Matrix Metalloproteinase-2 Cleavable Peptide-Based siRNA Delivery System for Cancer Treatment

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

Among all kinds of gene therapy, siRNA, a class of 20 to 25 nucleotide-long double-stranded RNA molecules, is one of the promising therapeutic solution to regulate post transcriptional process for cancer treatment. However, naked siRNA is easily degradable in the body circulation system and cannot efficiently being consumed by cells. To overcome this problems, cell-penetrating peptides (CPPs) have received much attention due to their ability to translocate through plasma membranes along with a low toxicity. In past years, our group has developed a CPP called NP1 (Stearyl-HHHHHHHHHHHHHHHHRRRRRRRR-NH2), aiming to provide highly efficient siRNA delivery. However, although NP1 has outstanding transfection results on various cell line on in vitro tests, it could not provide promising results on serum environment since the presence of serum largely reduces the transfection efficacy and the overall positively charged surface of NP1/siRNA complex is not favored in systematic application.

Matrix metalloproteinase-2, a category of gelatinase subgroup of MMPs, has been confirmed playing a critical role in tumor progression, angiogenesis, and metastasis. It has the ability to degrade the surrounding ECM to help cancer cell migrate inside the body. Thus,

relatively larger amount of MMP-2 secretion can be detected at tumor site which makes them an universal stimulus for bio-responding.

Herein, this thesis focus on increasing the stability of NP1 while maintaining the high transfection efficiency in the presence of serum. The complex surface will be sheltered with polyethylene glycol (PEG) to screen the surface charge and avoid serum protein binding; Furthermore, the linker between NP1 and PEG, with composed of 8 specific amino acid sequence (GPLGIAGQ), will be recognized by matrix metalloproteinase-2 to achieve sensitive cleavage of PEG.

In this study, the following objective has been examined: (i) success cleavage of the designed linker and the existence of MMP2 in the cultured environment ; (ii) the physicochemical characterization of the modified peptides, and the interaction between peptides and siRNA molecules; (iii) the evaluation of the silencing efficiency, and toxicity of peptides/siRNA complexes in cultured cells in serum environment versus the results from NP1; (iv) *in vitro* biocompatibility study of the peptides/siRNA nanocomplexes, and (vi) the stability along with the RNase resistance ability of new modified peptide carrier comparing with NP1.

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List of Abbreviations

Acronym	Full name
AIDs	Acquired immune deficiency syndrome
Ago-2	Argonaute-2
ATCC	American Type Culture Collection
AMD	Age-related macular degeneration
CPP	Cell-penetrating peptide
DOX	Doxorubicin
DLS	Dynamic light scattering
dsRNA	Double-stranded RNA
ECM	Extracellular matrix
EPR	Enhanced permeability and retention effect
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBV	Hepatitis B virus

HIV	Human immunodeficiency viruses
LDH	Lactate dehydrogenase
LNPs	Lipid nanoparticles
LPS	Lipopolysaccharide
LRT	Long terminal repeat
mRNA	Message RNA
MMP	Matrix metalloproteinase
MR	Molar Ratio
NPs	Nanoparticles
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Polydispersity index
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PLGA	Poly(lactic-co-glycolic acid)
PNAs	Peptide nucleic acids

PTDs	Protein transduction domains
PTGS	Post-transcriptional gene silencing
RSV	Respiratory syncytial virus
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-PCR	Reverse transcriptase PCR
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNALP	Stable nucleic-acid-lipid particle
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

Chapter 1

Introduction

1.1 Overview

1.1.1 Matrix metalloproteinase-2 (MMP-2)

Matrix metalloproteinase-2 (gelatinase A, M_r 72,000 type IV collagenase), along with MMP-9 (gelatinase B, M_r 92,000 type IV collagenase) are proteases that belong to a gelatinase subgroup of the MMPs family [1,2]. In particular, MMP-2 shares a strong tie with cancer. There are several steps that involve in the process of cancer cells invasion and metastasis: (i) tumor cell detachment from the primary site; (ii) extracellular matrix (ECM) degradation and blood vessels invasion; (iii) adhesion to blood vessels at another site; and (iv) invasion of the organs. Among all the steps, degradation of surrounding ECM (step (ii)) is considered the essential step in tumor invasion and metastasis [3], and MMP-2 has been discovered as an important role in this step [4–7]. It is a biomarker of malignancy since its overexpression is in almost all types

of tumors [8]. Therefore, it has been widely used as drug delivery target or release stimulus for different kinds of carriers.

Based on the specific protease activity of MMP-2, it can be utilized for enzymatically metabolized drug delivery, in which therapeutic drugs or protection composition are covalently bound to MMP-2 substrate peptides [9,10], and activated upon cleavage of the peptide by MMP-2 afterwards. For example, a specific tumor-targeted prodrug, comprised of MMP-specific peptide and methotrexate, has been developed and tested by Robert Langer's group for chemotherapeutics in human tumor xenograft models [11,12]. Similarly, Lin and etc. have tried co-delivery of siRNA and hydrophobic drugs into A549 lung cancer cells using a MMP2-sensitive multifunctional micelles formed by the PEG-peptide-PEI conjugation [13]. All in all, the specialty of MMP-2 secretion at tumor site and the MMP-2 specific peptide provides a new strategy for cancer targeting therapy.

1.1.2 RNA interference (RNAi)

RNA interference is considered as one of the most exciting and enlivening phenomenon that has been discovered in the past decade by many biologists [14]. It is a highly efficient regulatory process in which the expression of specific genes can be forbidden by the

degradation of its corresponding mRNA sequence in the cytoplasm by short double-stranded RNA (dsRNA) in most eukaryotic cells [15–18]. Two American scientists Andrew Z. Fire and Craig C. Mello, who discovered RNA interfering phenomenon in the 1990s, shared the Nobel Prize for Physiology or Medicine in 2006 for their work.

In general, short interfering RNA (siRNA) are produced from the cleavage of dsRNA, which are the precursors that derived from either convergent transcription or hairpin-structured RNAs, by the RNase III like enzyme, endonuclease Dicer, or synthesized by chemical or biochemical methods [19–21]. They are normally 21-23 nucleotides in length with a stabled double stranded structure. After the formation of siRNA, they will be loaded onto an RNA-induced silencing complex (RISC), which has Argonaute 2 (Ago-2) as the catalytic core of RISC[21]. Upon binding to double-stranded siRNA, RISC will be activated by the cleavage and release of the "passenger" strand, and the remaining single-stranded RNA molecule that called "guide" strand will direct the specifically recognition of the target complementary mRNA by intermolecular base pairing [22–25]. Ultimately, Ago-2 cleaves the target mRNA for preventing protein formation from its corresponding genes [26]. Tuschl and his colleagues also noticed that the introduction of chemically synthesized siRNA into mammalian cells can performed its

silencing functionality as same as the long dsRNA [17]. The entire RNA interference pathway is illustrated by Figure 1.1 [27].

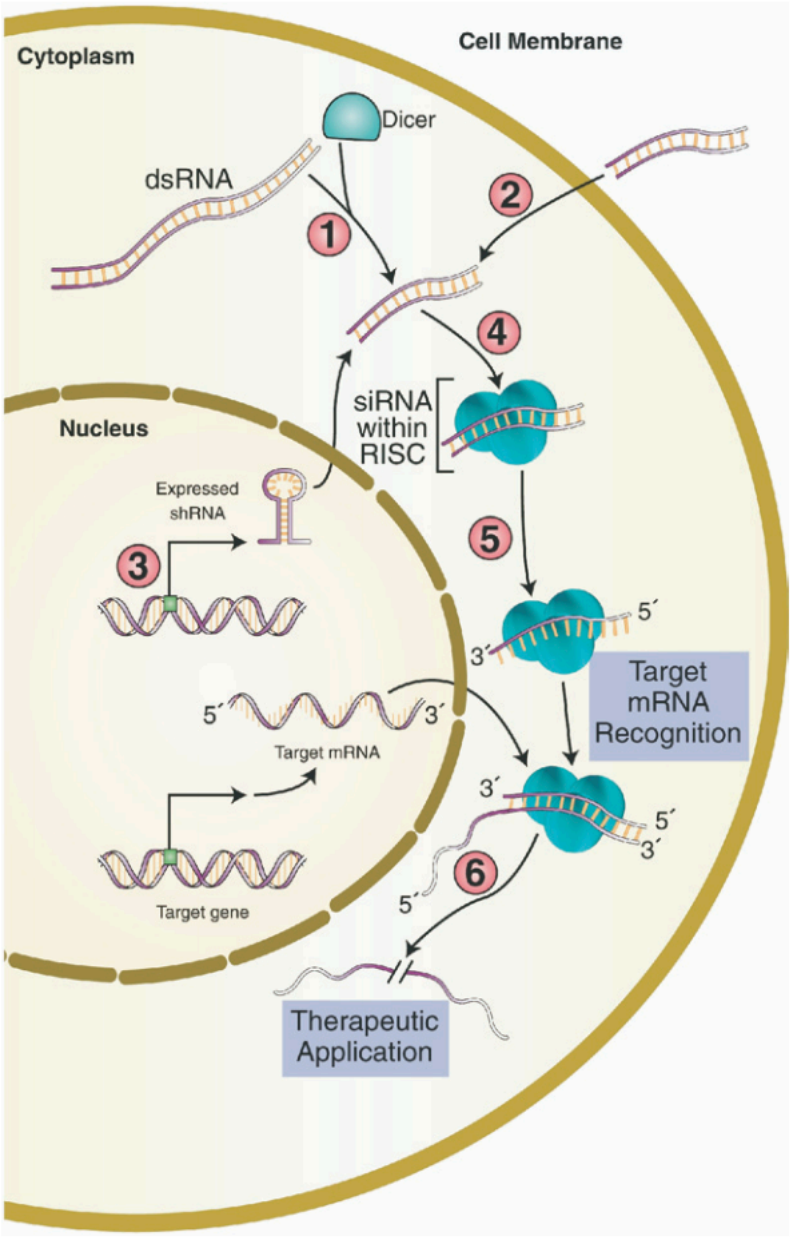


Figure 1.1 Illustration of siRNA gene silencing mechanism [27].

RNA interference has been widely exploited in basic biological research and clinical applications. Developing gene-specific drugs based on short interfering RNA therapy is

becoming one of the prominent means for functional genomics study [28,29]. In biology area, examining the phenotype of organisms that lack of or contain mutations in its encoding gene is the straightforward way for biological function or pathway determination of a protein [30]. In this case, siRNAs are powerful tools on specific silencing the expression of certain genes. Due to its high efficiency and diversity, it become one of the latest additions to gene-silencing reagents. Meanwhile, RNA interference has been an attractive choice for future therapeutics due to its ability to control the disease-associated genes. Switching off problematic genes using RNA interference therapy is promising for a large number of human diseases. Ideally, every human disease caused by the activation of one or a few genes could be eliminated contributed from RNAi-based intervention. The first clinical trial of RNAi therapy has been directed in patients at the treatment of age-related macular degeneration (AMD) back in 2004 [31]. Millions of adults suffer from blindness or limited vision causing by this disease every year [32,33]. At the same time, the extended clinical evaluations of siRNA in other genetic and viral diseases were performed due to its relatively low safety concerns in humans [34]. Moreover, RNA interference technology suggests a powerful gene therapy for people as an non-harmful cancer treatment that tremendous side effects of traditional surgery, chemotherapy and radiation due to the non-

specificity to the tumor cells can be avoid [35]. Some in vivo experiments on xenograft mouse model have already shown the successful inhibition of tumor cell growth by siRNA [36–40].

Although *in vivo* studies have shown great potential and promises of RNAi-based therapies, there are various of issues that need to be considered for siRNA-based therapeutics. Among them, the most essential consideration is the efficient delivery of siRNA to its target site in the cytoplasm. All the unfavorable physicochemical properties such as its hydrophilicity, large molecular weight, negative charge and easily degradation by nuclease and instability with plasma [41] impede significantly on the uptake of siRNA into the target cells, especially after intravenously injection [42]. Therefore, a safe and efficient delivery which can secure the biological functionalities of siRNA is crucial for them to be a potentially successful therapeutic agent. An ideal delivery system should bear the following properties: (1) prevent siRNA from serum degradation, (2) reduce clearance rate in the human body and increase the retention time of siRNA in the circulatory system, (3) facilitate the delivery of siRNA to specific cell types, (4) promote cellular uptake, (5) successful siRNA release in the cytoplasm after internalization, (6) be composed of well characterized, biocompatible and easily prepared material and (7) showing minimized level of toxicity to the human body.

1.1.3 Carriers for siRNA Delivery

Many non-viral carriers, which can be classified into 3 main categories — lipids, polymer and peptides, have been developed to deliver siRNAs into the cells for years, and all three have achieved varying degrees of success. Lipid-based delivery systems, such as micelles, liposomes, emulsions, and solid lipid nanoparticles, represents a mature technology for all kinds of drugs delivery and were introduced as carriers for RNA over 20 years ago [43,44]. The development of cationic lipids is one of the key factors in the success of lipid-based siRNA delivery systems owing to their simple formation manner, stability, high transfection efficiency and enhanced pharmacokinetic properties.

Cationic lipids, the main component for lipid-based delivery system, have been wildly used to encapsulate negatively charged siRNA molecules by electrostatic interaction and deliver them into cells. In an aqueous environment, lipid bilayer, which are automatically formed by amphiphilic lipid particles, can further become a sphere with an aqueous core. Therefore, siRNA can be protected from enzymatic degradation and renal clearance reduction. Moreover, the physical and chemical properties of liposomes can be optimized with additional flexibility using multiple types of lipid [45]. However, the relatively large amounts

of the lipids required for siRNA transfection can results in toxicity [46]. It has been reported that cationic lipids can modify cellular signaling pathways and further stimulate specific immune or anti-inflammatory responses [47]. These toxic/immunogenic features limit the use of lipidic carriers *in vivo*.

Another class of carrier systems for siRNA therapeutics is synthetic/natural polymers, which consisting of repeated units of covalently bonded monomers. The structural and chemical properties of the polymers are well established, siRNAs can bind to cationic polymers through electrostatic interactions. Dendrimers, a relatively new class of cationic polymers, have been studied extensively in recent years [48–51]. Various of polymers such as polyethyleneimine (PEI), poly-(lactic-co-glycolic acid) (PLGA), poly-l-lysine, poly (alkylcyanoacrylate), chitosan, and gelatin has been investigated as well [52]. Typically, PEI is the most widely used polymer and PLGA has been used for decades in pharmaceutical applications since it is biodegradable and biocompatible. Similar to lipidic carriers, the development of polymeric delivery systems also restricts to relatively high cellular toxicity and immunological rejection [53].

