080301 was well-tolerated and can last up to 10 days [80].

Table 2.1 Examples of RNAi therapeutics

Type of disease	Conditions	Target	Delivery system	Therapeutic name	Phase	Reference
Viral infections	Hepatitis B (HBV)	HBV gene	GalNAc-siRNA conjugate	ARO-HBV	I/II	[90]
	Respiratory Syncytial Virus Infections (RSV)	RSV nucleocapsid "N" gene	Naked siRNA	ALN-RSV01 (Asvasiran sodium)	II	[81, 91]

	HIV/AIDS	CCR5	Lentivirus vector, Benitect's DNA-directed RNAi technology	Cal-1 (LVsh5/C46, Cal-1 modified HSPC, Cal-1 modified CD4+ T lymphocytes)	I/II	[78, 79]
	Ebola virus infection	VP35, Zaire Ebola L-polymerase	LNP	TKM-Ebola-Guinea (TKM-130803)	II	[82]
Hereditary diseases	Transthyretin (TTR)-mediated amyloidosis	TTR	LNP	ONPATTRO (Patisiran, ALN-TTRO2)	Approved	[76, 88]
	Acute Hepatic Porphyria (AHP)	ALAS1	GalNAc-siRNA conjugate	GIVLAARI (Givosiran, ALN-AS1)	Approved	[86]
	Primary hyperoxaluria type 1	HAO1	GalNAc-siRNA conjugate	Lumasiran (ALN-GO1)	III	[85]
	Hypercholesterolemia	PCSK9	GalNAc-siRNA conjugate	Inclisiran (ALN-PCSsc)	III	[83]
Cancers	Hematological malignancies	PD-L1/L2	Ex vivo transfection	PSCT19 (MiHA-loaded PD-L-Silenced DC caccination)	I/II	[84, 89]
	Pancreatic ductal carcinoma	PKN3	AtuPlex	Atu027	I/II	[77, 87]
	Clear cell renal cell carcinoma (ccRCC)	HIF-2α	TRiM (RGD-siRNA conjugate)	ARO-HIF-2	I	
	Hepatocellular carcinoma (HCC)	PLK1	LNP	TKM-080301	I/II	[80]

Abbreviation: GalNAc, N-Acetyl-D-galactosamine; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; CCR5, C-C chemokine receptor type 5; VP35, viral protein 35; LNP, lipid nanoparticle; ALAS1, 5′-aminolevulinate synthase 1; HAO1, hydroxyacid oxidase 1; PSCK9, proprotein convertase subtilisin-kexin type 9; PD-L,

programmed death-ligand; PKN3, protein kinase N3; HIF-2α, hypoxia-inducible factor-2 α; PLK1, polo-like kinase 1.

In sum, with the in-depth research of RNAi mechanism and the improvement of RNAi technique, it has the potential to become a convenient and practical genome research method and gene therapy drug, heralding a new era of RNA.

This ancient and multi-potent cell-level supervision system in cells has been tested for thousands of years of natural selection and evolution, and it is undoubtedly an invaluable treasure. This technology will be widely used in agriculture, forestry, animal husbandry, fishery, and other fields, and has huge value on scientific research, economy and society [108].

2.2 Current peptide-based delivery system

2.2.1 Design principle of self-assembling cell-penetrating peptide

In designing an amino acid pairing peptide that can self-assemble and serve as a cell-penetrating peptide, three significant interactions are considered between adjacent peptides: electrostatic interaction, hydrophobic interaction, and hydrogen bonding.

The electrostatic interactions between two oppositely charged residues can form ionic pairing between peptides. Regardless of the charge position, electrostatic interaction can occur in most conditions. In the meantime, the charged residues can enhance the peptide solubility, assisting

peptide self-assembly and possibly facilitating cell adhesion for tissue engineering applications [162].

The hydrophobic amino acid pairing utilizes non-polar side chains, depending on their structural similarity for better matching and hydrophobicity strength for self-assembly tendency. The arrangement of the hydrophobic residues in the sequence will directly impact the amphiphilic structure of the peptides and their self-assembled nanostructures [163].

Hydrogen bonds can be formed by most naturally occurring amino acids via the side chains, either as hydrogen donors or acceptors. The pairing can occur at more localized domains than electrostatic interactions [161].

The appropriate utilization of these interactions is the core for designing self-assembling peptides and constructing functional nano-biomaterials.

2.2.2 Cell-penetrating peptide-based siRNA delivery system

Cell-penetrating peptides (CPPs) are short peptides capable of assisting cellular internalization of macromolecules such as peptides, proteins, ribonucleic acids, quantum dots, and other therapeutic molecules [109, 152]. Over the last 30 years, CPPs have been continuously and extensively studied as delivery agents for molecular therapies. The discovery of CPP can be traced back to the end of last century, the first observation was made by Frankel and Pabo in 1988 that the HIV-1 transcription-transactivation (TAT) protein could shuttle within the cells and translocate in the nucleus [110]. Three years later, Prochiantz's team confirmed the

internalization of Drosophila Antennapedia homeodomain by neuronal cells [111]. In the year of 1994, the first protein transduction domains (PTDs) or CPPs was uncovered, a short peptide sequence with 16 amino acids (RQIKIYFQNRRMKWKK) derived from the third helix of the homeodomain of Antennapedia, and this CPP was termed penetratin [113, 118]. By developing the work of Frankel and Pabo, the group of Lebleu identified the shortest peptide sequence of TAT (YGRKKRRQRRR) required for cellular uptake in 1998 [117, 125]. Since then, the application of the process for efficient intracellular drug delivery was on trend. Studies confirmed the ability of CPP to translocate various types of molecules into cytoplasm, making it a great candidate for theranostic application [127]. In most cases, CPPs possess low cytotoxicity and can transport nonpermeable cargos into living cells, thus, they are of significant pharmaceutical interest. Generally, siRNA mediated by CPP can enter cells by endocytosis or direct crossing through cell membrane [128].

CPPs are categorized into three major groups according to physicochemical properties and amino acid composition: hydrophilic (cationic or anionic), hydrophobic and amphipathic [129].

Cationic CPP, such as TAT from HIV-1, should carry a minimal number (8) of positive charges to function as a proper penetrating peptide. Among many DNA/RNA-binding motif-based CPPs, oligoarginines exhibits remarkable penetrating ability. It is revealed that the length of

oligoarginine affects its internalization ability. When the number of arginine is 8 or higher, peptides are efficiently taken into cells. For delivery purposes, the optimal length of oligoarginines is 8- to 10-mer for the maintenance of cell viability and interaction with plasma membrane [130, 153, 154].

Amphipathic CPPs typically include hydrophilic domain and hydrophobic region. 40% of CPPs developed so far belong to the class of amphipathic CPPs. They can associate with neutral and anionic membranes, and possibly induce rearrangement of membrane organization when inserted into membranes. Additionally, the stearylation of CPP is considered to increase the quantitative uptake of the cargo, and more importantly, the stearylation promotes a more efficient endosomal escape, which is a critical improvement for amphipathic CPPs [131, 155].

Hydrophobic CPPs are derived from signal peptide sequences and contain either only nonpolar or possess very few charged amino acids, and not commonly used for cargo delivery. Compared to the other two groups, the mechanism and function of hydrophobic CPP are less studied [132].

CPP can form covalent conjugate with the cargo, which is achieved by chemical cross-linkage. Different chemistries have been proposed for cleavable conjugation including disulfide bonds and thio-esters linkages. Covalent conjugations have been commonly studied for the delivery

of DNA mimic molecules or steric block oligonucleotides, which is advantageous for rationalization and the control of CPP-cargo stoichiometry. Nevertheless, several limitations exist for covalent CPP strategy like risks of interfering the biological activity of the cargo, especially in the case of oligonucleotide or siRNA [133, 156].

CPP can also interact non-covalently with the cargo through electrostatic interactions, commonly occurs with short amphipathic peptide carriers. By mixing siRNA and CPP in a proper molar and charge ratio, complexes with positive charges can be formed, facilitating cell membrane translocation. For instance, in 2003, a primary amphipathic CPP, MPG, adapted the non-covalent strategy and efficiently delivered siRNA into cells. MPG is a peptide derived from HIV-1 gp41 protein and nuclear localization signal (NLS) of SV40 large T antigen, which formed stable complexes with siRNA through non-covalent electrostatic and hydrophobic interactions [134].

Table 2.2 Examples of selected CPPs

Type	Name	Sequence	Cargo	Ref.
Hydrophilic peptides	Penetratin	RQIKIWFQNRRMKWKK	Insulin	[2,7]
	Oligoarginines	RRRRRRRRR/ RRRRRRRRRRRR	Protein	[4,5,9]

	HIV-TAT (47-57)	YGRKKRRQRRR	Plasmid DNA	[6,14]
Hydrophobic peptides	Hepatitis B virus translocation motif	PLSSIFSRIGDP	ovalbumin	[1]
	Kaposis sarcoma fibroblast growth factor	AAVALLPAVLLALLAP	NLS of NF-kappa B p50	[8]
	Human calcitonin partial sequence	LGTYTQDFNK(X)FHTFPQTAIGVGAP-amide	Nucleic acid drug	[11,12]
Amphipathic peptides	Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-amide		[13]
	PepFect14	Stearyl-AGYLLGKLLOOLAAAALOOLL-amide	Oligonucleotide	[3]
	KLA sequence	Acetyl-KLALKLALKALKAALKLA-amide	Nucleic acids	[10,15]

Abbreviation: NLS, nuclear localization sequence

2.2.3 Internalization mechanism of CPPs

CPP uptake mechanism varies from different CPP groups. Recent advances have reported three main mechanisms for CPP translocation.

The first pathway is direct penetration, which is energy independent. Early studies showed that Tat has the same cell-penetrating efficiency at different temperatures, and the absence of ATP did not affect the internalization process [135].

Unlike direct penetration, endocytosis-mediated translocation is energy-dependent. According to Richard's observation, polyarginine entered the cells via endocytosis during which cells

obtained energy from outside of the membrane. Endocytosis can be further divided into phagocytosis and pinocytosis. Phagocytosis is adapted in the absorption of macromolecules whereas pinocytosis is used for solute absorption [136].