Peptide-based carriers are emerging as an alternative for safer *in vitro* and *in vivo* delivery

due to the existence of safety concerns and efficacy issues with current drug delivery systems. As of now, more than 100 peptides with the cell-penetrating ability, which called cell-penetrating peptides (CPPs), have been identified [54]. Cell-penetrating peptides can deliver a variety of cargos including small molecule pharmaceuticals, proteins and oligonucleotides into cells through cellular membrane efficiently and specifically [54]. Particularly, these CPPs can reach area of the body that are not easily accessible, such as the blood-brain barrier, and successfully deliver active substances.

Our lab has been studying and developed a self-assembled CPP called NP-1 which can be used as drug or gene delivery vehicle [55]. It has been proven to successfully used to encapsulate siRNA and deliver it across the cell membrane in a controlled manner *in vivo*. However, as the environment gets increasingly severe with the addition of serum, the transfection efficiency was dramatically decreased. The non-ideal results were considered to have been caused by the instability of the peptide/siRNA complexes in the serum systems where a lot of unfavored components, such as various of ions, have the effect of destabilizing the complexes. Thus, the idea of peptide conjugated with polyethylene glycol (PEG) and controlled MMP-2 sensitive detachment has been receiving growing attention. PEG can serve several purposes at the same

time: (1) masking the CPP activity to inhibit contacts with cell membranes [56] (2) prolong the half-life of therapeutic molecules in the bloodstream and preventing the interactions with blood components (3) enhances passive tumor targeting following the enhanced permeability and retention (EPR) effect [57]. Meanwhile, a specific designed, 8 amino acids sequence was applied as the MMP-2 responding cleavage site, and 3 different molecular weight of PEG were conjugated with the linker then linked with NP-1. The new designed peptides were called NP1-peg3, NP1-peg9 and NP1-peg18, in abbreviation peg3, peg9 and peg18, according to the difference in size of PEG. Physicochemical characterizations were first applied to study the formed peptide/siRNA complexes including size and zeta potential. Then, the optimal concentration ratio for peptide versus siRNA to form complexes was determined and their cellular uptake, cytotoxicity and transfection tests were all performed in A549 cancer cells under this specific ratio. The design principle for MMP-2 related cleavage was also be tested and confirmed. Finally, the stability of the complexes against RNase were determined qualitatively and quantitatively. Based on the results reported in this study, strategies could be referenced to construct more functional peptide-based nanocarriers for *in vitro* siRNA delivery and cancer treatment.

1.2 Research Objectives

The goal of this research is to improve the functionality of NP-1 as a more efficient and safer carrier for *in vitro* siRNA delivery (with serum). To achieve this goal, polyethylene glycol (PEG) was introduced as the particle protector and matrix metalloproteinase-2 cleavable amino acid sequence was conjugated as a controlled release switch for PEG detachment. Particle stability, siRNA conservation ability, silencing efficiency and cultured cells toxicity were studied for the new designed peptide and siRNA complexes. The specific objectives of this thesis are listed in the following:

(1) Design of modified peptide based on NP-1 sequence that can remain high siRNA transfection efficiency and low cytotoxicity while increasing stability in serum environment.

(2) Characterization of peptide/siRNA complexes; This including the particle size, zeta potential, the optimal peptide (positive charge)/siRNA (negative charge) molar ratio, thereby providing basic information for better-formed siRNA/peptide complexes.

(3) Verification of the specific amino acid, which can be recognized by MMP-2, performance with A549 lung cancer cell.

(4) Investigation of the transfection efficacy, toxicity and biocompatibility of the complexes *in vitro* with serum in A549 cells along with the particle stability and resistivity towards RNase degradation.

1.3 Outline of the Thesis

The thesis consists of eight chapters. The following are the scopes of each chapter:

Chapter 1 gives an overview of the thesis, including a brief introduction to matrix metalloproteinase-2, a highly employed drug release stimulus, and RNA interference with its mechanism, potential clinical applications along with existing gene delivery options and the promising future of peptide based delivery system. The research objectives and the outline of the thesis are also given in this chapter.

Chapter 2 provides an overview of the application of matrix metalloproteinase-2 and the advantages of RNAi as potential new pharmaceutical drugs following with therapeutic applications of siRNAs and current siRNA delivery systems. Uptake pathways and subsequent intracellular trafficking of cell-penetrating peptides (CPPs) are also mentioned at the end of this chapter.

Chapter 3 introduces all the instruments with clear photos that have been used during this research and the experimental procedure.

Chapter 4 presents a series newly modified NP-1, amphiphilic and cationic cell-penetrating peptide peg3, peg9 and peg18. Physicochemical characterizations, transfection efficacy, cytotoxicity, particle stability and resistivity towards RNase degradation of the complexes in serum environment were conducted in A549 lung cancer cells. Performance of the specific amino acid linker was also been evaluated. Finally in this chapter, discussion and conclusions of this study from this research and recommendations for future work were stated.

Chapter 2

Literature Review

2.1 Functionality and application of matrix metalloproteinase

2.1.1 Functionality of matrix metalloproteinase

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes, which play a central role in a range of numerous physiological and pathological processes such as cartilage and bone repair, morphogenesis, cell migration, wound healing, angiogenesis and cancer invasion [58]. There are more than 25 structurally related enzymes categorized into four main classes according to their cellular localization and substrate specificity: gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-12), collagenases (MMP-1, MMP-8 and MMP-13) and membrane-type MMPs (MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP) [59–62]. Although they are best known for remodeling of the extracellular matrix (ECM) and degradation of extracellular proteins in the context of cancer cell invasion and metastasis, they also been found involving in cell survival,

differentiation, proliferation, migration, adhesion and in cell-cell interactions as well as their importance in regulating the entire extracellular signaling milieu [63,64]. Among all MMPs, the most popular enzyme is matrix metalloproteinase-2 (MMP-2 or gelatinase A), a constitutive enzyme that can be found in almost all cell types with its ability for collagen type IV (a component of the basement membrane) and denatured collagen (gelatin) as well as other degradation [65,66]. Without the adhesion and support from extracellular matrix proteins, cancer cells can easily transfer from original lesion location to a distant site through circulation system in the body. Since they are highly participated in tumor invasion, MMP-2 overexpression can be employed as a trigger for developing self-localized, on-demand drug delivery systems.

2.1.2 Matrix metalloproteinase triggered release

Substantial interest has been attracted on developing MMPs responsive drug delivery systems that have resulted in numerous remarkable published designs and various of different applications. Since MMPs exhibit high efficiency on typical substrates and are frequently overexpressed in specific disease states or /and tissues, “smart” linkers between the drug and a carriers can be designed for controlled cleavage [67]. They are ensured to be stable during administration, storage, in the blood circulation system and under physiologic conditions but

are enzymatically broken upon contact with specific MMPs. The best performed linker is a peptide linker with high sensitivity of MMP recognition, biocompatibility and simple synthesis process. The sequence of peptide linker is unique for specific kind of MMP, as Kratz et al. has proved in their research that the incorporated octapeptide with a sequence of Gly-Pro-Gln-Arg–Ile-Ala-Gly-Gln cannot be cleaved by activated MMP2 and MMP9 in contrast to the sequence which only two amino acids change of Gly-Pro-Leu-Gly–Ile-Ala-Gly-Gln that is cleaved efficiently by activated MMP2 and MMP9 [68].

Meanwhile, with different MMP that been chosen as stimulus, MMP sensitive systems can accommodate different drug types, such as cytotoxic drugs like DOX or protoporphyrin, low molecular weight chemical drugs and high molecular weight nucleic acids drugs like DNA or siRNA with appropriate synthetic carrier molecules. Various synthetic carriers have been developed instead of natural carriers such as albumin, which can be synthesized at high purity and modified further in a simple manner. Additional functionalities can be added to the delivery systems such as solubility tailoring, which improves intracellular or pharmacokinetics delivery of the cargo [12,69]. As one of the synthesis carrier — cell penetrating peptides(CPP), its activity is reduced or entirely inhibited as long as the conjugate is intact. As soon as the MMP-

specific peptide linkers cleaves at the target site, CPP will be released and facilitate cellular uptake of the cargo [9,70–72].

MMP-specific PEG or alternative polymer shells disassembly or de-shielding is a strategy frequently employed for stabilizing and protecting drug delivery carriers by several research groups, either alone or in combination with RGD motifs, cell penetration enhancers or other targeting moieties[73–78].

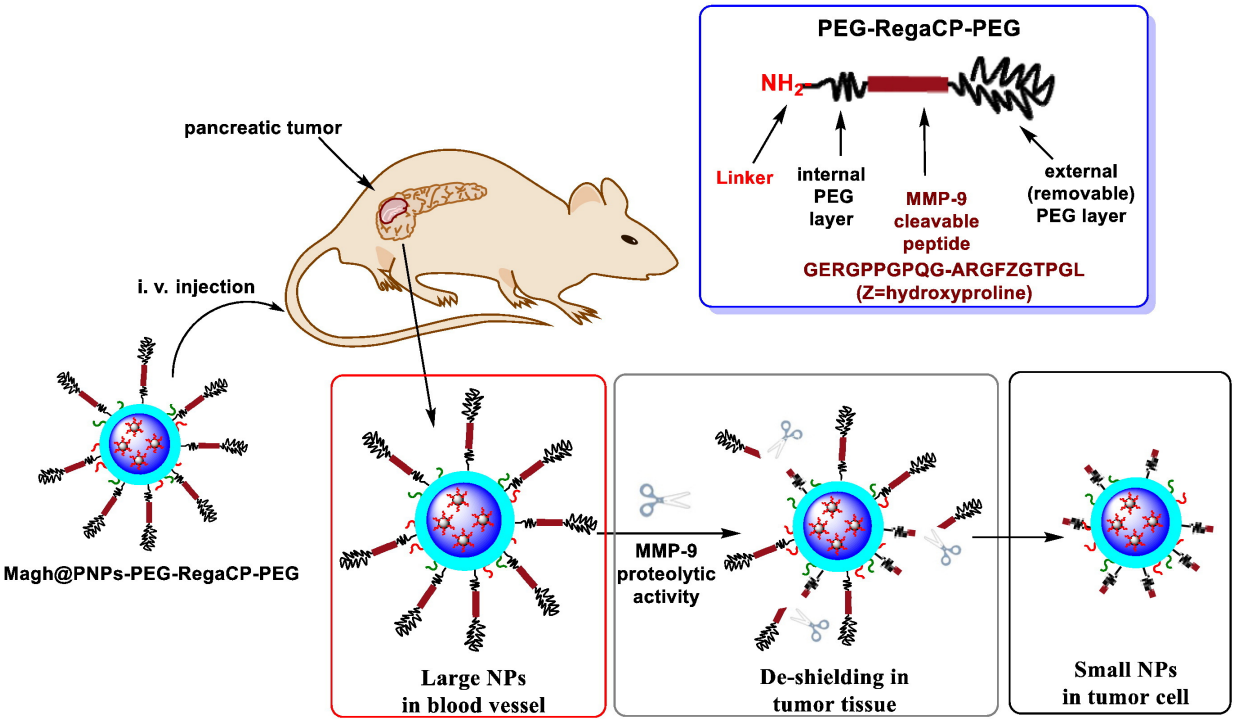


Figure 2.1 schematic of *in vivo* de-shielding of PEG induced by MMP-9 [80].

This concept can be exemplified by the research from Grünwald et al., who prepared poly (lactic-co-glycolic acid) (PLGA) as backbone with MMP-sensitive PEG coating for efficient tumor targeting [79]. The targeting strategy relied on (1) elongated circulation half-life by PEGylation; (2) particles passive accumulation in the tumor due to the EPR effect and (3) subsequent specific de-shedding of the PEG corona by tumor-secreted MMPs (Fig. 2.1). Overall, a successful MMP responsive drug delivery requires optimal incorporation of MMP sensitive elements concerning the conjugation to the main carrier and/or drug, minimized interaction of MMP sensitive elements with scaffold and/or drug that provides accessibility for MMPs along with thoughtful selection on materials and synthetic strategies [80].

2.2 Pharmaceutical potential of RNAi as a drug

2.2.1 siRNA therapy versus traditional drugs

Most approved drugs have the ability to bind to proteins or alter protein function with similar features like apolarity and relatively small in size (molecular weight< 500 Da) [81]. Although traditional drugs designing and their modification for *in vivo* efficacy improvement are well known, the actual development of small molecule drugs is often announced abortive in preclinical trials even with this sufficient experience [82]. However, siRNA drugs are not the

same comparing with traditional drugs. Sharon Engel, director of genomic data at Compugen Ltd., Tel Aviv, Israel said " The process for creating traditional drug was done by trial and error—applying random materials to cells until the desired phenotype is obtained, this may results in extensive side effects and non-specificity. On the contrary, it's possible to minimize the side effects by identifying the reason for a phenotype to develop, validating your identification, and attack the exact location in the cell where responsible for disease with siRNA. The specificity RNAi's sequence can fulfill different kinds of treatment you would demand from a next-generation drug." As an advanced therapeutic approach, RNAi may overcome the major difficulties from traditional pharmaceutical drugs. Key features of RNAi as a therapeutic approach compared with small molecules and proteins and antibodies, two major classes of traditional pharmaceutical drugs, are shown in Figure 2.2 [83].

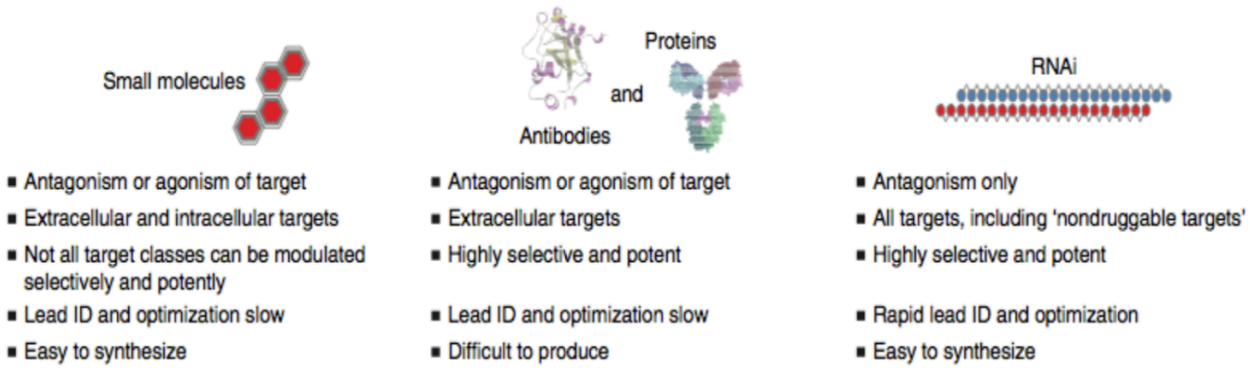


Figure 2.2 Key features of RNAi as a therapeutic approach comparing with small molecules and proteins and antibodies.

The most outstanding advantage of RNAi over other drugs is that the targets are can be "non-druggable", where they have not been known to or predicted to bind with high affinity to a certain drug. It is a time-consuming and difficult process to identify highly selective and potent compounds for small molecule drugs [83]. For RNAi, highly selectivity and potent targets make it a promising pharmaceutical drug in modern medicine.

2.2.2 Therapeutic applications of RNAi

RNAi therapy has outstanding merits including therapeutic precision, broad applicability and minimized side effects owed to its highly selectivity. Currently, advanced technologies have broadened its applications for multitudinous human diseases where the diseased gene has been reported silenced by administration of siRNAs. Figure 2.3 shows the organs in the human body for which RNAi silencing has been proved [83]. Direct RNAi, a way for siRNA delivery at local site, has been carried out successfully to a lot of tissues and organs like eye, skin, nose,

lung, the nervous system and the digestive system. Furthermore, intravenous delivery of siRNA molecules, the systemic RNAi, has been found efficient into lung, liver, joint and tumors.

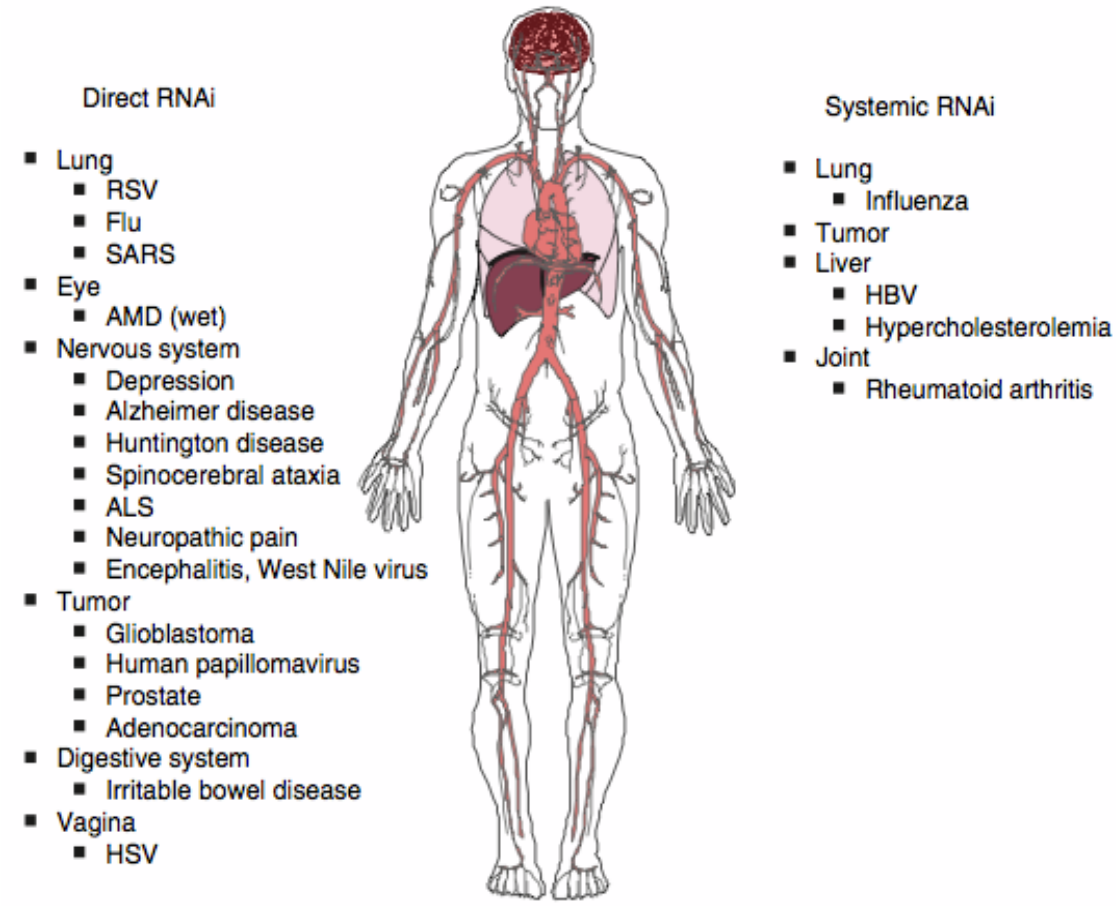


Figure 2.3 Diseases and organs for which the RNAi effect has been proved [83].

Several diseases that are widely studied and common for siRNA treatment will be discussed as following.

Infectious disease

One of the major causes of death worldwide are the diseases caused by bacteria and viruses. Out of many of the diseases including AIDS, hepatitis and influenza for virus infection and sepsis and pneumonia for bacterial infections, can emerge resistant strains against medication which have become a rising concerns for people [84]. Fortunately, the inhibition of infectious agents' cellular uptake or replication have been demonstrated in cell culture studies for RNAi, eg. HBV gene virus [85,86].

With the help of a genomic RNA intermediate and virally encoded reverse transcriptase, HBV, as a DNA virus, can replicate itself in the cell automatically. Many studies have shown that specific siRNAs exhibited various levels of efficacies on inhibition of the gene expression and viral DNA replication on virus [86–88]. For example, intravenous injections of a stable nucleic-acid-lipid particle (SNALP) which can reduce serum HBV DNA concentration in mice has been reported by Morrissey *et al.* [87]. McCaffrey *et al.* demonstrated a significant reduction of viral mRNAs and protein expression in mouse liver by injection of HBV specific shRNAs (small heparin RNA) along with a large volume of plasmids encoding the HBC gene [89]. Table 2.1 shows more examples of siRNA treatment for HBV virus.

Table 2.1 Examples of siRNA targeting towards HBV virus

Gene	Host	Transfection/Delivery	Results
S, C	HepG2.2.15 cells	Liposomes	Reduction in HBsAg secretion by 80% in cell culture
C	Mice	Tail vein injection	Significant reduction in HBsAg and HBeAg expression
S	Mice	Hydrodynamic injection	Three daily intravenous injections of 3 mg/kg day reduced serum
P, C, S	HepG2.2.15 cells	oligofectamine	HBeAg expression decreased by 73.8% and 72.8% after siRNAs targeting Pre C region
C	HuH7 and HepG2 cells	oligofectamine	HBeAg levels in the cell culture medium decreased to 4.6 fold and 4.9 fold
NA	HepG2.2.15 cells	DOTAP liposomes	Inhibition rate is approximately 80-90% in treated cells

Abbreviation: C, core antigen; S, surface antigen; P, polymerase; S, X protein; NA, not report; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane

Another infectious agent targeted by RNAi is the HIV virus which is well known for its gene expression pattern and lifecycle. A number of siRNAs and shRNAs has been tested on targeting HIV genome regions such as tat, pol, env, vpr, rev, gag, vif, nef, and the long terminal repeat (LTR) showed promising results in inhibition of viral production in infected cells [90]. Due to the high viral mutant rate that may let mutants escape from being targeted, targeting the virus directly encounters a substantial challenge for clinical application [91]. Therefore, RNAi therapy suggests an alternative approach of cofactors which associated with virus infection

knockdown. Qin *et al.* successfully developed a lentivirus-based vector for siRNAs delivery into human peripheral blood T lymphocytes against the HIV-1 co-receptor CCR5 [92]. The results showed a 10-fold inhibition of CCR5 expression on the cell surface over two weeks and a 3-7 fold dropping in infected cells since the lymphocyte populations was protected substantially from the HIV-1 virus infection. Surabhi and Gaynor addressed in their research that siRNAs directed silencing NF-κB p65 subunit could significantly decrease the corresponding protein levels and thus inhibiting HIV-1 replication [93].

Cancer

Cancer is the second fatal disease leads to human death after cardiovascular disease and it is estimated to cause 13.1 million human casualties in 2030 [94]. It can cause growing burden to the patients, families and even society. Current cancer treatment commonly rely on surgery, chemotherapy and radiation which has been strongly limited by critical side effects. Luckily, recent understanding of the genetic causes of cancers provides the prospective for gene therapy as an alternative approach. There are a lot of anti-gene approaches such as anti-gene oligonucleotides [95], ribozymes [96], peptide nucleic acids (PNAs) and antisense oligonucleotides. Among them, siRNAs, which apply a post-transcriptional gene silencing (PTGS) mechanism,

Table 2.2 Comparison of advantages and disadvantages for different gene therapy

Strategy	Advantages	Disadvantages
Low-molecular-weight agents	Easy to administrate	Often nonspecific
	Often inexpensive	May not be available
		Off-target effects
Antisense ODNs	Easy to synthesize	Only exogenous delivery
	Inexpensive	Protein binding
	Modification to improve selectivity and efficacy	Off-target effects
		Induction of interferon response
Ribozymes	Simple catalytic domain	Requirement of specific cleavage triplets
	Delivery as free molecules and by expression vectors	Protein binding
	Tissue-specific delivery if vector established	
	Administration to subcellular compartments	
DNAzymes	Inexpensive	Only exogenous delivery
	Good catalytic properties	Off-target effects
	Modification for systemic delivery	
RNAi	Highly effective at low concentrations	Off-target effects
	Delivery as free siRNA molecules and by short hairpin RNA expression vector	Induction of interferon response
	Tissue-specific delivery if vector established	Expensiveness of siRNAs

ODN: Oligodeoxynucleotide; siRNA: Short interfering RNA

perform much better in target gene silencing comparing with the antisense RNA alone since the transcript of the gene will be rapidly degraded before accumulation. Pros and cons of different gene silencing strategies are summarized in Table 2.2 [35].

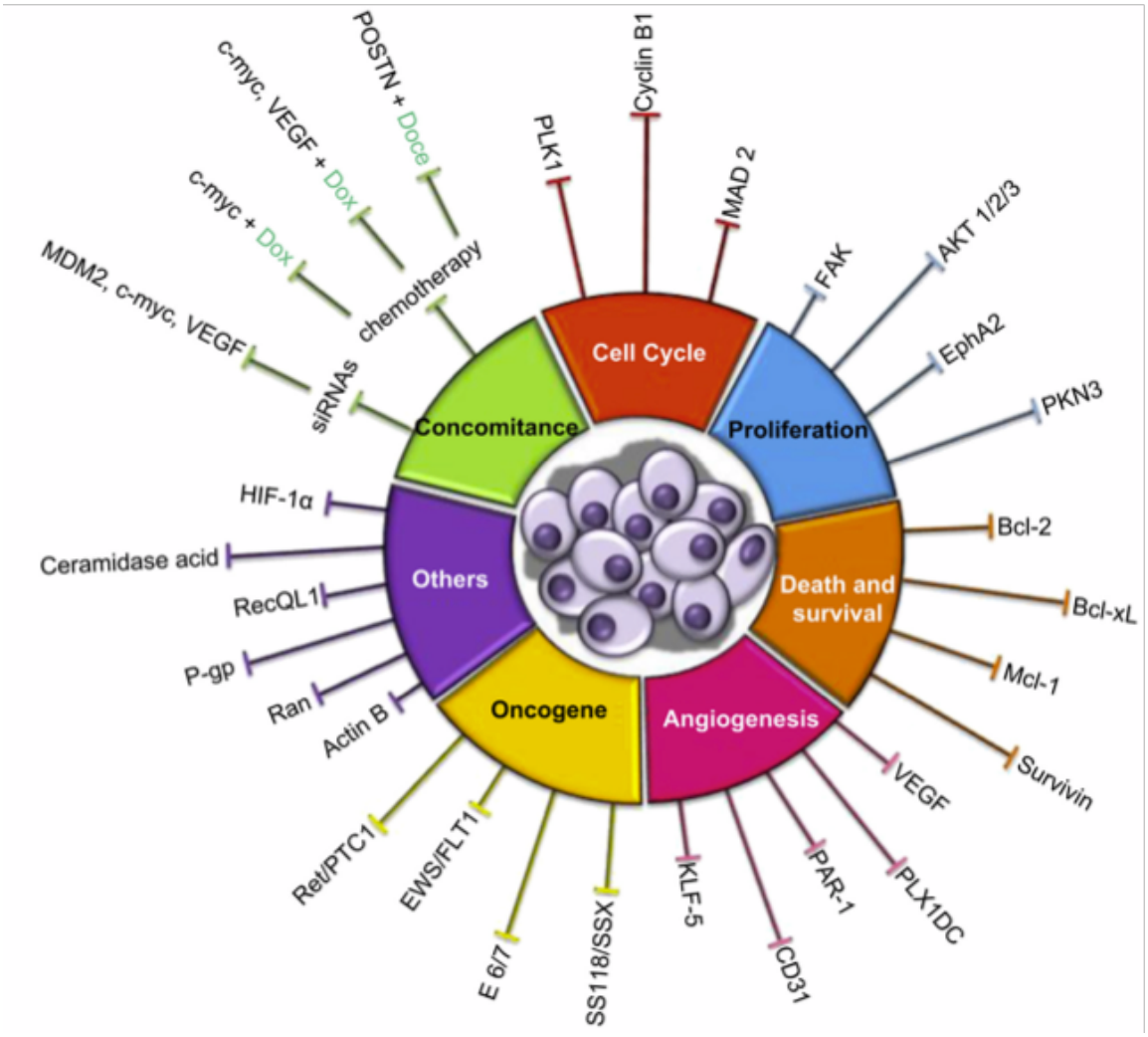


Figure 2.4 Representation of siRNA molecules targeting pathways and genes used in preclinical studies to develop an anti-cancer treatment. AKT 1/2/3: KLFisoforme of serinethreonin kinase; Bcl: B-cell lymphoma; CA: ceramidase acid; Doce: docetaxel; Dox:

doxorubicin; EphA2: receptor of ephrins; FAK: focal adhesion kinase; HIF-1a: hypoxia inducible factor 1a; KLF-5: Kruppel-like factor 5; MAD2: mitotic-arrest deficient 2; Mcl-1: myeloid cell leukemia; MDM2: murine double minute 2; PAR-1: protease activated receptor 1; PKN3: protein kinase N3; Plk1: polo-like kinase1; PLX1DC: plexin domain containing 1; POSTN: periostin; VEGF: vascular endothelium growth factor.