The translocation via the formation of a transitory membrane structure relies on the structure of inverted micelles, allowing the CPP to adapt a hydrophilic condition. For example, arginine-rich CPP would combine with the phospholipids possessing negative charges, resulting in the formation of an inverted micelle inside the lipid bilayer [137, 157].

The uptake mechanism is particular for each CPP, and it can still be altered by different physicochemical parameters, concentration, temperature, and time. Endocytosis is considered the major mechanism for CPP internalization [138].

2.3 Protein phosphorylation-induced drug release

In 1955, protein phosphorylation was stated as a regulatory mechanism of great importance for regulation processes in mammalian cells [139]. This mechanism is instructed by protein kinases, and it allows cells to respond to external stimuli. The family of protein kinases is huge and diversified. Almost 2% of human genes are transcribed into protein kinases, it is a kinase capable of selective modification of proteins [140]. The eukaryotic protein kinases include a large number of diverse enzymes essential for regulation and cell growth. Within the large and

diverse family of protein kinases, protein kinase A (PKA) is one of the most widely studied protein kinases due to its simplicity and uniqueness. PKA, more precisely known as adenosine 3',5'-monophosphate (cyclic AMP)-dependent protein kinase, was discovered by Edmond H. Fischer and Edwin G. Krebs in 1968. They were awarded the Nobel Prize in Physiology or Medicine for 1992 for the discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism [141].

The simplicity of PKA is due to the structural composition, only comprised of two different types of subunits that can dissociate upon activation by cAMP. In the absence of cAMP, the inactive holoenzyme consists of two regulatory and two catalytic subunits, abbreviated as R and C, respectively [142]. The cooperative binding of cAMP with high affinity to tandem cAMP-binding domains in the R subunit induces a conformational change that unleashes the active monomeric C subunits. The simplicity of the dissociated catalytic subunit makes it an ideal framework for viewing the other more complex protein kinases, where the regulatory and catalytic moieties are typically either part of a contiguous polypeptide chain or part of a stable multi-subunit structure The R subunit serves not only as inhibitors of the C subunit and receptors for cAMP, but it also functions as adapters which can link the C moiety through a dimerization/docking domain to scaffold proteins A-kinase anchoring proteins (AKAPs) [143].

AKAPs target PKA to specific sites within the cell. The unleashed C subunit is the key to protein/peptide phosphorylation. C subunits functions by transferring the gamma phosphate from ATP to serine residue, resulting in phosphoserine [144].

The dissociation of cargo from carrier induced by carrier phosphorylation is a novel direction to achieve drug release. Phosphorylation-depending binding of a synthetic peptide to calmodulin has been studied by Brian's group. The synthesized peptide containing 25 amino acids, consists of four protein kinase C (PKC) phosphorylation sites and the calmodulin (CaM) binding domain of the myristoylated alanine-rich C kinase substate (MARCKS) proteins. This peptide is produced to determine the effects of phosphorylation on its binding and regulation of CaM. PKC phosphorylation of the peptide produced a significant decrease in its affinity for CaM. The peptide inhibited CaM's binding to myosin light chain kinase and CaM's stimulation of phosphodiesterase and calcineurin. The phosphorylation of the peptide occurred primarily at Ser 8 and 12 which regulated peptide affinity for CaM resulting in a rapid release of bound CaM [145].

Chapter 3

Physicochemical Characterizations and Mechanism of Self-Assembly of CPPs

3.1 Materials and Methods

3.1.1 Peptide and siRNA

Three stearylated peptides were synthesized in bulk by NPC (Qingdao, China): NP1 (Styl-H16R8-NH2), NP1-SS (Styl-H16R3SR5S-NH2) and NP1-SS'(Styl-H16R3SR3S-NH2) as shown in Figure 3.1. Silencer GAPDH siRNA (Life Technologies, Carlsbad, USA) was used to target the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. This gene encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family which is a very important enzyme involved in metabolic processes that catalyzes the sixth step of glycolysis and serves to break down glucose for energy. The sense sequence of the silencer was 5'-GGU CAU CCA UGA CAA CUU Utt-3' and antisense sequence was 5'-AAA GUU GUC AUG GAU GAC Ctt-3'. The silencer Cy3-labeled GAPDH siRNA (Life Technologies, Carlsbad, USA) was used in Fluorescence Activated Cell Sorting (FACS).

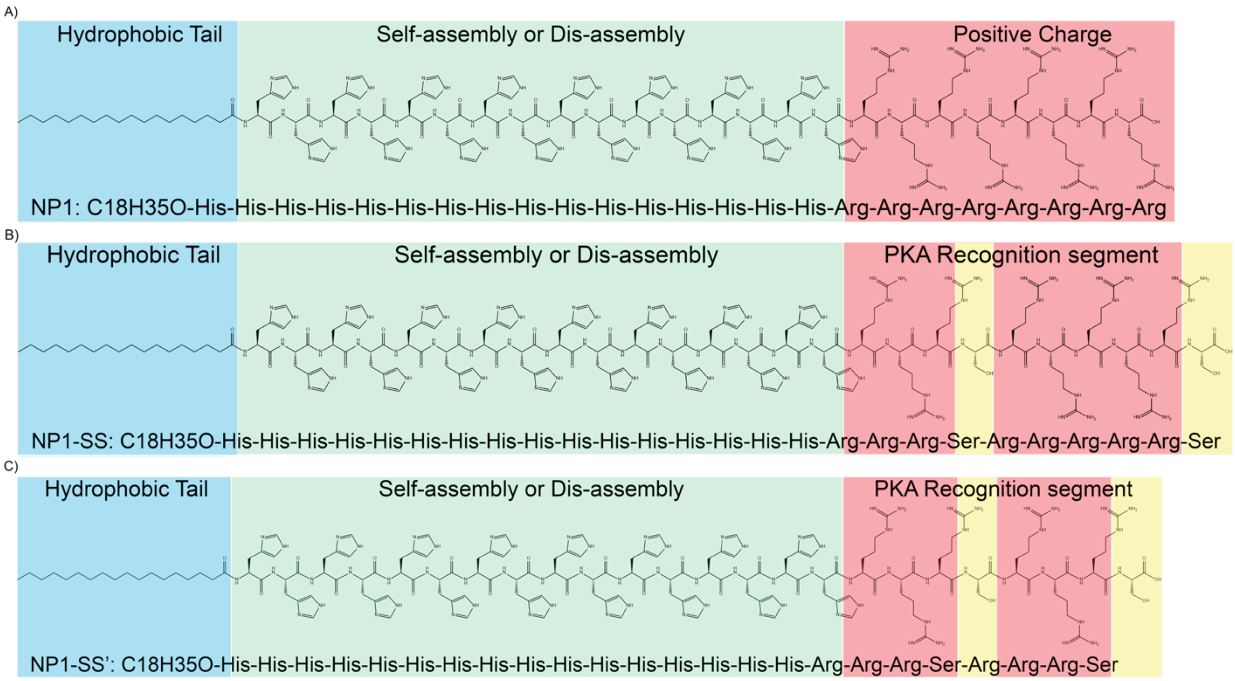

Figure 3.1 Structures of designed CPPs. A) NP1; B) NP1-SS: NP1 modified with addition of serine amino acid to form RRRS as PKA substrate; C) NP1-SS': NP1 modified by replacing arginine with serine to form RRRS as PKA substrate.

3.1.2 Preparation of peptide-siRNA complex

Peptides and siRNA were dissolved in filtered Ultra-pure water (filtered Milli Q water), respectively. Peptides were prepared at pH 6.0 and pH 8.0 to study the influence by pH. The complexes were formed by mixing different types of peptide and siRNA solutions at various molar ratios of CPP:siRNA, which were incubated for 20 minutes at room temperature before

characterization and used in siRNA release studies. The final peptide and siRNA concentrations varied in order to adapt to the different experiments.

3.1.3 Fluorescence Spectroscopy

The critical aggregation concentrations (CAC) of three CPP solutions were determined by an ANS fluorescence assay. Different concentrations of peptides (0.625, 1.25, 2.5, 5, 10, 20, 30, 40, 60, and 100 μM) were prepared for the measurement. 40 μL peptide solution was mixed with 40 μL ANS (Thermo scientific, Ottawa, Canada) solution (10 μM) using vortex for 10 s. For measuring the fluorescence, the mixture was transferred to a quartz cell and then measured by a Photon Technology International spectrofluorometer (Type QM4-SE, London, Canada). The baseline used in this experiment was ANS in Ultra-pure water. The excitation wavelength was set to 360 nm while the fluorescence spectrum was collected from 420 to 660 nm. The CAC of each peptide was determined by 475 nm ANS fluorescence intensity plotted against the peptide concentration from low to high.

3.1.4 Agarose gel-shift assay

The complexation of peptide and siRNA was studied using an agarose gel-shift assay on a nucleic acid electrophoresis instrument. 0.5x Tris-Base Electrolyte (TBE) (150mL) was used

as the gel base, and Agarose (Sigma-Aldrich, Oakville, Canada) (2g) was added and mixed with TBE solution. The mixed solution was placed in a microwave and heated in four steps (30s-20s-10s, then 7s) until large bubbles burst at the surface and no filament of agarose could be seen. While the solution was still hot, GelRed Nucleic Acid Stain (Sigma-Aldrich, Oakville, Canada) was added into the solution and mixed well. The heated solution was poured into a gel mode glass tray to solidify with the combs in place and allowed to sit for 30 minutes. After it solidified, the glass tray was placed in the gel box, and 0.5x TBE was added into the gel box until the top of the gel was fully covered with 0.5x TBE. After the gel was ready, samples were mixed with DNA loading dye and added into each respective well in the gel (20 μl/well). Covering the gel box with the lid, the gel was run under 1hour with 100 V. Since the dye was dual color, the gel would be ready for imaging when the two colors separated by a short distance (less than 1 hour). The resulting gel was imaged in an image reader using GelRed as the signal source. The running protocol was set to nucleic acid gel – GelRed, and the gel was positioned at the middle of the reader to be imaged.

3.1.5 Particle size and zeta potential

The hydrodynamic diameter of the peptides and the complexes were measured on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 4 mW He-Ne laser operating

at 633 nm. A disposable cuvette (50uL, ZEN0040) with a 3 mm light path was used and the scattered light intensities were collected at an angle of 173º. The measurement parameter was set to size, using protein as material, waster as solvent, equilibrium time was set to 20 s, and 3 cycles per measurement. Zeta potential measurements were also performed on the same machine using clear disposable zeta cells (700 μL) with the measurement parameter set to zeta potential.

3.1.6 Transmission Electron Microscopy (TEM)

Peptides (6 μM) and their corresponding peptide-siRNA (6 μM of peptide, 60:1 as CPP:siRNA molar ratio) complexes were observed using TEM. 10 μL peptide solution was incubated on a 400-mesh Formvar-coated copper grid (Canemco-Marivac, Canton de Gore, Canada) for 5 min, then 10 μL of freshly filtered 1% phosphotungstic acid was added to stain the samples for 30 s. And then the samples were washed three times by 30 μL water to remove excess phosphotungstic acid. After drying, a HT7800 TEM (Hitachi, Japan), with 120 kV accelerating voltage, was used for imaging.

3.1.7 Atomic Force Microscopy (AFM)

The designed CPPs (30 μM) and their corresponding CPP-siRNA (30 μM of peptide, 60:1 as CPP:siRNA molar ratio) complexes were loaded onto a mica surface (50 μL), washed twice (DI water, 50 μL), and air-dried for 24 h before imaging. Using a Dimension ICON AFM (Bruker Corporation, MA, USA). The Scanasyst static mode was used for imaging. The scale of the image was set to 1.5 μm to maintain resolution ratio.

3.1.8 Circular Dichroism spectroscopy (CDs)

The peptides (80 μM) and peptide-siRNA (80 μM of peptide, 60:1 as CPP:siRNA molar ratio) complexes secondary structures were characterized using a J-816 Circular dichroism spectroscopy instrument. The peptide solutions (150 μL) and complex solutions were added into a 1 mm quartz cell (Hellma, Concord, Canada). Filtered Milli Q water was used as baseline, and the spectra were collected from 190 nm to 260 nm with a 1 nm bandwidth at a scanning speed of 100 nm per minute. The mean residue molar ellipticity of the peptides and corresponding complexes was calculated according to the following equation:

Equation 3.1

$$Ellipticity\ (\theta) = \frac{(millidegrees\ \times\ mean\ residue\ weight)}{(path\ length\ in\ millimeters\ \times\ concentration\ of\ peptide\ in\ mg/mL)}$$

3.2 Results and Discussion

3.2.1 Modification of NP1

It has been proved by earlier research from our group members that the designed peptide sequence NP1 (Stearyl-H16R8) could facilitate cellular uptake and endosomal escape [147, 158]. However, it is still hard for siRNA to be disassociated from the complex due to the strong electrostatic interaction between negatively charged siRNA and positively charged peptides. For this reason, serine was introduced into the peptide sequence, constituting a subsequence, RRRS, as the substrate of PKA. Two stearic CPPs, NP1-SS and NP1-SS', were designed and investigated to study the influence of peptide phosphorylation on siRNA release. The new sequences and molecular weights of the designed CPPs are listed in Table 3.1, respectively.

Table 3.1 Sequences and molecular weight of the designed CPPs.

Name	Sequences	MW / (g/mol)

NP1	Stearyl-HHHHHHHHHHHHHHHHHRRRRRRRR-NH2	3724
NP1-SS	Stearyl-HHHHHHHHHHHHHHHHHRRRSRRRRRS-NH2	3934
NP1-SS’	Stearyl-HHHHHHHHHHHHHHHHHRRRSRRRS-NH2	3586

3.2.2 Critical aggregation concentration of CPPs

The ANS is a fluorescent probe used to determine the formation of molecular hydrophobic environments in solution. The critical aggregation concentration (CAC) of CPP at pH 6.0 and pH 8.0 were determined by ANS fluorescence to indicate the strength of interactions among the self-assembling peptides (Figure 3.2). Accumulation of aggregates in an aqueous solution led to the formation of hydrophobic cores. The fluorescence intensity of ANS increases when incorporates with the hydrophobic core, which indicates the assembly of a peptide. No evident variation of ANS fluorescence intensity was detected for protonated peptides until beyond ~10 μM shown in Figure 3.2C; in contrast (Figure 3.2D), the deprotonated peptides could be self-assembled at a relatively low CAC of ~1 μM. The result showed that deprotonation of oligohistidine could promote the tested CPPs to form aggregation/nanostructures.

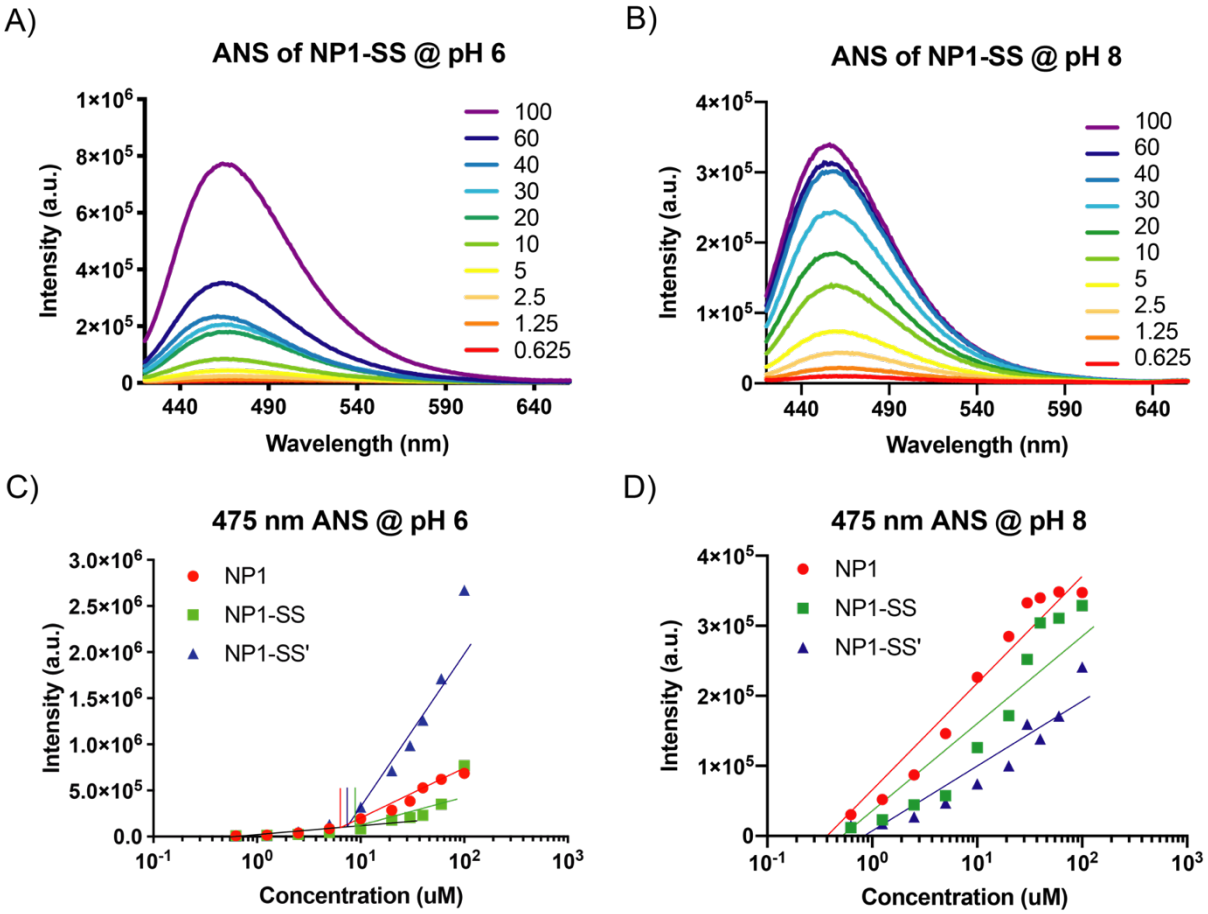

Figure 3.2 ANS fluorescence of CPPs at gradient concentrations at pH 6.0 and pH 8.0. A&B) ANS intensity of NP1-SS at pH 6.0 and pH 8.0, respectively; C&D) CACs of three CPPs at pH 6.0 and pH 8.0.

3.2.3 Peptide and siRNA complexation – binding capacity

In order to dig deeper into the interactions occurring between CPPs and siRNA, siRNA binding capacities for complete complexation onto peptides were determined by agarose gel-shift assay.

The principle of this assay was the fluorescent interaction between RNA and GelRed nucleic acid stain.

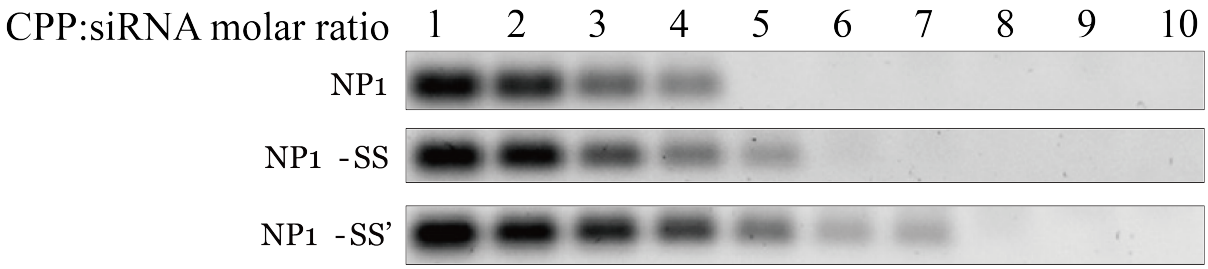

Figure 3.3 siRNA binding capacity of CPPs displayed by agarose gel-shift assay. siRNA concentration is fixed at 300 nM. Different volumes of peptide stock solutions were added to siRNA to obtain CPP:siRNA molar ratio from 1 to 10.