Proto-oncogenes have been frequently been activated in cancer cells by various mechanisms. Cancer cells are different from normal cells in uncontrolled growth due to an abnormality in the cell cycle and long survival time caused by the dysfunction of the proteins that mediate cell apoptosis. In this circumstance, they can gradually gain resistance to many anti-cancer drugs. Thus, the downregulation of tumor progression gene, such as an anti-apoptotic gene and/or expression of the oncogenes in cell cycle in cancer cells through siRNA treatment suggests a better solution for cancer treatment compared to traditional drugs. There are a lot of other tumor formation genes also widely studied, eg. vascular growth factor that can facilitate cancer metastasis and mutated genes in cancer cells that developed drug-resistivity. Figure 2.4 represents successful *in vivo* animal studies of different target genes and pathways for cancer treatment [97]. At the meantime, many studies have confirmed the

anticipated tumor inhibition effect of RNAi *in vivo* and showed its therapeutic potential. Detailed information were summarizes in Table 2.3 [97].

Table 2.3 Various studies based on siRNA delivery for cancer treatment

Pathway	siRNA target	Cancer model	Injection route	Inhibition of tumor volume	Other effects
Cell cycle	Cyclin B	Prostate	s.c	70%-92%	50% long survivial
	PLK1	Breast	s.c	70%	Caspase 3 activation
	MAD2	Colon	s.c	35%	Increased apoptosis
Proliferation	EphA2	Ovarian	i.p	55%	/
	AKT1	Prostate	s.c	66%	/
	AKT2	Prostate	s.c	89%	/
	AKT3	Prostate	s.c	57%	/
	PKN3	Prostate	Orthotopic	60%	75% for metastasis
	FAK	Ovarian	i.p	60%	/
Cell death and Survival	Bcl-2	Prostate	s.c	65%	/
	Mcl-1	Breast	s.c	75%	no toxicity for max dose
	Survivin	Prostate	s.c	30%	/

Angiogenesis	VEGF	Prostate	s.c	87%	No toxicity
	PAR-1	Melanoma	s.c	77%	/
		Melanoma meta	i.v	/	Decreased metastase number 81%
	CD31	Prostate	Orthotopic	65%	/
	KLF-5	Lung carcinoma	s.c	60%	/
		Prostate	s.c	48%	Prolonged survival median
	PLX1DC	Ovarian	i.p	87%	Increased apoptosis 35%
Oncogene protein	E6/7	Renal	s.c	55%	No synergic effect with cisplatin
	ret/PTC1	Fibroblast	s.c	90%	/
	EWS-FLI1	Ewing sarcoma	i.v	80%	No inflammatory response
	SS18-SSX	Synovial sarcoma	s.c	83%	/
Hypoxy	HIF-1a	Glioblastoma	s.c	50%	/
Metabolism	CA	BT474	s.c	75%	Increased apoptosis,non-immunogenic
DNA repair	RecQL1	Colorectal meta	Spleen	43%	/
		Pancreatic meta	Spleen	31%	/
		Lung	s.c	46%	/
Nuclear transport	Ran	Neuroblasto-ma	s.c	49%	Increased apoptotic cell

Resistance	P-gp	Breast	s.c	60%	/
Migration	Actine B	HT-1080	s.c	65%	No inflammatory response
siRNA concomitance	MDM2, c-myc, VEGF	Melanoma	i.v	30%	No inflammatory response
Chemotherapy Concomitance	c-myc	Colon	s.c	60%	Increased apoptosis
	c-myc, VEGF	Ovarian	s.c	57%-60%	Increased apoptosis, no inflammatory response
	POSTN	Ovarian	i.p	90%	Increased apoptosis 45%, decreased proliferation 60%

s.c: sub-cutaneous injection, i.p: intraperitoneal injection, i.v: intravenous injection. AKT 1/2/3: KLFisoforme of serinethreonin kinase; Bcl: B-cell lymphoma; CA: ceramidase acid; EphA2: receptor of ephrins; FAK: focal adhesion kinase; HIF-1a: hypoxia inducible factor 1a; KLF-5: Kruppel-like factor 5; MAD2: mitotic-arrest deficient 2; Mcl-1: myeloid cell leukemia; MDM2: murine double minute 2; PAR-1: protease activated receptor 1; PKN3: protein kinase N3; Plk1: Polo-like kinase1; PLX1DC: plexin domain containing 1; POSTN: periostin; VEGF: vascular endothelium growth factor.

However, in order to avoid side effects that occur in traditional treatment, precise selectivity for cancer cells destruction solely need to be ensured without damaging normal cells. To achieved this purpose, the siRNA should be designed to target specific genes involved in cancer cell growth and delivered to the desired tumor site directly.

2.2.3 siRNA therapeutics in clinical trials

RNAi has rapidly upgraded from research level to clinical trials within a short period. Numerous financial investment and manpower have contributed in introducing siRNA technology into actual drug market. VEGF pathway for the wet form of AMD, a leading cause

Table 2.4 siRNA drugs in clinical trials

Drug	Target	Disease	Phase	Company
ALN-VSP02	KSP and VEGF	Solid tumors	I	Alynylam Pharmaceuticals
siRNA-EphA2-DOPC	EphA2	Advanced cancers	I	MD Anderson Cancer Center
Atu027	PKN3	Solid tumors	I	Silence Therapeutics
TKM-080301	PLK1	Cancer	I	Tekmira Pharmaceutical
TKM-100201	VP24, VP35, Zaire Ebola,	Ebola-virus infection	I	Tekmira Pharmaceutical

ALN-RSV01	RSV nucleocapsid	Respiratory syncytial virus infections	II	Alynylam Pharmaceuticals
PRO-040201	ApoB	Hypercholesterolaemia	I	Tekmira Pharmaceutical
ALN-PCS02	PCSK9	Hypercholesterolaemia	I	Alynylam Pharmaceuticals
ALN-TTR02	TTR	Transthyretin-mediated amyloidosis	II	Alynylam Pharmaceuticals
CAKAA-01	RRM2	Solid tumors	I	Calando Pharmaceuticals
TD101	K6a (N171K mutation)	Pachyonychia congenita	I	Pachyonychia Congenita Project
AGN211745	VEGFR1	Age-related macular degeneration, choroidal neovascularization	II	Allergan
QPI-1007	CASP2	Optic atrophy, non-arteritic anterior ischaemic optic neuropathy	I	Quark Pharmaceuticals
I5NP	p53	Kidney injury, acute renal failure	I	Quark Pharmaceuticals
PF-655	RTP801	Choroidal neovascularization, diabetic retinopathy,diabetic macular oedema	II	Quark Pharmaceuticals
siG12D LODER	KRAS	Pancreatic cancer	II	Silenseed
Bevasiranib	VEGF	Diabetic macular oedema,macular degeneration	II	Opko Health
SYL1001	TRPV1	Ocular pain, dry-eye syndrome	I, II	Sylentis
SYL040012	ADRB2	Ocular hypertension, open-angle glaucoma	II	Sylentis

CEQ508	CTNNB1	Familial adenomatous polyposis	I, II	Marina Biotech
Rxi-109	CTGF	Cicatrix scar prevention	I	Rxi Pharmaceuticals
ALN-TTRsc	TTR	Transthyretin-mediated amyloidosis	I	Alynylam Pharmaceuticals
ARC-520	Conserved regions of HBV	HBV	I	Arrowhead Research

of blindness and on the RSV genome, and RSV infection, the leading cause of pediatric hospitalizations, have been chosen as the therapeutic targets in the initial trials [98]. They have both towards non-toxicity in Phase I trials. A total number of 14 RNAi therapeutic programs initiated by different companies have been reported to enter clinical trials in 2010 [99], and promptly, this number increased to 22 untill 2013, as shown in Table 2.4 [100]. Among them, the QP1-1007 from Quark and ALN-PCS02, with a new name called Inclisiran, from Alnylam and he Medicines Company have been reported proceeding from phase I to phase III in 2018. Similarly, the siRNA drug invented by Sylentis and PharmaMar called SYL-1001 with the name changing to Tivanisiran, have made promising progress from phase I, II to phase III last year [101].

The exciting potential of siRNAs can be demonstrated according to the promising results in these clinical trials. However, there are still several extracellular and intracellular challenges that strongly limit the use of RNAi in the actual therapy. Some target organ and tissues may requires unique treatment since gene silencing approaches may not be able to completely get rid of the mRNA, which could led to off-target silencing. Moreover, siRNA can induce potential unwanted effects such as activating an innate immune response or shutting off defense systems in the body which has the functionality of viruses elimination.

2.3 Current delivery systems

siRNA molecules cannot activate the RNAi pathway unless entering the target cells. However, in some cases that have been described in many literatures, eye, lung and central nervous system can directly uptake naked siRNA without any delivery facilitation [98], but the mechanism behind this uptake is still not well explained. Other than this particular circumstance, siRNAs require specific delivery strategy to assist their cellular uptake and protect them from degradation since they are too large and hydrophilic to pass through cell membranes by themselves in most cases. The barriers the delivery system may encounter *in vivo* are shown in Figure 2.5 [102].

To solve these issues, many types of delivery strategies have been created. Physical internalization method, such as hydrodynamic injection [103,104], which a large volume of siRNA solution is rapidly inject into a mouse via the tail vein, particle bombardment and

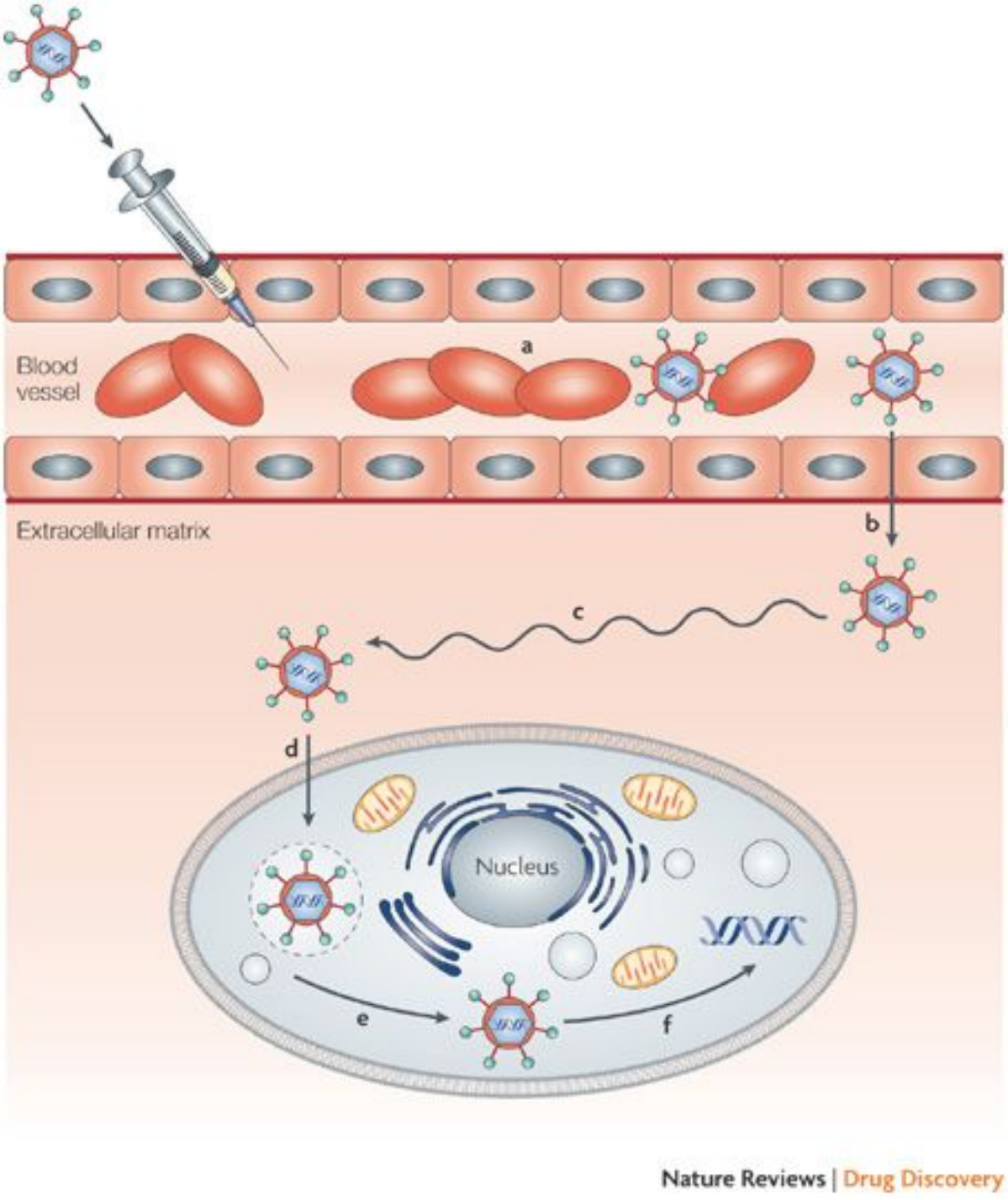


Figure 2.5 Systemic delivery barriers for siRNA *in vivo*. An injected nanoparticle must have the ability to prevent siRNA from filtration, phagocytosis and degradation in the bloodstream (**a**); transport across the vascular endothelial barrier (**b**); diffuse through the extracellular matrix (**c**); be taken up by the target cell (**d**); escape from the endosome (**e**); and release the siRNA in cytosol (**f**).

are well established [103,104]. Although these methods may avoid possible immune system stimulations that often arise in normal chemical based delivery systems, they have not been investigated as extensively as chemical delivery systems such as viral vectors, the most powerful transfection tools due to their high efficiency. Yet, they are difficult to produce in a large scale and more problematic, their inflammatory and immunogenic nature stop them from clinical administration. In this case, a few non-viral vectors that benefited from their relatively low immunogenicity and high biocompatibility have been studied as an safer alternative for siRNA delivery to overcome these limitations. Three major types of delivery system for siRNA will be discussed as following.