When free siRNA bond to GelRed, it showed a strong fluorescence at around 600 nm (excitation at around 300 nm). However, if siRNA molecules were bond thoroughly with CPPs, they cannot be detected by GelRed. Thus, no signal of fluorescence would be displayed. Generally, all three types of CPPs showed no fluorescence intensity above the CPP:siRNA molar ratio of 10, indicating these CPPs could complex with siRNA efficiently above this ratio (Figure 3.3). Under the ratio of 10, NP1 showed the complete binding with siRNA at a minimum CPP:siRNA molar ratio of 5, while the minimum ratio is 7 for NP1-SS and 9 for NP1-SS'. This phenomenon suggests that adding neutral amino acid or reducing the length of

oligoarginine would cause a decreased binding capacity to siRNA. An oversaturated CPP:siRNA molar ratio 60 was selected for use in further experiments.

3.2.4 pH triggering CPP-siRNA complex disassembly

Theoretically, the protonation of oligohistidine from pH 8.0 to pH 6.0 can alternate the assembly structure of CPPs by providing repulsive force between cationic histidine residues, leading to the disassembly of nanostructures, resulting in the exposure of siRNA. As shown in Figure 3.4, as pH reduced from 8 to 6, all three designed CPPs formulated with siRNA displayed stronger GelRed signals when the CPP:siRNA molar ratio was lower than 10:1, suggesting the released siRNA from the complex; while no apparent signal was detected at higher CPP:siRNA molar ratios. This result showed that pH triggered peptide protonation would facilitate the exposure and release of siRNA, while the effect is not strong enough when CPP is excessive.

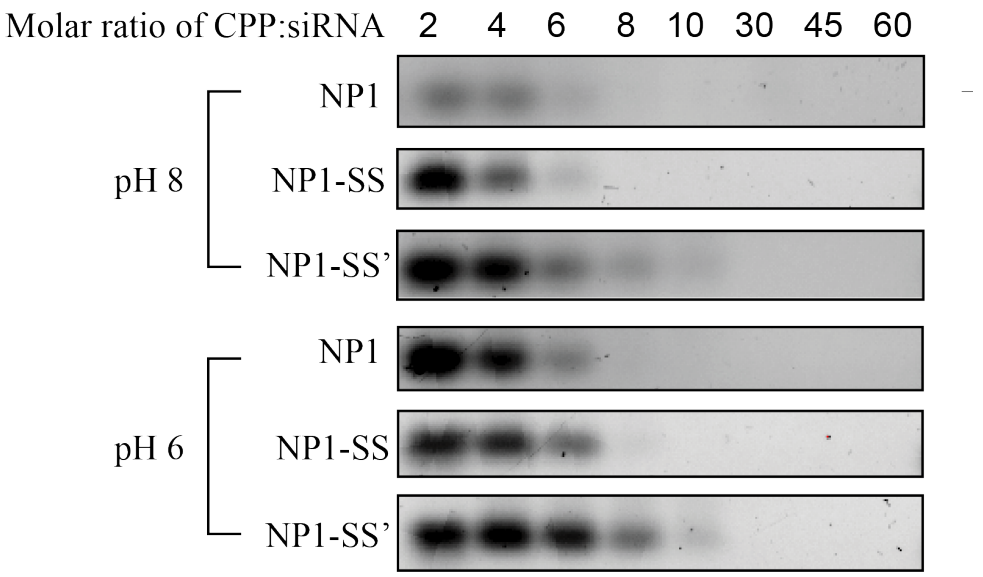

Figure 3.4 Gel electrophoresis of CPP-siRNA complexes in gradient molar ratios from pH 8.0 to pH 6.0.

3.2.5 Particle size and potential

The ability to form nano-sized complexes with siRNA was first examined. Due to histidine protonation at different pH, CPP solutions and CPP-siRNA complexes were formed in both mildly acidic (pH 6.0) and basic (pH 8.0) environments. The final concentration of CPPs was fixed at 30 μM, and CPP:siRNA molar ratio was set to 60:1 to form complexes in both environments. As hypothesized, CPP with cationic subsequence should ideally form complexes smaller than 200 nm in diameter to reduce the risk of recognition by phagocytic cells and to guarantee enhanced permeation retention effect (EPR) could optimize the diffusion

of nanoparticles through tumor tissues. The formation of the CPP-siRNA complex was driven by a) the electrostatic interaction between cationic CPP subsequence and negatively charged siRNA, and b) the hydrophobic interactions provided by the stearic acid moiety. The forces facilitated the condensation of the CPP-siRNA complex into the size suitable for transfection. The theoretical length of the CPP monomer could be calculated by the addition of stearic acid moiety and amino acids, which is approximately 6 nm per monomer. At both pH, the DLS size measurement showed somewhat different sizes of CPPs and their complexes. For CPP only samples, the size was evidently large and unstable, while for all complex samples, the sizes were stabilized around 100 nm. This result confirmed the ability of all three CPPs to condense siRNA into stable nanostructures of about 100 nm. In the meantime, DLS potential measurement exhibited the change of CPP self-assembly altered by environmental conditions. At pH 6.0, histidine subsequence was protonated, providing enhanced electrostatic repulsive force among the peptides, preventing CPP to cluster together, resulting in less densely charged surfaces. At pH 8.0, though the size did not change much, the potential increased significantly, indicating a higher degree of assembly of CPPs and CPP-siRNA complexes. The modification of the oligoarginine subsequence caused some differences in the size and potential. For peptide subsequence containing 6 arginine residues like in NP1-SS', the potential of NP1-SS' was

observably higher than others with 8 arginine residues. The potential difference was due to the more potent cationic repulsive force between R8 than NP1-SS' with R6. Moreover, a positively charged nanoparticle is preferable to interact with the negatively charged cell membrane. Thus, CPP with R8 subsequence had higher membrane affinity and can facilitate nanoparticles to cross the cell membrane. The details of self-assembly behaviors of all CPPs were further discussed in CDs result.

Table 3.2 Particle sizes (in diameter), zeta potentials at pH 6.0 and pH 8.0 of three CPPs and CPP-siRNA complexes, determined by dynamic light scattering (DLS) measurements. The complexes were formed by mixing the solutions of stock CPPs and stock siRNA at molar ratio 60:1.

Peptide/ complex	pH 6.0		pH 8.0	
	Diameter/ nm	Zeta potential/ meV	Diameter/ nm	Zeta potential/ meV
NP1	383±26	7.0±7.3	334±172	29±1
NP1-siRNA	121±3	37±4	99±4	43±3
NP1-SS	265±57	5.4±4.9	280±51	30±5
NP1-SS-siRNA	121±2	33±2	125±1	43±7
NP1-SS'	654±160	23±9	363±60	46±2
NP1-SS'-siRNA	117±2	33±6	125±3	45±4

3.2.6 Particle morphology

TEM images of the three CPPs and their corresponding complexes were taken and shown in Figure 3.6. The samples were prepared at the peptide concentration of 6 μM at pH 8.0. The CPPs and complexes formed spherical structures with diameters similar to the DLS measurements. Furthermore, the sizes of NP1 nanoparticles were less uniform than the other two (Figure 3.5A). After formulation with siRNA, the uniformity of nanoparticle size has improved (Figure 3.5B). The serine modified CPPs had improved uniformity without complexing with siRNA (Figure 3.5A), which could be caused by the interfered oligoarginine, weakening the repulsive forces in between. The conformations of CPP-siRNA complexes were quite similar for all three peptides, indicating the formulation with siRNA could stabilize the complex.

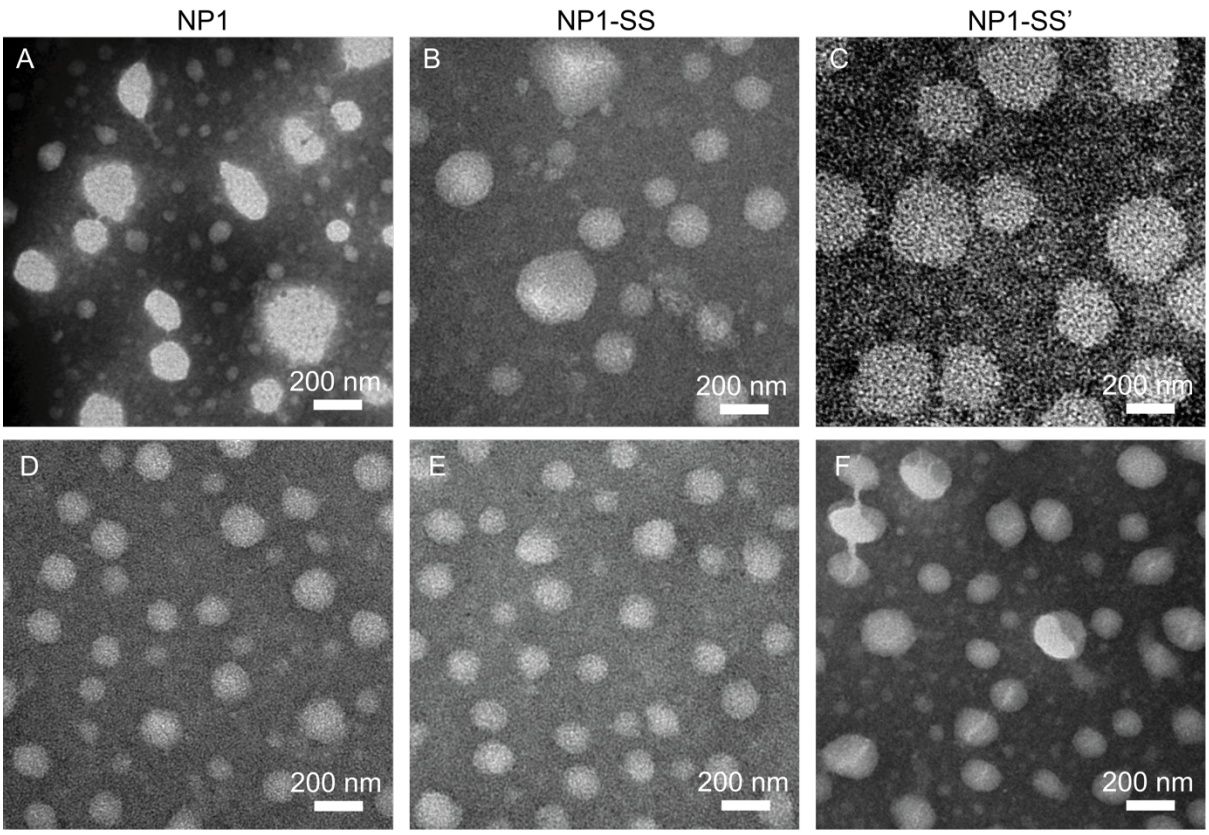

Figure 3.5 TEM of CPPs and corresponding complexes with siRNA at CPP:siRNA molar ratio 60:1, where CPP concentration is 6 μM. A) CPPs only; B) CPP-siRNA complexes

Three-dimensional measurement of CPPs (30 μM) and CPP-siRNA complexes at pH 6.0 and pH 8.0 were conducted by AFM. Figure 3.6 showed the morphology of NP1-SS in various conditions. At pH 8.0, deprotonated NP1-SS self-assembled into micelle-like structure, and NP1-SS and siRNA formulated into similar structure. At pH 6.0, protonated NP1-SS barely

formed assembled structure, and with siRNA, smaller sized spherical structures were detected, indicating the dissociation of complexes.

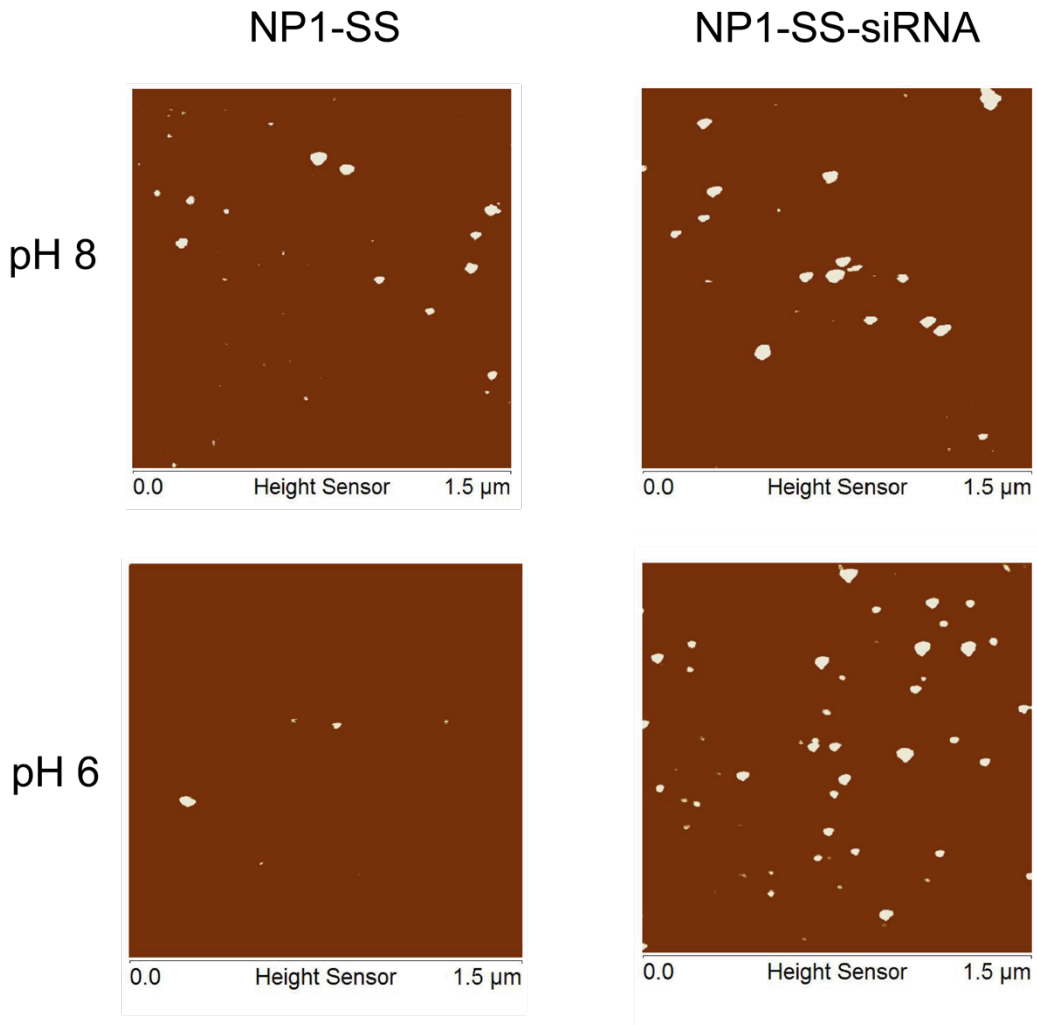

Figure 3.6 AFM images of NP1-SS at 30 µM and NP1-SS-siRNA at 60 CPP:siRNA molar ratio in pH 8.0 and pH 6.0 solution.

3.2.7 Secondary structure of CPPs and CPP-siRNA complexes

Detailly, the secondary structures of CPPs and their complexes were determined by CD spectra, as shown in Figure 3.7. When histidine was deprotonated (pH 8.0), two characteristic peaks were observed, positive peaks at ~205 nm and negative peaks at ~218 nm (Figure 3.7A), indicating the existence of secondary structure, helixes and sheets. When histidine subsequence was protonated (pH 6.0), the negative peaks at ~ 198 nm and positive peaks at ~220 nm indicated that all peptides and their complexes existed as random coils (Figure 3.7B). However, in the case of NP1-SS'-siRNA, a positive peak ~205 nm was observed, suggesting the existence of helix and sheet (Figure 3.7B). The CDs results suggested that the (de)protonation of oligohistidine could alter the conformation of CPPs and complexes.

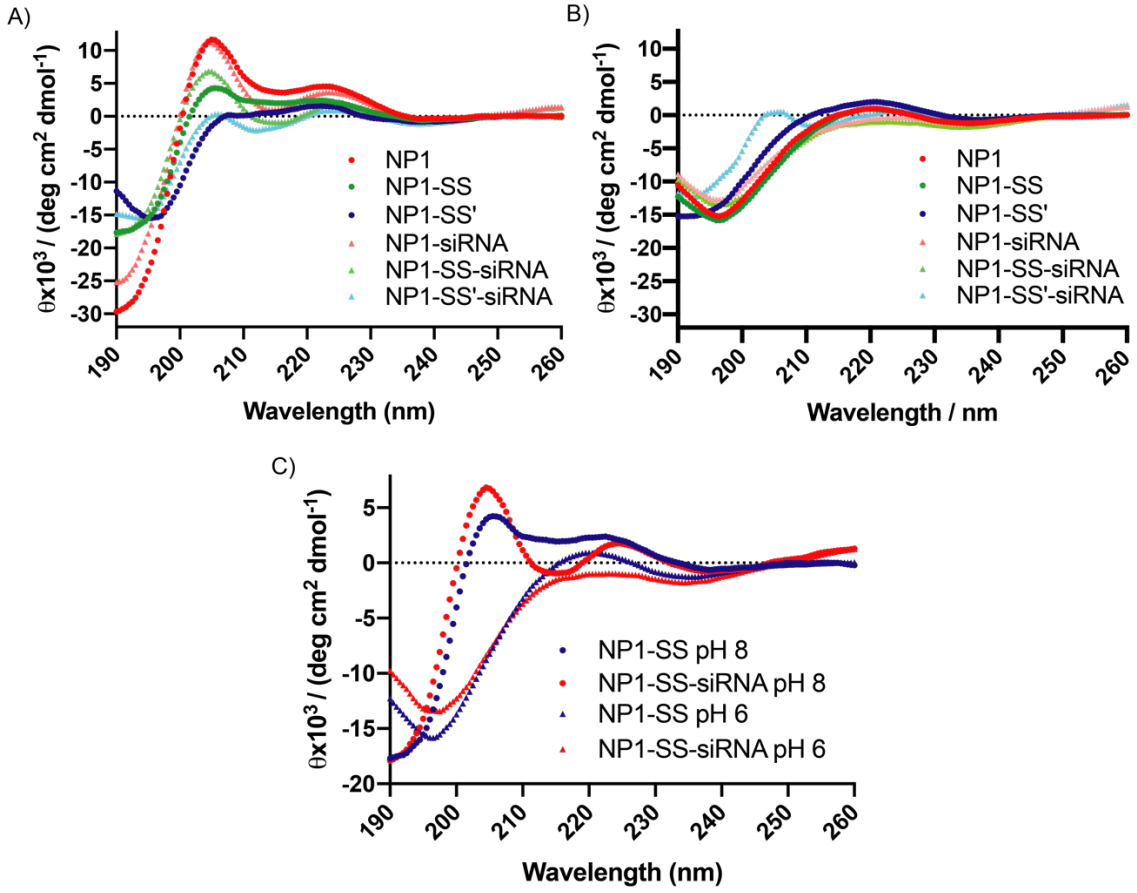

Figure 3.7 CDs of CPPs and CPP-siRNA complexes at pH 6.0 and pH 8.0. A) secondary structures of CPPs and complexes at pH 8.0; B) secondary structures of CPPs and complexes at pH 6.0; C) comparison of NP1-SS and complex secondary structure at pH 6.0 and pH 8.0.

3.3 Conclusion

Considering the assembly of peptides, internalization of the complex and intracellular release of siRNA, NP1 was rationally modified with serine amino acid to improve siRNA release

without impairing the self-assembly ability and internalization capability. In the characterization of physicochemical properties of CPPs and CPP-siRNA complexes, peptides and complex solutions were firstly prepared for all the measurements. Agarose gel-shift assay determined the binding capacity of each peptide to siRNA. With the addition of neutral amino acid or reduction of the length of oligoarginine, the binding capacity would be decreased.