2.3.1 Lipid-based siRNA delivery system

Lipid carriers has attracted tremendous interest worldwide since the first discovery and active research of in 1965 [105]. Liposomes, the most versatile supramolecular assemblies with respect to size variety, composition and capacity to capsulate a variety of compounds, has made significant progress in the pharmaceutical industry, especially in drug and gene delivery. Several liposomes have been proved their biocompatibility and efficiency for small molecule drugs delivery in patients. Their superiority perfectly exemplified by Doxorubicin liposome (Doxil; Orthobiotech) which has received FDA approval for breast cancer, ovarian cancer and other solid tumors treatment [106].

With different structures and formulations, a variety of LNPs including liposomes, microemulsions, micelles and solid lipid nanoparticles have been generated [107]. Cationic liposomes and lipoplexes emerged as the most promising vehicles among these synthetic carriers. The ideal liposomes can encapsulate siRNA with high affinity, prevent siRNA from enzymatic degradation in serum, and form a narrow size distribution around 100 nm to ensure the accession to extravascular regions. They are normally form a phospholipid bilayer constructed with an aqueous core for entrapping hydrophilic drug in the contrary to lipoplexes,

which can spontaneously formed through static interaction [108]. There will be electrostatic interaction between the positive charges of the cationic lipid and the negatively charged phosphate backbone of the siRNA upon mixing of the two species in solution, then the overall complexes will be neutralized due to the siRNA being condensed with the lipid.

Lipid-based carriers have been proven to be successfully delivering siRNA into cells. For instance, mRNA and protein levels of CD31 and Tie2 genes *in vivo* was downregulated by the complex of cationic and fusogenic lipids and corresponding siRNA after intravenous injection [109]. Intraperitoneal injection of cationic liposomes DOTAP complexed with anti-TNF ∝ siRNA can inhibit LPS induced anti-TNF ∝ gene expression in mice [110]. Besides, Lipofectamine, Oligofectamine, RNAifect are lipid-based delivery system that are routinely used in the laboratory and have been commercialized for years [111].

There are still inherent difficulties that exist although satisfactory results have been obtained using lipid based siRNA delivery system. The relatively large amount of the lipids required for efficient siRNA transfection can results in severe toxicity. Moreover, they can easily adsorb to serum proteins and trigger unexpected immune system response in the body [46].

2.3.2 Polymers-based siRNA delivery system

Cationic polymers, made up of repeated units of covalently bonded monomers in linear or branched structure is another category of siRNA delivery systems that have been widely studied. Some typical polymer nanocarrier structures designed for siRNA delivery are shown by Figure 2.6 [112]. Similarly, negatively charged siRNA can bind to positively charged polymers through electrostatic interaction [113].

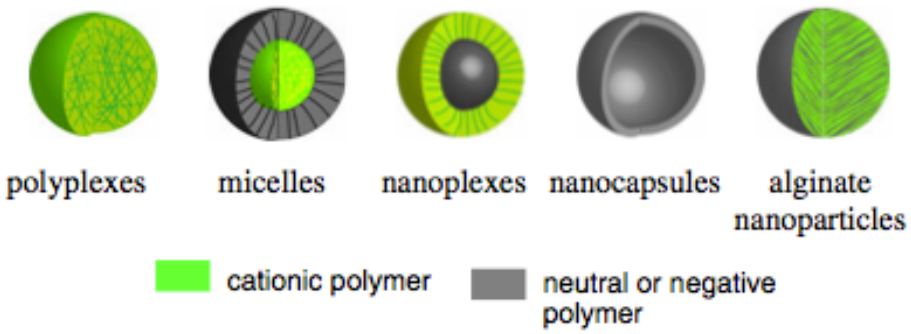


Figure 2.6 Schematic structure of various polymer nanocarrier for siRNA delivery [112].

Numerous polymers have been investigated including poly(ethyleneimine)(PEI), chitosan [114], gelatin [115], poly-(L-lysine)(PLL) [116], poly-D,L-lactide-co-glycolide (PLGA) [117], poly (dimethylaminoethylme-thacrylate) (PDMAEMA) [118,119] and poly (trimethylamino-

ehylmethacrylate) (PTMAEMA). Among them, PEI is the most popular polymer for siRNA delivery due to its higher transfection efficiency offered by the branched structure. Successful internalization by the cells and significant silencing effect was achieved by non-covalently complexed PEI/siRNA. Unfortunately, the drawback came along with the advantage, as there is its increased toxicity caused by the high molecular weight. The systemic application of PEI complexed with HER-2-specific gene has proved inhibition of the established tumors growth [120]. PEI-siRNA complex that targets the pain receptor NR2B subunit with intrathecal injection was shown to have a decrease of mRNA level and its associated protein expression [121].

2.3.3 Cell-penetrating peptide based siRNA delivery system

Cell penetrating peptides are named because their ability to penetrate through cytoplasmic and/or other cellular compartments after internalization [122]. The initial discovery of cell penetrating peptide was when the HIV-1 transactivating protein Tat was found to be taken up by mammalian cells about twenty years ago [123,124], following by the discovery of the homeodomain of *Drosophila melanogaster* transcription factor Antennapedia which also share the same property among some "non-nature" peptides [125]. According to the mutation and deletion analysis, only small domains within these proteins are actually responsible for the

cellular uptake instead of the full-length peptide, and these small peptide sequences are now referred as CPPs or protein transduction domains (PTDs). The CPPs, normally amphipathic and net positively charged at physiological pH [126], can be directly obtained from nature sequences such as Tat and penetratin, they can also be artificially designed and constructed with critical feature of already known CPPs [127,128]. siRNAs can be linked to CPPs by expression as a fusion or by chemical coupling which led to less peptide requirement since higher peptide amount could result in higher toxicity to the cells. Same as the lipid and polymer delivery system, the most popular way to form siRNA/peptide complex is binding each other through ionic interactions. This approach has the advantage of being fast, as there is a reduction in the purification procedure and nature structure of RNAs conservation since it is not necessary for siRNAs chemical modification [129]. Some positively charged amino acid such as arginine, lysine and histidine are often utilized for CPP regarding to this purpose. Figure 2.7 presents the pathways of peptide mediated siRNA delivery [130].

The first CPP/siRNA complex is called MPG which derived from the nuclear localization sequence (NLS) of SV 40 T antigen and the fusion peptide domain of HIV-1 gp41 protein [131] and the Luciferase activity was shown a decrease by 80% after transfection. The siRNA

binding ability and transfection efficiency were enhanced by the derivatives MPGΔ^{NLS} and MPGα [132]. Furthermore, information and current development related to CPPs can be found in a variety of reviews [133–135].

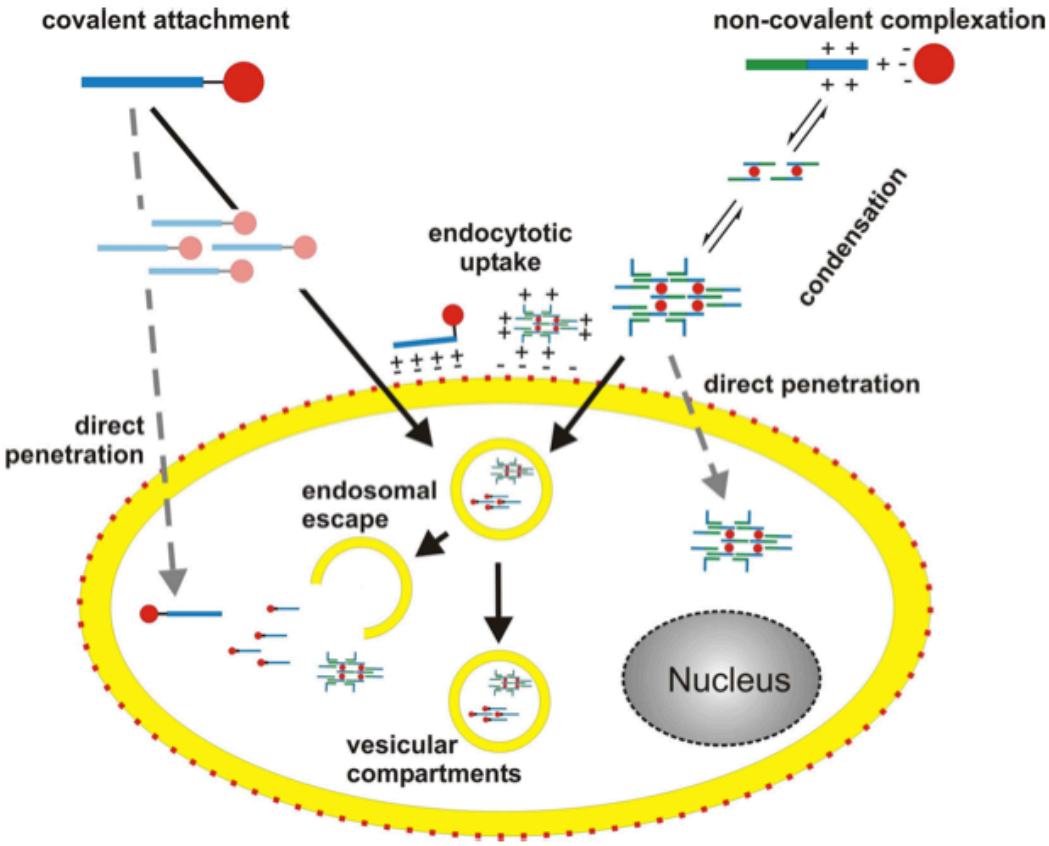


Figure 2.7 Pathways of peptide mediated siRNA delivery [130].

Chapter 3

Equipment Introduction and Experimental Procedure

3.1 Zetasizer Nano ZS

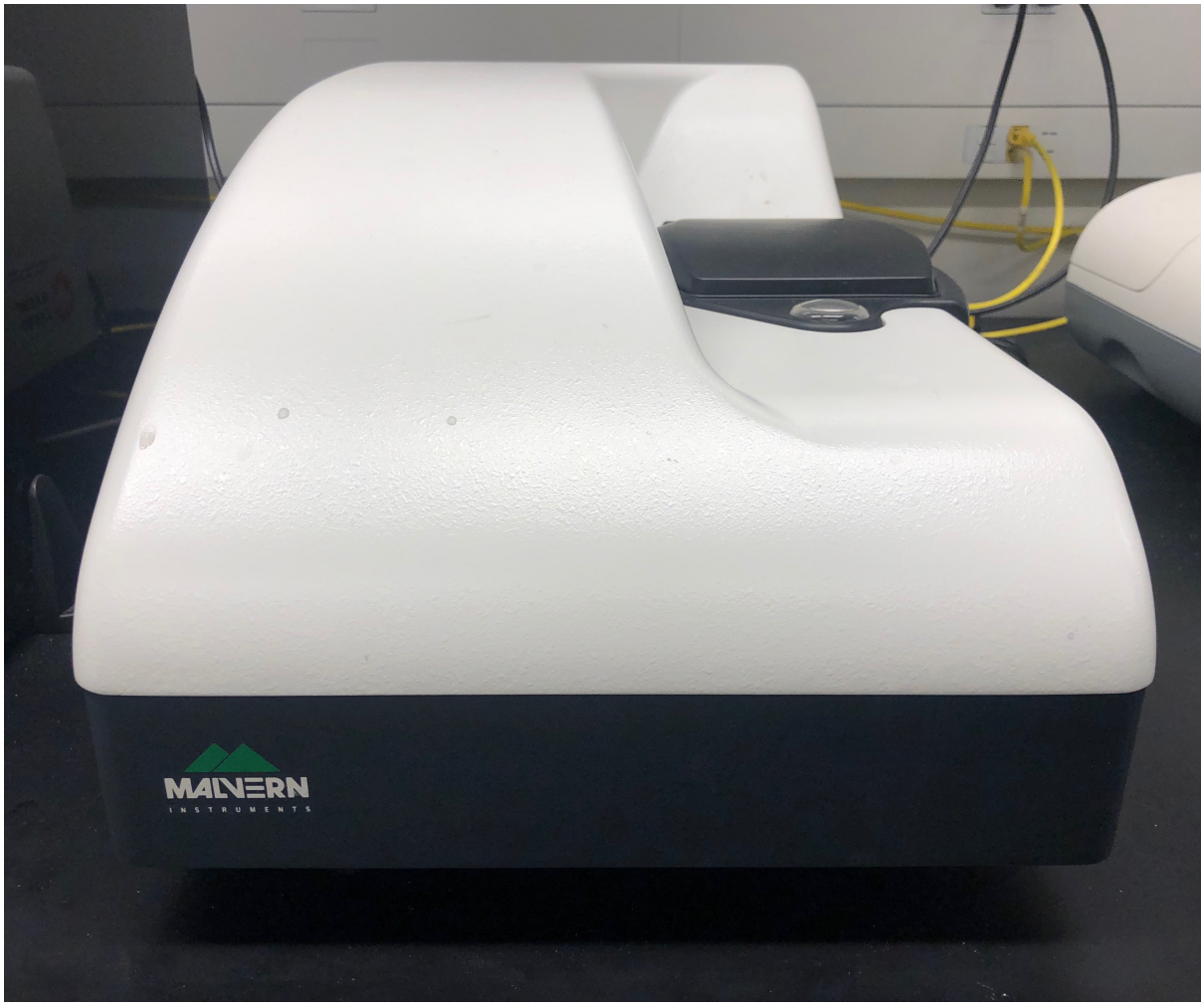


Figure 3.1 Picture of Zetasizer Nano ZS instrument in the lab.

The Zetasizer Nano ZS was mainly used for particle size and zeta potential determination. The hydrodynamic diameter of the peptide/siRNA complexes was measured by dynamic lighter scattering (DLS) on this machine that bought from Malvern Instruments, Malvern, UK equipped with a 4 mW He-Ne laser operating at 633 nm. The measuring cell was a quartz microcell (45 μL) with a 3 mm light path and the scattered light intensities were collected at an angle of 173°. Zeta potential measurements of the complexes were also performed on the same machine using DTS1070 zeta cells. The zeta potential results were obtained with the multimodal algorithm CONTIN, Dispersion Technology Software 5.0. Three measurements were performed to generate the intensity-based size and zeta potential plot reported herein.