All three peptides could self-assemble with siRNA into small nanoparticles (about 100 nm) with helix and sheet secondary structures due to: a) hydrophobic interaction of stearic acid moiety, b) self-assembly of deprotonated oligohistidine, and c) electrostatic interaction between oligoarginine and siRNA. When pH was reduced, the complex could disassemble to random coils and trigger the first stage of siRNA release.

Chapter 4

Peptide Phosphorylation-induced siRNA Release in vitro

4.1 Materials and Methods

4.1.1 Cell Culture

Adenocarcinoma (A549) cells were purchased from American Type Culture Collection (ATCC CCL-61). Cells were cultured in F-12K medium (Thermo scientific, Ottawa, Canada) supplemented with 10% fetal bovine serum FBS (Sigma-Aldrich, Oakville, Canada). Cells were incubated at 37ºC in a humidified atmosphere containing 5% CO2. Culture medium were removed and replaced every two days.

4.1.2 Cell Cytotoxicity assay

A549 cells (8,000 cells/well) were seeded in a 96-well cell culture plate in F-12K medium with 10% FBS at day 1. 24 hours later, the confluence of cells reached 50%. Cells were dosed with three types of peptides with various concentration (3 μM, 6 μM, 10 μM, 20 μM and 25 μM) and complexes with various peptide to siRNA molar ratios (peptide concentration kept at 6uM, 15:1, 30:1, 45:1, 60:1) and incubated at 37ºC in a humidified atmosphere containing 5% CO2 for 24 hours. After the incubation, the sample-containing medium was removed, and cells were

treated with diluted Cell Counting Kit-8 (CCK8) reagent (Dojindo, Maryland, USA) (11 times dilution) for 20 minutes or until the reading of non-treated cells reached 0.7 and higher. In the presence of the electron carrier 1-Methoxy PMS, CCK8 is reduced by dehydrogenase in the mitochondria to a highly water-soluble orange-yellow formazan product. The faster and more cells proliferate, the darker color the solution appears; the more cytotoxic, the lighter the color. For the same cell, the color depth is proportional to the number of living cells, so this feature can be used to directly analyze cell proliferation and toxicity [148]. Eventually, cell viability was assessed by measuring the CCK8 absorbance at 570 nm with a FLUOstar OPTIMA microplate reader and expressed as the ratio of the cells treated with the carriers over the non-treated cells (negative control). Data was analyzed using Microsoft Excel.

4.1.3 siRNA release measured by Nanodrop

In order to measure the siRNA release from CPP-siRNA complex, an indirect method was used to measure the concentration of siRNA before and after the addition of PKA and ATP to CPP-siRNA complex solution. Firstly, siRNA only, CPP-siRNA complexes were prepared for Nanodrop measurement. For nucleic acid samples, there would be an absorbance at 260 nm, and for protein samples, there would be an absorbance at 203 nm. Concentration of each sample was measured before the addition of ATP and PKA. After the measurement, PKA and

ATP were added to complex samples and incubated in 5% CO2 at 37°C for 5 min, which would allow the reaction to reach equilibrium. After the phosphorylation reaction, chloroform was added to samples to remove proteins and waste residues. 2-propanol was then added to precipitate released siRNA, followed by 75% ethanol washing twice. Finally, the concentration of siRNA only and complexes samples were measured again to qualify the release of siRNA.

4.1.4 Fluorescence-Activated Cell Sorting (FACS)

The working principle of the flow cytometer is to put the cells to be tested into a sample tube after being stained with specific fluorescent dyes and enter the flow chamber filled with sheath fluid under the pressure of gas. Under the restriction of the sheath liquid, the cells are arranged in a single row and ejected from the nozzle of the flow chamber to form a cell column, which intersects the incident laser beam perpendicularly, and the cells in the liquid column are excited by the laser to produce fluorescence [149]. Cellular uptake of peptide-Cy3-labeled GAPDH siRNA complexes was studied with fluorescence-activated cell sorting (FACS) using flow cytometry (BD Biosciences, BD FACS Vantage SE Cell Sorter, USA). Cells were seeded at a density of 100,000 cells/well in 24-well plates and incubated in 5% CO2 incubator at 37°C. 24 hours later, the confluence of cells reached 80%, and then the cells were transfected with 3 different peptide-Cy3-labeled siRNA complexes (100 nM) at a molar ratio of 60:1 (N/R). Cells

treated with Lipofectamine RNAiMAX (Thermo scientific, Ottawa, Canada) in coupling with 100 nM siRNA as well as siRNA (100 nM) alone were also included in the experiments as controls. The dosed cells were transfected for 3 hours at 37°C and then processed for FACS. In detail, the cells were rinsed with Opti-MEM (Thermo scientific, Ottawa, Canada) containing heparin for 20 minutes in the incubator at 37°C for three times followed with PBS (Thermo scientific, Ottawa, Canada) washes twice with occasional shaking. Thereafter, the cells were detached with 0.25% trypsin/EDTA (Thermo Fisher Scientific, Ottawa, Canada) for 5 minutes and fixed with 2% paraformaldehyde (PFA) (Sigma-aldrich, Oakville, Canada) in PBS and then collected in tubes, wrapped in tinfoil and stored at 4ºC prior to FACS. Data was analyzed by Flowjo software afterwards.

4.1.5 Gene Silencing

A549 cells (40,000 cells/well) were seeded in a 24-well cell culture plate in F-12K medium with 10% FBS at day 1. After 24 hours, the medium was removed from the plate, and then the plate was washed with PBS. Opti-MEM medium (200 µl/well) was added to the plate, and the plate was prepared to be treated with samples. In sample preparation, siRNA solution and peptide solution were initially prepared separately in Opti-MEM medium, and solutions were incubated for 5 minutes. Then peptide and siRNA solutions were mixed to form complexes at

molar ratios 60:1 and 30:1 for 20 minutes. Thereafter, the cells were treated with sample solutions continuing the complexes of the peptides or Lipo/siRNA (100 µl/well). The dosed cells were transfected for 3 hours at 37°C in a 5% CO2 atmosphere. After 3h transfection, F-12K medium with 20% FBS (300 µl/well) were added to the plate, and the plate was incubated for 48 hours in the incubator as well.

Total RNA was extracted from the cells with TRIzol reagent (Life Technologies, Carlsbad, USA), then treated with chloroform (Sigma, Oakville, Canada) to remove any protein and waste residue and 2-propanol (Sigma-aldrich, Oakville, Canada) to precipitate RNA as recommended by the manufacturer. RNA concentrations were measured by Nanodrop spectrophotometer ND-1000 (Therno scientific, Ottawa, Canada). all RNAs were reverse transcribed with Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Mississauga, Canada). The cDNA synthesis was primed with a unique blend of oligo (dT) and random primers. The following pairs of primers were used for qPCR: 5'-TTGCTGTTGAAGTCGCAGGAG-3'and 5'-TGTGTCCGTCGTGGATCTGA-3' (Sigma, Oakville, Canada). In the experiment, the housekeeping gene cyclophilin was chosen as an internal control to normalize the GAPDH gene. The normalization was performed by the amplification of mouse/rat cyclophilin mRMA with the following primers: 5'-AGGGTTTCTCCACTTCGATCTTGC-3' and 5'-

AGATGGCACAGGAGGAAAGAGCAT-3' (Sigma, Oakville, Canada). qPCR reaction was performed with Brilliant II Fast SYBR Green QPCR Master Mix (Agilent Technologies, Wilmington, USA) on an Mx3005P™ Real-Time PCR System (Agilent Technologies, Wilmington, USA). Data was exported to Excel and analyzed afterwards.

4.2 Results and Discussion

4.2.1 In vitro cytotoxicity evaluation

It is critical for oligoarginine- and oligohistidine-based CPPs to maintain low cytotoxicity when used as siRNA delivery agents. To evaluate the cytotoxicity of three CPPs and their complexes on the A549 cell line, the cells were treated by varying concentrations of CPPs and CPP-siRNA complexes with different CPP:siRNA molar ratios for 24 h. The cell viability of the treated A549 cells was determined by Cell Counting Kit-8 (CCK8) assay, as shown in Figure 4.1.

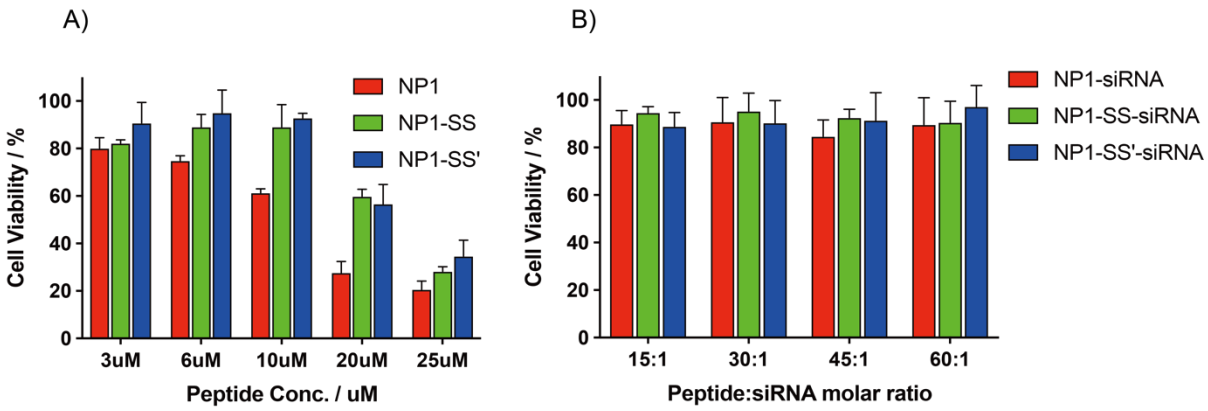

Figure 4.1 In vitro cytotoxicity evaluation of CPP only and CPP-siRNA complexes on A549 cells. A) cell viability of A549 cells treated with different concentrations of CPPs; B) cell viability of A549 cells treated with different CPP:siRNA molar ratios, CPP concentration was fixed at 6 μM.