3.2 Mini-PROTEAN Tetra cell

MMP2 existence in cultured medium for A549 lung cancer cell was determined using the electrophoresis cell. The cultured medium was collected from A549 cultured plate after 24 hours growth in the incubator at 37 degree, then concentrated by Amicon Ultra-0.5 centrifugal filter device (30K MWCO) at 7,200 × g for 20 mins and eventually load and run on the precast

4-20% SDS-PAGE gel with sample loading dye. The gel was stained by self-made 0.25% wt. brilliant blue for 2 hours and distained for 72 hours.

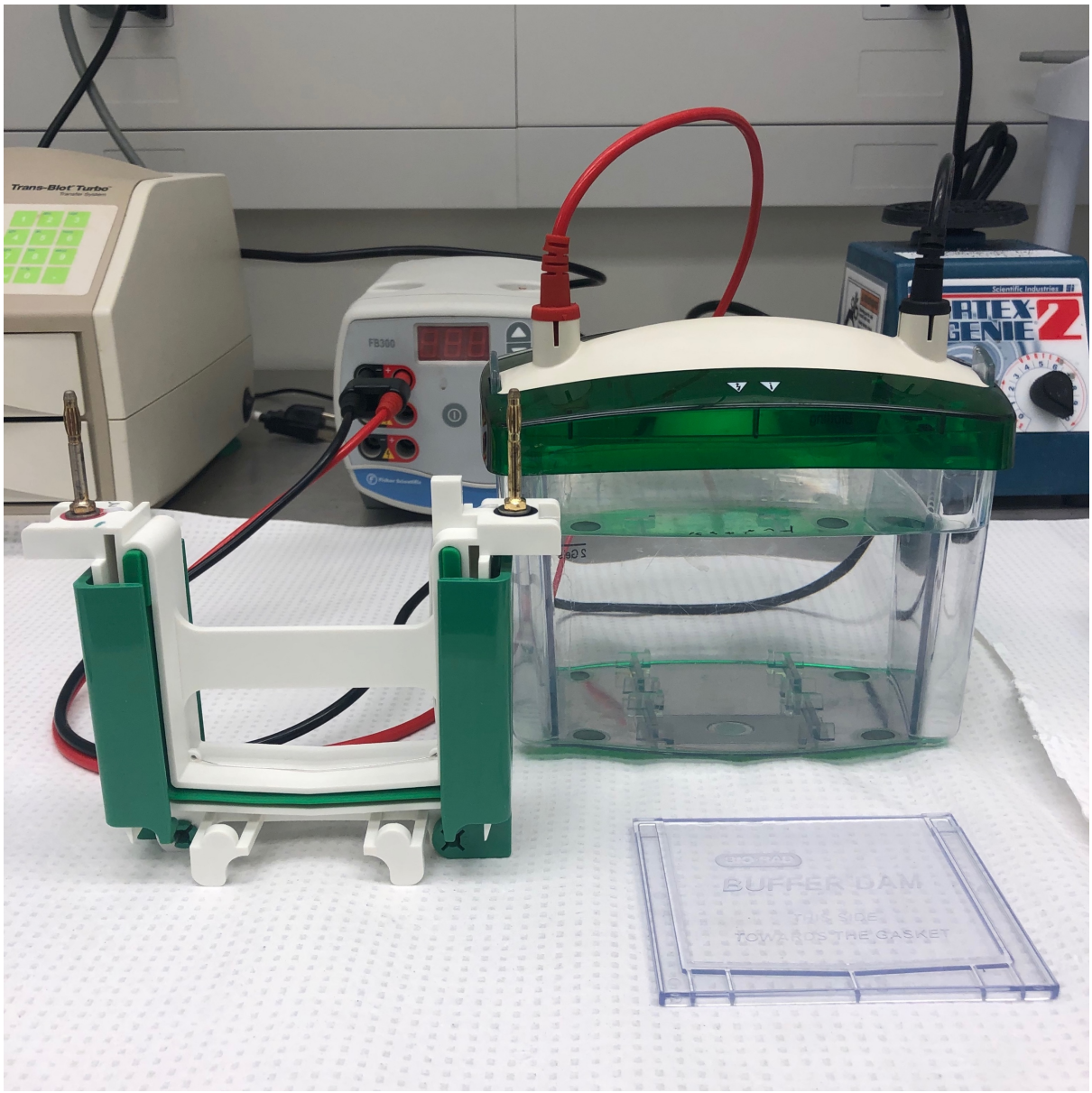


Figure 3.2 Picture of Mini-PROTEAN Tetra cell and its components in the lab.

The Precision Plus Protein Standards (Dual Color) was used as molecular weight markers.

3.3 BD FACSAria Fusion Special Order System

Figure 3.3 Picture of BD FACSAria Fusion Special Order System in the lab from biology department.

Fluorescence-activated cell sorting (FACS), which measuring the cellular uptake of Cy-3 labeled GAPDH siRNA (100 nM), was performed using BD FACSAria Fusion Special Order System (BD Biosciences, Mississauga, Canada) shown above. A549 cells were transfected with the complexes (molar ratio 60:1) according to the protocol listed below. Nontreated cells

served as a negative control. After 3 h incubation, the culture medium was discarded and cells were washed with PBS. 2 μL of Trypsin-EDTA was then added into each well to detach the cells from the plate, eventually the cells were suspended in 4% PFA solution and collected.

3.4 Mx3005PTM Real-Time PCR System (only for cDNA now)

cDNA, the second step after RNA extraction for gene silencing assay, was conducted by Mx3005PTM Real-Time PCR System from Agilent Technologies, Wilmington, USA.

A549 lung cancer cells (40,000/well) were plated in a 24-well cell culture plate in F-12K medium with 10% FBS for 24 hours. The medium was removed and washed with PBS afterwards. 30 μL of the sample solution was prepared in RNase free water containing the complexes of GAPDH siRNA (100 nM) formulated with peg3/peg9/peg18 at molar ratio 1:60 or Lipofectamine 2000 and diluted to 300 μL F-12K with 10% FBS medium. The final solution was add to the cells and 300 μL of F-12K with 10% FBS was added after 3 hours transfection. The cells were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. Afterwards, the cultures were then washed with PBS. Total RNA was extracted from the cells with 200 μL/well TRIzol

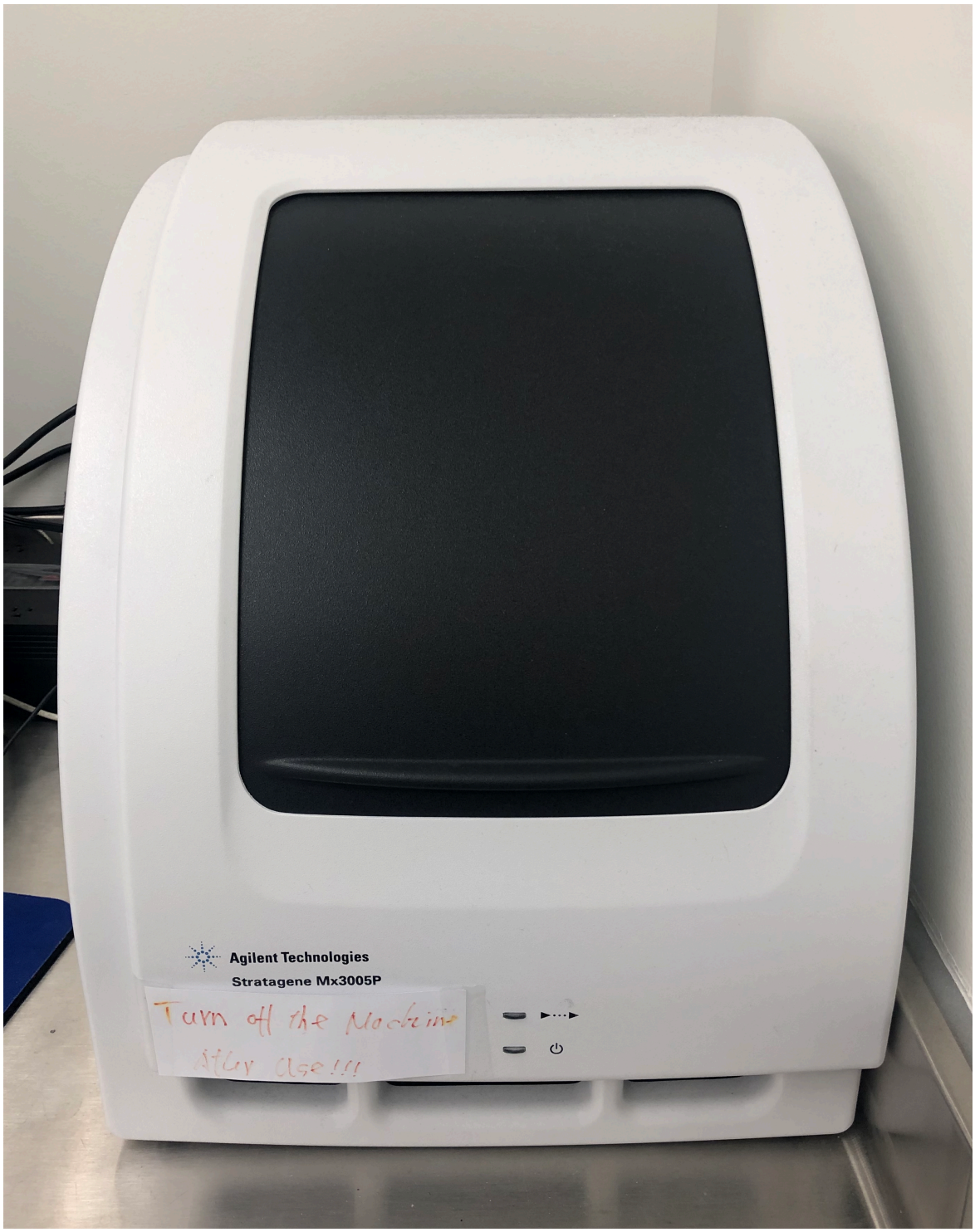


Figure 3.4 Picture of Mx3005PTM Real-Time PCR in the lab.

reagent (Life Technologies, Carlsbad, USA), then treated with 40 µL/well chloroform (Sigma, Oakville, Canada) and 100 µL/well 2-propanol (Sigma-Aldrich, Oakville, Canada) as recommended by the manufacturer. Final RNA concentrations were measured by Nanodrop spectrophotometer ND-1000 (Thermo scientific, Ottawa, Canada). All RNAs were reverse transcribed with Quantabio qScript cDNA SuperMix (Quantabio, Beverly, USA). The cDNA synthesis was primed with a unique blend of oligo (dT) and random primers. The thermal profile of cDNA is comprising of 2 continuously segments: 1 cycle at 25 °C for 5 mins in segment 1 and 1cycle of 30 mins at 42°C then rising to 85 °C for 5 mins in segment 2.

3.5 C1000 Touch Thermal Cycler

Polymerase chain reaction (PCR) which is the final step of gene silencing assay, was performed with PerfeCTa SYBR Green FastMix (Quantabio, Beverly, USA) on the instrument shown below.

The following pairs of human GAPDH gene primers were used for PCR: forward 5′-GAAATCCC ATCACCAT CTTCCAGG-3′, reverse 5′-GAGCCCCA GCCTTCTC CATG-3′ (Sigma, Oakville, Canada). Here, the housekeeping gene cyclophilin was chosen as an internal

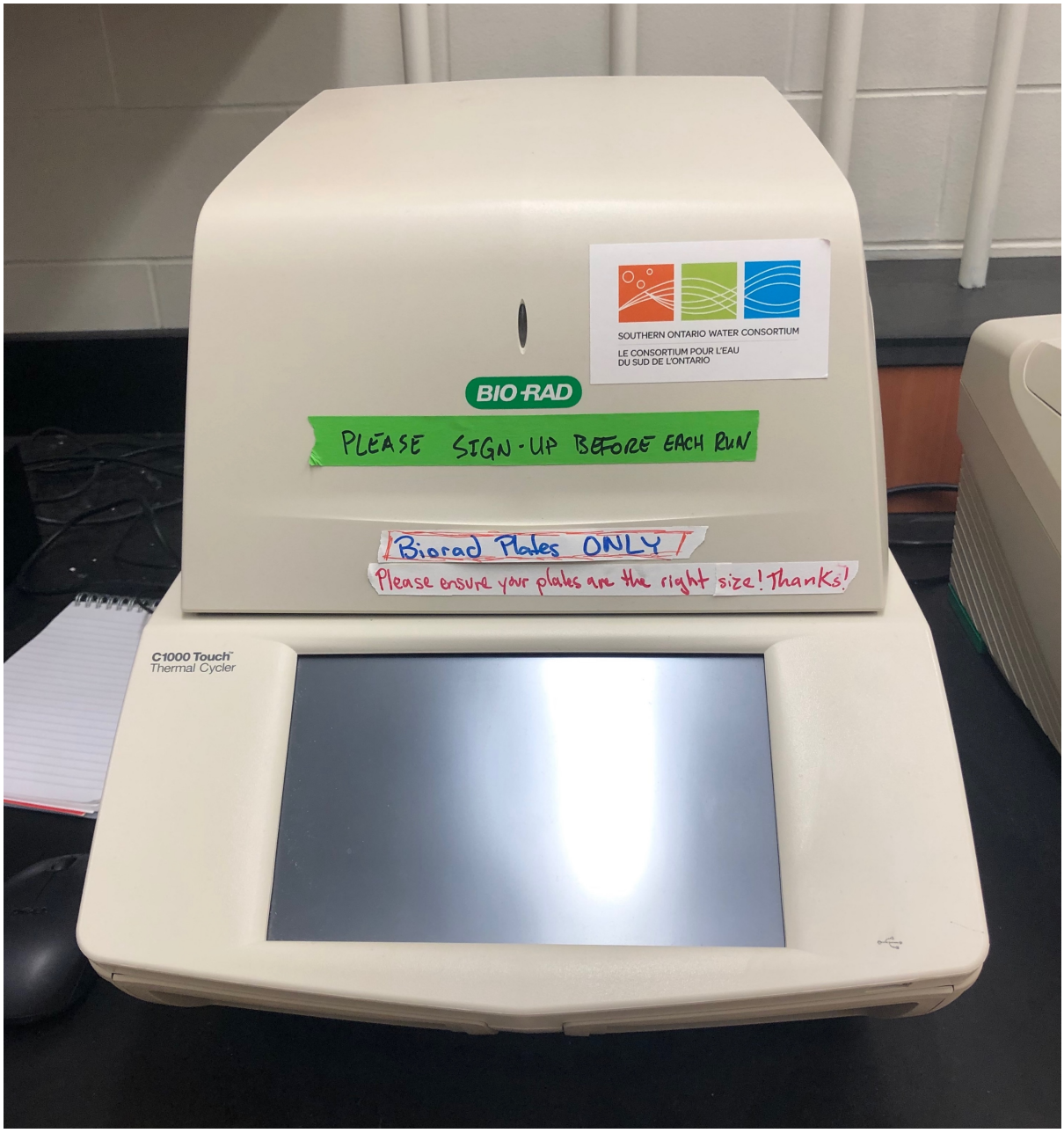


Figure 3.5 Picture of C1000 Touch Thermal Cycler in the lab from Professor Paul Craig.

control to normalize the GAPDH gene. The normalization was performed after the amplification of human cyclophilin mRNA with the following primers: forward 5′-GGTGATC TTTGGTCT CTTCGG-3′ and reverse 5’-TATATGC TCTTTCC TCCCTGTG-3′ (Sigma,

Oakville, Canada). The thermal profile of PCR was comprising of 3 continuously segments: 1 cycle at 90 °C for 30 seconds in segment 1; 40 cycles of 5 seconds at 95°C then decreasing to 60 °C for 30 seconds in segment 2 and 1 cycle of 1 mins at 95°C then decreasing to 55 °C for 30 seconds finally rising back to 95°C for 30 seconds in segment 3.

3.6 FLUOstar OPTIMA microplate reader

Cytotoxicity assay was conducted using this equipment. A549 lung cancer cells were plated in to 96-well plates (8000 cells/well) in F-12K medium with 10% FBS. The medium was removed and washed with PBS after 24 h hours. The solutions containing peptides or Lipo were prepared in RNase free water and diluted by F-12K medium with 10% FBS medium to the concentrations which were the same as those in gene silencing assay. 60 μL of the solution was added to the cells, and 60 μL F12-K medium with 10% FBS was added after 3 hours.

After incubation for 3 and 24 hours at 37 °C in a 5% CO_2 atmosphere, the cultures were removed. 80 μL of F12-K medium with CCK-8 reagent was then added to each well. Cell viability was assessed by measuring the absorbance at 570 nm with the FLUOstar OPTIMA

microplate reader showed above and expressed as the ratio of the treated cells with the carriers over the nontreated cells (negative control).

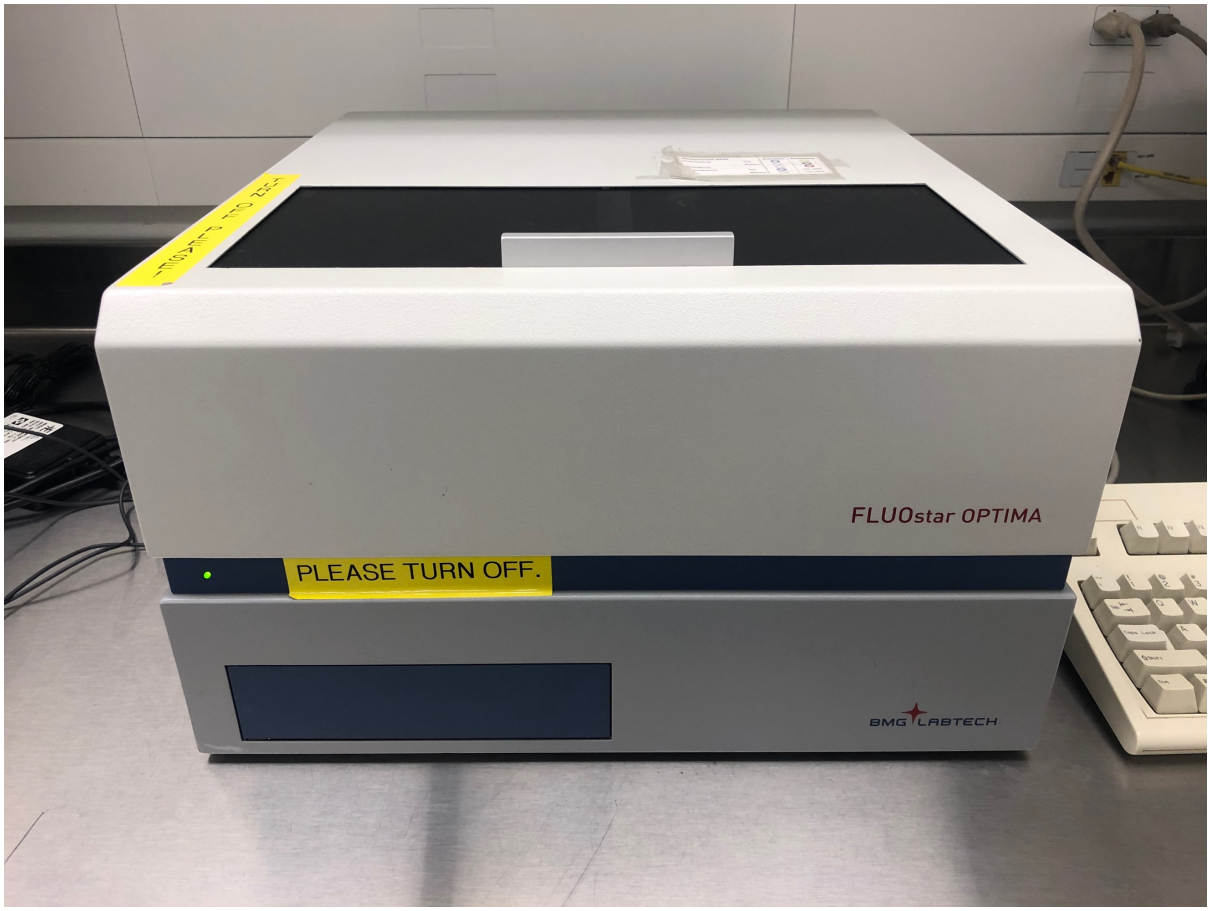


Figure 3.6 Picture of FLUOstar OPTIMA microplate reader in the lab.

3.7 Electrophoresis System

The optimal siRNA/peptide ratio and the qualitative RNase resistance assay along with the Heparin destruction assay were all conducted on this equipment from FisherBiotech. Since the

siRNA and peptides were complexed due to their contrary ionic charges, the optimal molar ratio for the peptide to fully wrap siRNA needed to be determined. Thus, samples of naked siRNA, peg3/peg9/peg18 to GAPDH siRNA on molar ratio of 3:1, 4.8:1, 9:1, 15:1, 30:1 and 60:1, were all loaded on the 1.8 wt%/vol preformed agarose gel stained with gel red and run under 100 constant volts for 1 hour. Then the gel was imaged on ChemiDoc MP Imaging System.

Heparin destruction assay was for the concentration determination of which heparin can disassociate the complexes. 0, 0.5, 2.5, 5 and 10 μg of heparin were added to different peptide to siRNA molar ratio of 15:1, 40:1, 60:1, 80:1 complexes and repeat for peg3, peg9 and peg 18 accordingly. All the samples were loaded and run on the same condition above, and eventually imaged on ChemiDoc MP Imaging System.

Agarose gel retardation assay was performed to examine the stability of siRNA in serum and RNase with and without the protection of peptide. Negative control siRNA at concentration of 5 μM was complexed with peg3, peg9, peg18 and NP1 at molar ratio 60:1 and diluted with RNase free water into 160 μL stock solution, all the stock solutions including siRNA only were incubated with 20% FBS/human serum or 0.08 μg/μL RNase at 37 °C. Every 20ul sample was

taken out from the corresponding stock solution at time 0, 30min, 1h, 2h, 4h, 8h, 12h and 24h and immediately put into -80 °C freezer. Heparin in the concentration of 1 μg/μL was added to the complexes to release siRNA before agarose gel analysis.

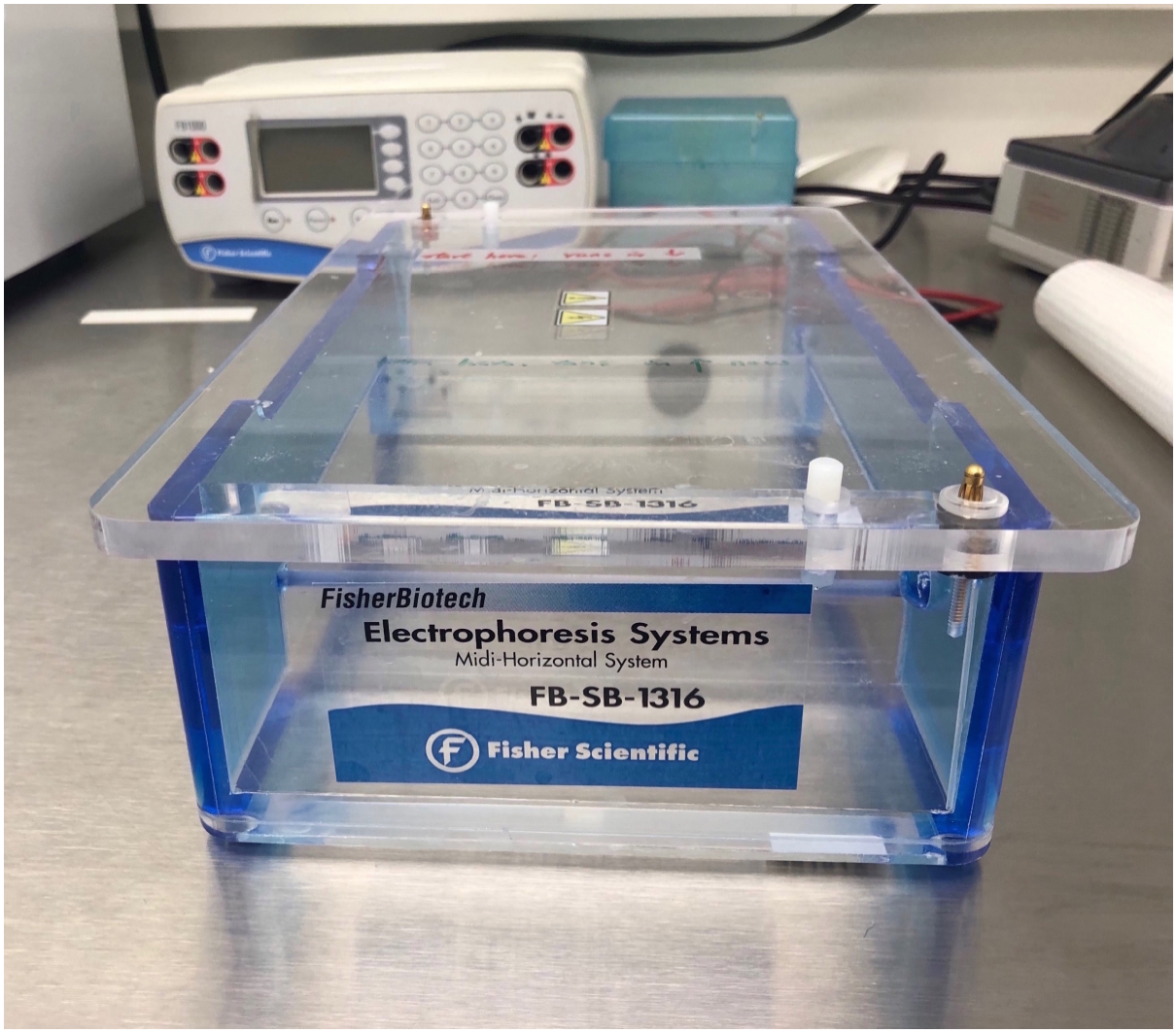


Figure 3.7 Picture of Electrophoresis System in the lab.

3.8 SpectraMax M3 Multi-Mode Microplate Reader

The quantitative assessment of particle stability against RNase along with the MMP2 sensitive linker cleavage assay were both measured by the microplate reader in different modes.

For the RNase resistance, RNaseA (RNaseA: siRNA = 10 ng: 1 μg) was added to the naked siRNA and to peg3/peg9/peg18 siRNA complexes (molar ratio = 60) solution. These solutions were incubated in room temperature at 37 °C for time 0, 30min, 1h, 2h, 4h, 8h, 12h and 24h and immediately put into -80 °C freezer. Before analysis, the siRNA was decondensed

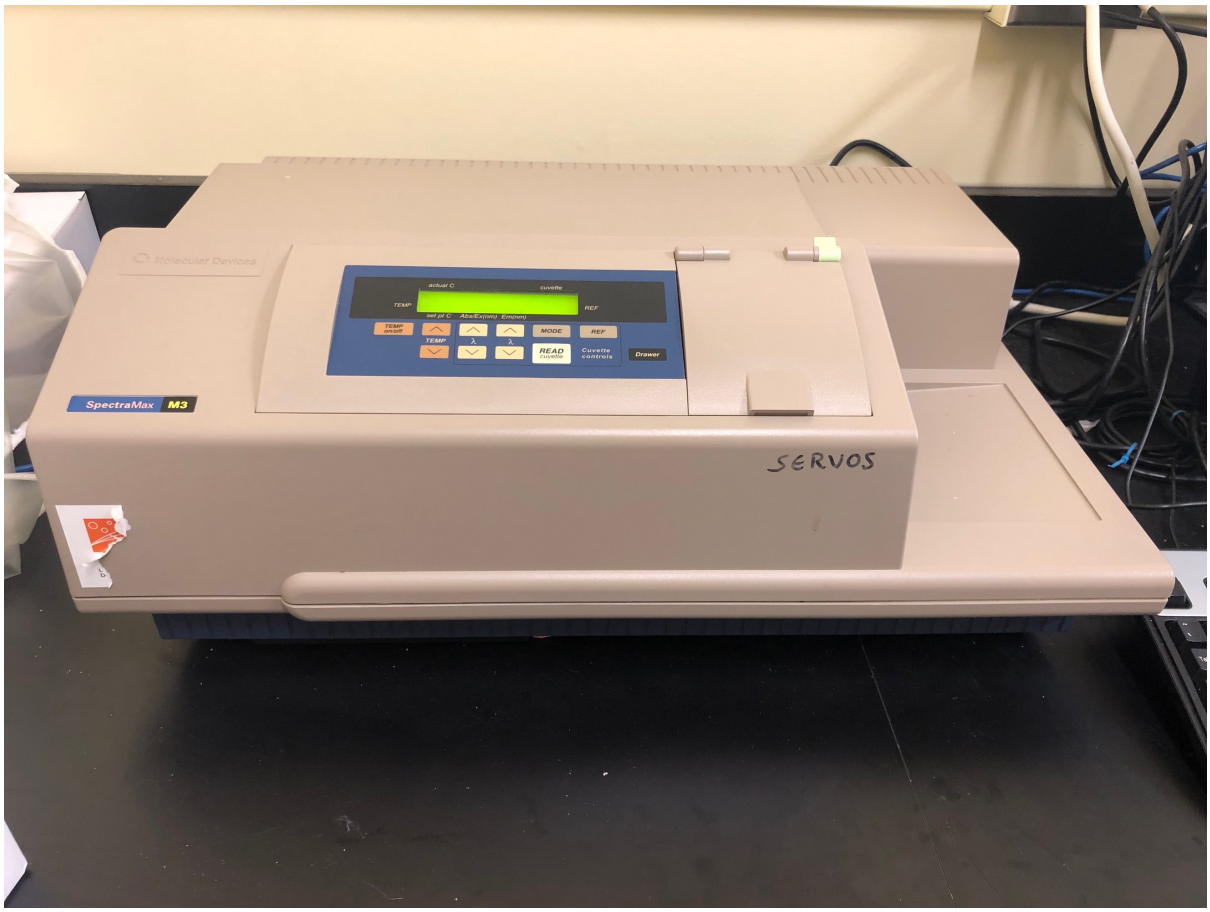


Figure 3.8 Picture of Microplate Reader in professor Mark Servos lab.

by 1 μg/μL Heparin and labeled by SYBR Green. The fluorescence intensity of each sample was collected from 497nm to 520nm by microplate reader. The relative fluorescence intensity result for each point was determined by the fluorescence intensity at 511nm for each point divided by the result at time 0.