As shown in Figure 4.1A, when peptide concentration was lower than 10 μM, all three peptides displayed low toxicity to A549 cells, with modified peptides exhibiting even lower toxicity than NP1. The lowered cytotoxicity of modified NP1 was due to the addition/replacement of neutral serine residues, and it weakened the membrane penetration of oligoarginine. For concentration range above 10 μM, cytotoxicity of NP1 to cells increased significantly while NP1-SS and NP1-SS' showed mildly increased toxicity to cells, demonstrating reduced toxicity caused by the peptide modification.

As for the corresponding complexes, peptide concentration was fixed at 6 μM because the cytotoxicity of CPP only samples was low, and it was also used in transfection while the concentration of siRNA varied to reach different CPP:siRNA molar ratio at 15:1, 30:1, 45:1, and 60:1. As illustrated in Figure 4.1B, all complexes at various ratios exhibited relatively low cytotoxicity to A549 cells, and all cell viability reached 80%. At low CPP concentration, the cytotoxicity of CPP-siRNA complexes was nearly identical, indicating that modification with serine at low CPP concentration would not alter much of the cell membrane penetration, which was ideal and critical for the carrier to be an effective siRNA delivery agent.

4.2.2 siRNA release by peptide phosphorylation

As mentioned in mechanism section 1.1.4, peptide phosphorylation would add a phosphate group on serine residue, alternating the electrical property of peptide, promoting the release of siRNA. By measuring the absorbance intensity of siRNA of CPP-siRNA complex samples before and after the addition of PKA and ATP, the siRNA intensity difference indicated the release after the phosphorylation reaction. As shown in Figure 4.2, naked siRNA had an absorbance at 260 nm, and peptide only and CPP-siRNA complex samples did not show absorbance at 260 nm, suggesting the complexation of CPP and siRNA. After the addition of PKA and ATP in NP1-siRNA and NP1-SS-siRNA complexes, siRNA absorbance of NP1-SS-

siRNA samples reappeared while no apparent siRNA absorbance of NP1-siRNA was detected. This result was consistent with the hypothesis that modified NP1 with RRRS motif can promote siRNA release while NP1 cannot, which indirectly exhibited siRNA release due to peptide phosphorylation based on the intensity difference measured w/o the addition of PKA and ATP.

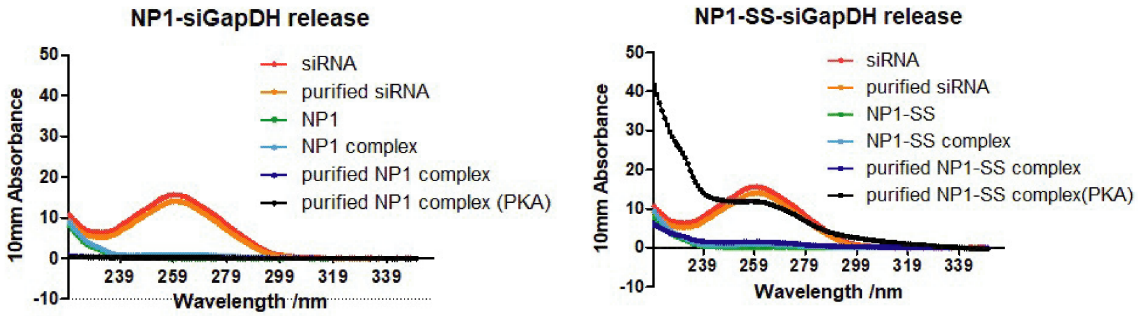

Figure 4.2 siRNA release triggered by peptide phosphorylation measured by Nanodrop. NP1-siRNA (left) and NP1-SS-siRNA (right) were treated with PKA and ATP for phosphorylation. All samples were purified by RNA extraction.

4.2.3 Cellular uptake of complexes into A549 cells

Cellular uptake of Cy-3 labelled siRNA formulated with CPPs at 60:1 CPP:siRNA molar ratio was measured by FACS as shown in Figure 4.3.

As shown in Cy3 fluorescence result in Figure 4.3B, naked Cy3-siRNA had an approximately 6 times lower absorbance intensity than the CPP-Cy3-siRNA complex. This phenomenon is

referred to as protein-induced fluorescence enhancement (PIFE). The enhancement of Cy3 fluorescence correlates with decreased efficiency of photoisomerization in conditions where the dye is sterically constrained by the protein [160]. Therefore, when siRNA was released from CPP and existed as naked siRNA, the absorbance intensity would be reduced to naked siRNA as well. The result of FACS was consistent with the result of Cy3 fluorescence. As shown in Figure 4.3A, non-treated A549 cells were used as negative control (NC). The fluorescence intensity of all complex samples is of the same difference at 1h, indicating the similarity of their internalization capability. However, the difference appeared when the transfection was completed at 3h. Among three CPPs, NP1-SS exhibited the lowest fluorescence intensity at 15.1, NP1-SS' showed higher intensity than NP1-SS at 23.4, while NP1 displayed highest at 26.8. The main assumption behind this was the different degree of siRNA release from each CPP. After transfected into cells for 3 h, most of NP1 was still formulated with siRNA as complex while more siRNA disassociated from NP1-SS and NP1-SS' and existed as free siRNA molecules whose fluorescence intensity would be reduced compared in complex form. Combining the release result described in 4.2.2, the FACS result helped prove that the RRRS sequence in NP1-SS can improve siRNA release after phosphorylation by PKA.

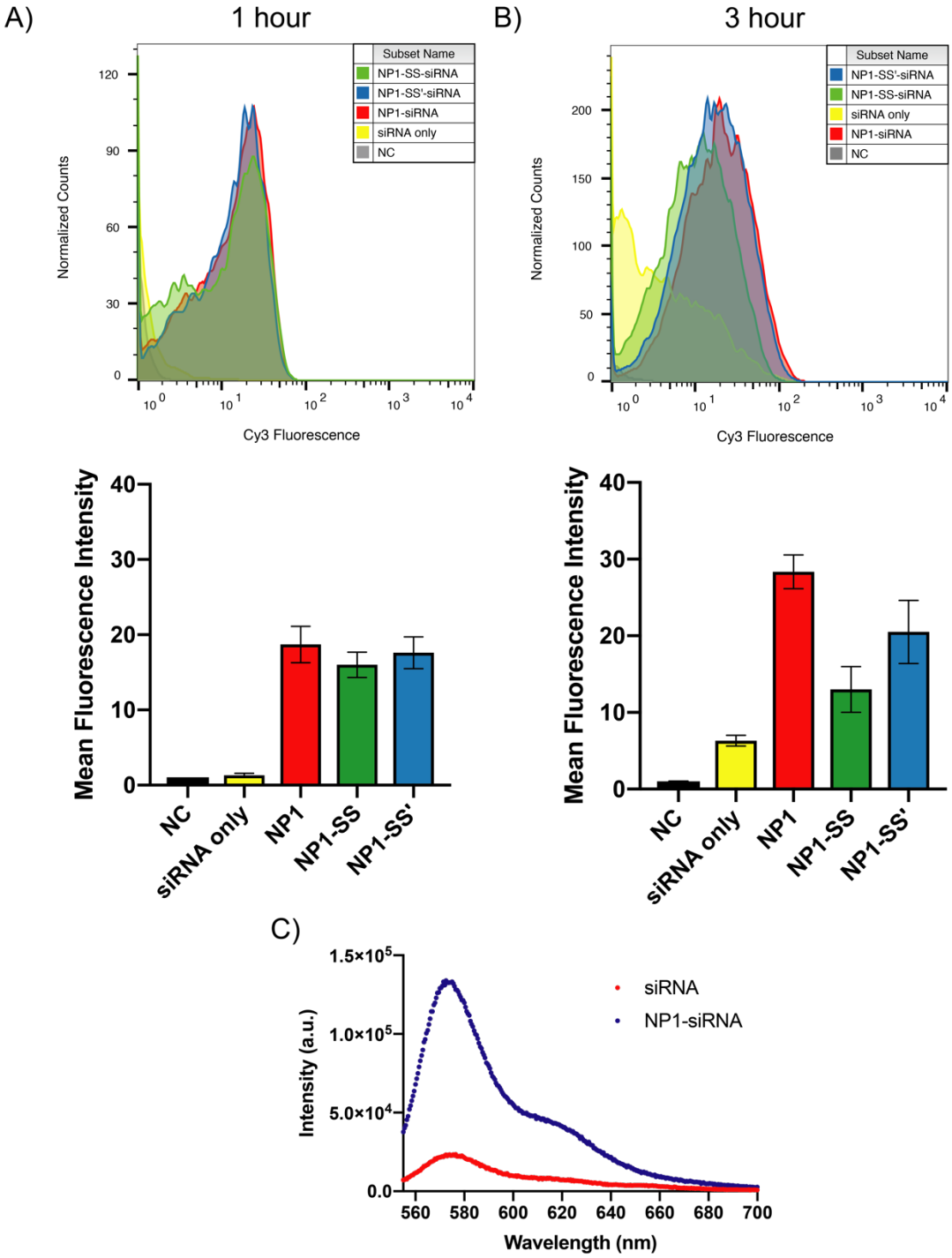
A)
1 hour
B)
3 hour
Subset Name
NP1-SS-siRNA
NP1-SS'-siRNA
NP1-siRNA
siRNA only
NC
Normalized Counts
Cy3 Fluorescence
Mean Fluorescence Intensity
NC
siRNA only
NP1
NP1-SS
NP1-SS'
C)
siRNA
NP1-siRNA
Intensity (a.u.)
Wavelength (nm)

Figure 4.3 Cellular uptake of Cy3-siRNA complexes formed with designed peptides after the treatment in A549 cells. A) FACS results of CPP-siRNA complexes at 1h; B) FACS results of CPP-siRNA complexes at 3h; C) fluorescence of Cy3-siRNA and NP1-Cy3-siRNA with 100 nM siRNA.

4.2.4 Gene knockdown efficiency

The gene knockdown efficiency of siRNA delivery by each designed CPP was determined using a GAPDH gene knockdown assay. The gene knockdown efficiency was expressed by calculating the removed mRNA % for each treatment. Higher mRNA % got removed by transfection indicated higher knockdown efficiency, showing that the siRNA carrier was more effective in delivering siRNA. The knockdown efficiency of the three CPPs is shown in Figure 4.4. The figure showed that all three peptides induced GAPDH mRNA knockdown at 30:1 and 60:1 CPP:siRNA molar ratios. Lipofectamine RANiMAX was used as the positive control (PC). Amongst three peptides, NP1-SS-siRNA formulations showed 80% gene knockdown efficiency, which was 14% and 22% higher mRNA knockdown levels than that of NP1-siRNA and NP1-SS'-siRNA at 60:1 molar ratio, respectively. While at a lower CPP-siRNA molar ratio of 30, the difference was not apparent. In consideration of the complex internalization, cell membrane affinity, and peptide phosphorylation, all three CPPs showed similar

internalization, while NP1-SS' showed weakened affinity to cell membrane due to the reduced oligoarginine subsequence; and comparing to NP1-SS and NP1-SS', NP1 did not interact with PKA and was incapable of changing electrical property through the reaction. The gene silencing result implied that serine addition to the peptide chain could improve the efficacy of siRNA delivery by promoting a second stage siRNA release triggered by peptide phosphorylation. However, replacing arginine with serine would lead to inefficient siRNA delivery, which was caused by the breakage of the R8 subsequence. When adding serine or replacing arginine with serine to the R8 sequence, the integrity of the R8 sequence was interrupted, hence, reducing the peptide affinity to the cell surface. Furthermore, with the affinity to surface decreased, the cellular uptake, internalization of the complex would also be impacted, leading to lowered siRNA utilization rate.

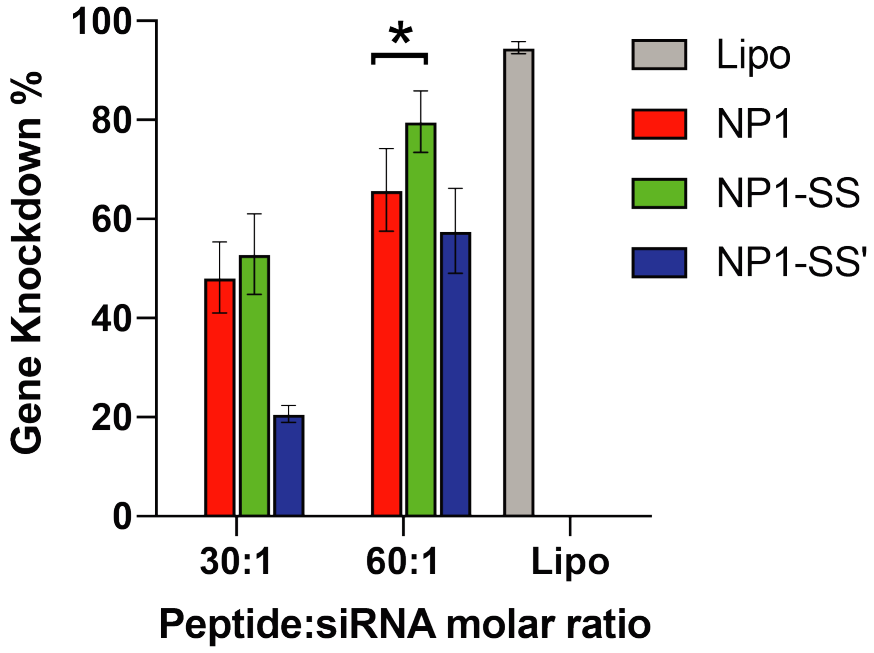

Figure 4.4 Gene knockdown efficiency of three CPP-siRNA complexes on A549 cells at CPP:siRNA molar ratio 30:1 and 60:1. Concentration of siRNA was fixed at 100 nM. Lipo was used as positive control. T-test of NP1 and NP1-SS showed significant difference ($P<0.05$)

4.3 Conclusion

Corresponding to the physicochemical properties of CPPs and CPP-siRNA complexes, the relationship between the structures and biological activities have been specifically investigated. By introducing serine residue and forming PKA recognition motif RRRS, cytotoxicity to A549 cells was reduced, intracellular siRNA release was enhanced, and gene silencing efficiency

was improved. However, minor drawback emerged when the length of oligoarginine was reduced, the intracellular siRNA release was attenuated, resulting in lowered gene knockdown. After the endocytosis of complex by cells, the oligohistidine segments induced proton sponge effect, histidine residues were protonated, which led to the structural change of complex and the first stage siRNA release during the endosomal escape, and this process would occur on all three types of complexes. When reached cytoplasm where ATP and PKA existed and functioned, the loosened complexes with RRRS motif, NP1-SS and NP1-SS', were recognized by PKA, transferring the gamma phosphate from ATP to serine residue, weakening the electrostatic interaction between CPP and siRNA, achieving the second stage siRNA release. As a result, NP1-SS reserved the function of oligoarginine, and with the improvement introduced by PKA phosphorylation, higher silencing of the GAPDH gene (80%) was achieved. The obtained results demonstrated that NP1-SS is an optimized carrier for siRNA delivery, and the modification by serine is valid, which could be branched out in future research.

Chapter 5

Summary of Thesis and Recommendation for Future Work

In this work, we modified a CPP, NP1, designed by our previous group member based on the current obstacles encountered by siRNA delivery. NP1 has shown the ability to self-assemble and co-assemble with siRNA, protecting siRNA from extracellular degradation, great cell membrane affinity for easy complex internalization, and low cytotoxicity to normal cells. Nevertheless, it caused another limitation on intracellular siRNA disassociation from CPP. To enhance siRNA release, protein phosphorylation by PKA was introduced to trigger a second stage dissociation after the first stage of pH-triggered release during the endosomal escape from the endosome. The substrate motif of peptide for PKA to identify is RRXS, ideal for NP1 because it already has an oligoarginine subsequence. Hence, by addition or substitution of serine residues into NP1, hypothetically, the modified peptide could undergo peptide phosphorylation and transfer an anionic phosphate group from ATP to serine, reducing the cationic nature of peptide, impairing the interaction between siRNA and CPP, and eventually facilitating the dissociation of siRNA and achieving an enhanced gene silencing. From the results, the addition of serine residues did not change the general structure and physicochemical properties of NP1, but the reduction of arginine had a negative impact on the

internalization of the complex. We confirmed that pH could alternate the secondary structure of CPPs, and NP1-SS had enhanced siRNA release than NP1 and NP1-SS' because of phosphorylation of peptide and reserved oligoarginine, respectively. Most importantly, an improved gene silencing efficiency was accomplished, proving this modification with protein phosphorylation is effective.

There is still much work that could be done to consummate this research. First of all, a direct method is needed to visualize the phosphorylation of peptides and determine the degree the phosphorylation, and the current results can indicate peptide phosphorylation but are not sufficient. Mass spectrometry can be used to detect the existence of the phosphate group. Specifically, liquid chromatography-mass spectrometry can be applied to qualitatively check the phosphorylation of CPPs and to calculate the fraction of the species. CPP solutions in the presence of excessive ATP can go through phosphorylation by adding PKA and react for an excessive amount of time. Samples before and after phosphorylation are then measured using LC-MS. 31P-NMR can also be used to record the phosphorylation process by monitoring the peak change during a long period of time for three peptide in the presence of ATP and PKA. Isothermal titration colorimetry can be used to measure the reaction heat change when phosphorylation occurs. Using ATP as the titration material and placing CPP with PKA in the

reaction chamber. The interval between the injections is set to allow the system to stabilize the thermal power to the new baseline after each injection. For phosphorylation reaction occurring in cells, western blot can be used to detect the intracellular phosphorylation product. Those different experiments will enrich the understanding of the properties and functions of the modified peptides.

For future research continuing the modification via peptide phosphorylation, and there are several ways to make progress. One way is to rearrange the position of serine insertion, there are still some forms of modifications was not included in this work. A parallel modification that could also be considered is replacing serine with threonine, which can group the PKA substrate motif. Another more advanced and branched direction is to apply other regulation processes involved in cell growth, taking advantage of the natural modifications, even could be used as targeting treatment.

Appendix A

Abbreviation

Acronym	Full name
CPP	Cell penetrating peptide
siRNA	Small/short interfering RNA
RNAi	RNA interference
dsRNA	Double-stranded RNA
CNS	Central nerve system
NP1	Stearyl-H16R8-NH2
Lipo	Lipofectamine
PA	Peptide amphiphile
ATP	Adenosine triphosphate
PKA	Protein kinase A
R	Arginine
S/Ser	Serine
NP1-SS	Stearyl-H16R3SR5S-NH2
NP1-SS’	Stearyl-H16R3SR3S-NH2
PTGS	Post-transcriptional gene silencing
Pre-miRNA	Precursor microRNA
RISC	RNA-induced silencing complex
Ago-2	Argonaute 2
HIV	Human immunodeficiency virus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
FACS	Fluorescence activated cell sorting
TBE	Tris-base electrolyte
DLS	Dynamic light scattering
CDs	Circular dichroism spectroscopy
CAC	Critical aggregating concentration

NC	Negative control
PC	Positive control
TEM	Transmission electron microscopy
AFM	Atomic force microscopy
MW	Molecular weight
EPR	Enhanced permeation retention effect
A549	Adenocarcinoma cell
CCK8	Cell count kit 8
Cy3	Cyanine 3
FBS	Fetal bovine serum
PBS	Phosphate-buffered saline
mRNA	Messenger RNA
qPCR	Quantitative polymerase chain reaction
CHB	Chronic hepatitis B virus
RSV	Respiratory
EVD	Ebola virus disease
TTR	Transthyretin
PDAC	Pancreatic ductal adenocarcinoma
PKN3	Protein kinase N3
LDL	Low-density lipoprotein
PCSK9	Proprotein convertase subtilisin-kexin type 9
HCC	Hepatocellular carcinoma
PLK1	Polo-like kinase 1
TAT	Transcription-transactivation
PTDs	Protein transduction domains
NLS	Nuclear localization signal
cAMP	Cyclic adenosine 3’,5’-monophosphate
AKAPs	A-kinase anchoring proteins
CaM	Calmodulin

PIFE	Protein-induced fluorescence enhancement
MARCKS	Myristoylated alanine-rich C kinase substate
PKC	Protein kinase C

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