Chapter 4

Experimental Results and Conclusion

4.1 Experimental Results

4.1.1 Structure of the peptide carrier after modification

The modified peptide carrier was designed utilizing NP1 as the basic structure. The end opposite to the stearyl acid modification was linked with the MMP2 sensitive amino acid sequence then conjugated with PEG in the outmost layer forming a final structure of Stearyl-HHHHHHHHHHHHHHHHRRRRRRRRRGPLGIAGQC-PEG. The thiol side group in amino acid cysteine was used as PEG coupling reaction site. Considering the hydrophilic-hydrophobic property of the entire particle after complexed with siRNA, the PEG, acts as a hydrophilic tale, cannot be too large as the complex will be outbalanced by the hydrophilicity leading to an unstable NPs. In this circumstance, the PEG was designed in different molecular weight, which were Peg3 (MW=4519.21), Peg9 (MW=4783.54) and Peg18 (MW=5180.02). All the peptides were purchased from WuXi App Tec (Shanghai, China). High performance liquid

chromatography (HPLC) analysis indicated that the synthetic peptide was 95% pure. Fig. 4.1 presents the schematic structure of PEG series peptide and the expected performance.

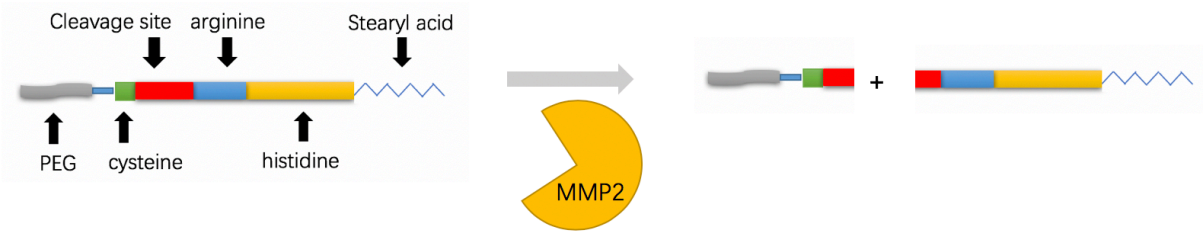


Figure 4.1 Schematic structure of PEG modified peptide and its expected performance.

4.1.2 Optimal modified peptide/siRNA ratio determination

Since the modified peptide peg3, peg9 and peg18 were elongated by several amino acid and PEG polymer chain, the optimal molar ratio for NP1 to fully protect siRNA may have been affected. So the agarose gel electrophoresis assay stained by gel red was used to determine the new molar ratio. Naked siRNA, peg3/peg9 and peg18 with GAPDH siRNA at molar ratio 3:1, 4.8:1, 9:1, 15:1, 30:1 and 60:1 were all tested at the same time. Gel red is an nucleic acid stain that will fluoresce with an orange color that strongly intensifies after binding to DNA. Similar appliance to RNA as DNA, the more well protected siRNA, there will be a decrease of fluorescence on the image. Surprisingly, the results showed that it was still 60:1 molar ratio group in different PEG modified groups showed the best condition that no signal was imaged

on the gel in or outside the well, indicating full coverage of siRNA. From the results, it can be concluded that the best molar ratio for peg3/peg9/peg18 to siRNA was still 60:1 since they complexed through electrostatic interaction and either the additional 9 amino acids nor PEG increased the positive charge overall. Under this situation, all the complexes were formed under the peptide to siRNA molar ratio of 60:1 in the entire research.

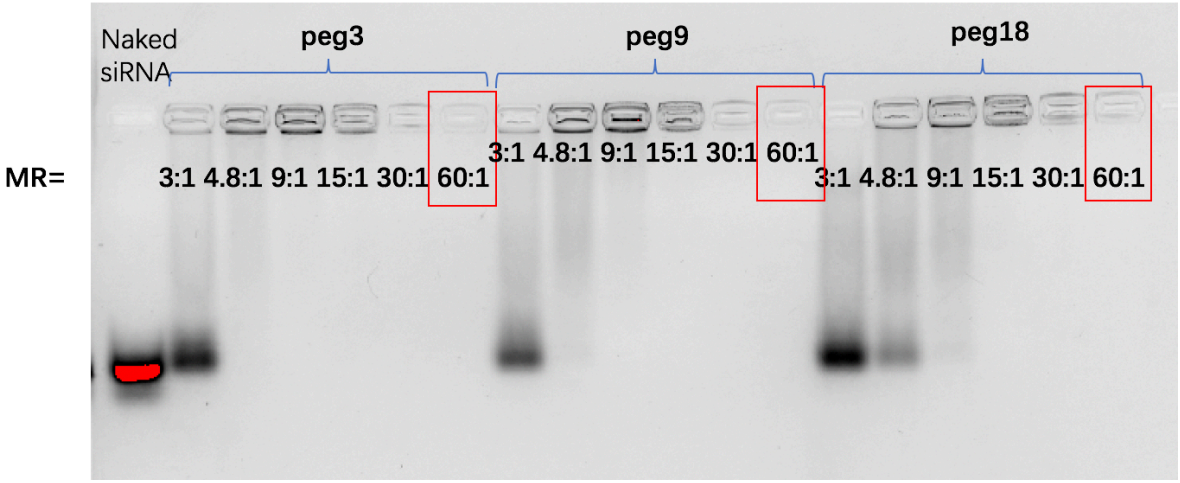


Figure 4.2 Image of agarose gel stained with gel red for optimal PEG modified peptide/siRNA molar ratio determination.

4.1.3 Particle size and zeta potential

The particle size and surface charge significantly affect nanoparticle circulation in the bloodstream, biodistribution, and uptake by the cells. It would be ideal for particle size ranging from 100 to 500 nm to target solid tumors based on the enhanced permeability and retention

(EPR) effect [136]. Therefore, those nanoparticles posses appropriate physical properties could remarkably enhance its therapeutic effect. The particle size and zeta potential of peg3/peg9/peg18/NP1 complexed with GAPDH siRNA were studied.

Table 4.1 Particle size of the modified peptide/siRNA complexes determined by DLS (n = 3) at time 0 and time 30 mins.

time	Name	Size (nm)	PDI [a]
Right after complexed	Peg3	448 ± 23	0.39 ± 0.02
	Peg9	282 ± 3	0.32 ± 0.10
	Peg18	272 ± 1	0.24 ± 0.01
	NP1	360 ± 15	0.48 ± 0.05
After 30 mins stabilization	Peg3	309 ± 50	0.31 ± 0.10
	Peg9	265 ± 20	0.21 ± 0.01
	Peg18	239 ± 5	0.17 ± 0.01
	NP1	141 ± 5	0.31 ± 0.01

a. PDI: Polydispersity index.

The peptides were complexed with GAPDH siRNA at molar ratio=60:1, then immediately measured, and measured once again after 30 minutes of stabilization in MilliQ water at room temperature. As shown in the particle size results in table 4.1, all the samples were below 500 nm which indicated they all appropriate for delivery size-wise. It's clear that the size were changed after 30 mins of stabilization since the peptide and siRNA were complexed through weak force — ionic interaction, as they became more uniform as the intensity and number-based DLS results showed the average size between 150 and 300 nm.

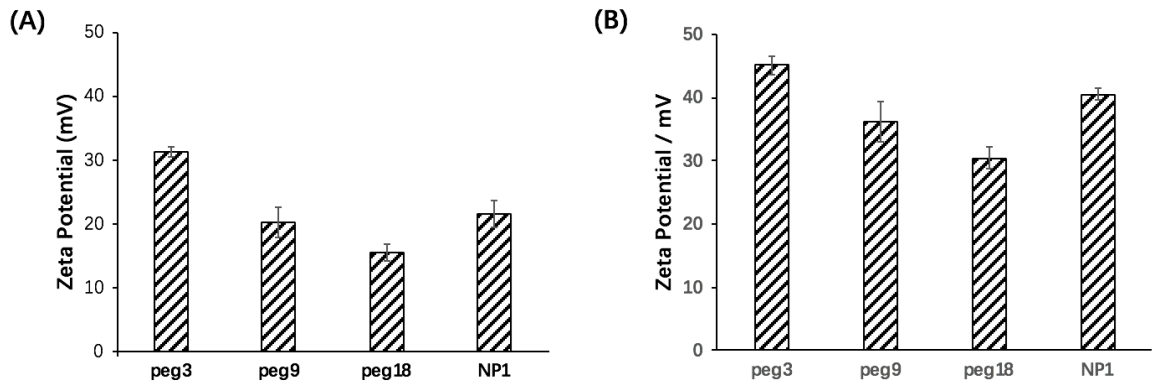


Figure 4.3 Zeta potential of peg3/peg9/peg18/NP1 GAPDH siRNA complexes at molar ratio 60/1 right after complexed (A), and after 30 minutes stabilized (B) in MilliQ water at room temperature.

Modified Peptide peg3/peg9/peg18 and NP1 were complexed with GAPDH siRNA at molar ratio=60:1, then measured for zeta potential immediately and once again after 30 minutes of stabilization in MilliQ water at room temperature. Zeta potential reflects the surface charge for the complex. It is crucial for nanoparticles to have net positive surface charge since it inhibits particle aggregation and enhances electrostatic interaction with the negatively charged phospholipids of the cell membrane upon siRNA delivery.

All the samples showed net positive charge upon contact, which meant that the peptides fully enwrapped siRNA and covered the surface of the complex immediately. After 30 mins of incubation, the zeta potential averagely increased 15mV in all the samples. This may due to more positively charged arginine residues being exposed to the environment through the stabilization process.

4.1.4 MMP2 existence

The MMP2 secretion by A549 cancer cells was testified by running SDS-PAGE gels. 20 μL of 1 μg/μL bovine serum albumin (BSA), which is band 1 shown in figure 4.4, was served as a quantity standard. Band 2 and 3 were 100 times dilution solution from the concentrated

culture medium collected after 2 days of cell seeding along with 50 times dilution solution of band 4 and 5. Band 2 and 4 were the F12K with 10 percent FBS cultured medium with cell growing in the system and band 3 and 5 were the same incubation condition only containing same culture medium with no cell seeding. Since secreted MMP2 has the same molecular weight around 66kD as one of the main proteins — BSA in FBS, in the same dilution level, there was a clear protein band showing at 66kD regardless of the existence of cells. However, there is an obvious difference in band width, which indicated the protein quantity difference, between the group with and without cells. The difference in protein quantity was the amount of MMP2 secreted by A549 cells comparing to the corresponding no-cell environment.

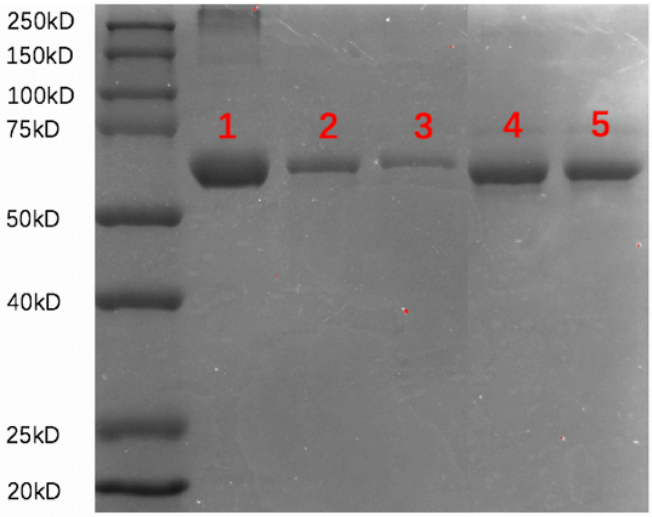


Figure 4.4 MMP2 levels in cell culture medium indicated by SDS/PAGE gel.

Table 4.2 Quantity analytical results of MMP2 level in cell culture medium.

Name	BSA (band 1)	100X dilution		50X dilution	
		With cells (band 2)	Without cells (band 3)	With cells (band 4)	Without cells (band 5)
Band intensity	100%	30%	20%	73%	61%
Absolute quantity	20μg	N/A	N/A	N/A	N/A
Loading volume	20μL	20μL	20μL	20μL	20μL

Setting the BSA band as reference, the relative quantity results of each band were directly analyzed by quantity tools in image processing software from Bio-Rad (table 4.2). The numerical results, which showed a higher relative quantity of 0.1 in cell-present sample compared to the corresponding system without cells.

4.1.5 MMP2 sensitive linker cleavage

The cleavage of the MMP2 sensitive amino acid linker has been tested by thin layer chromatography (TLC).

The cleavability of peg3, peg9 and peg18 was evaluated by enzymatic digestion. Briefly, 2.5 mg/mL peg3/peg9/peg18 was incubated with active human MMP2 (~5 ng/μL) in pH 6.4

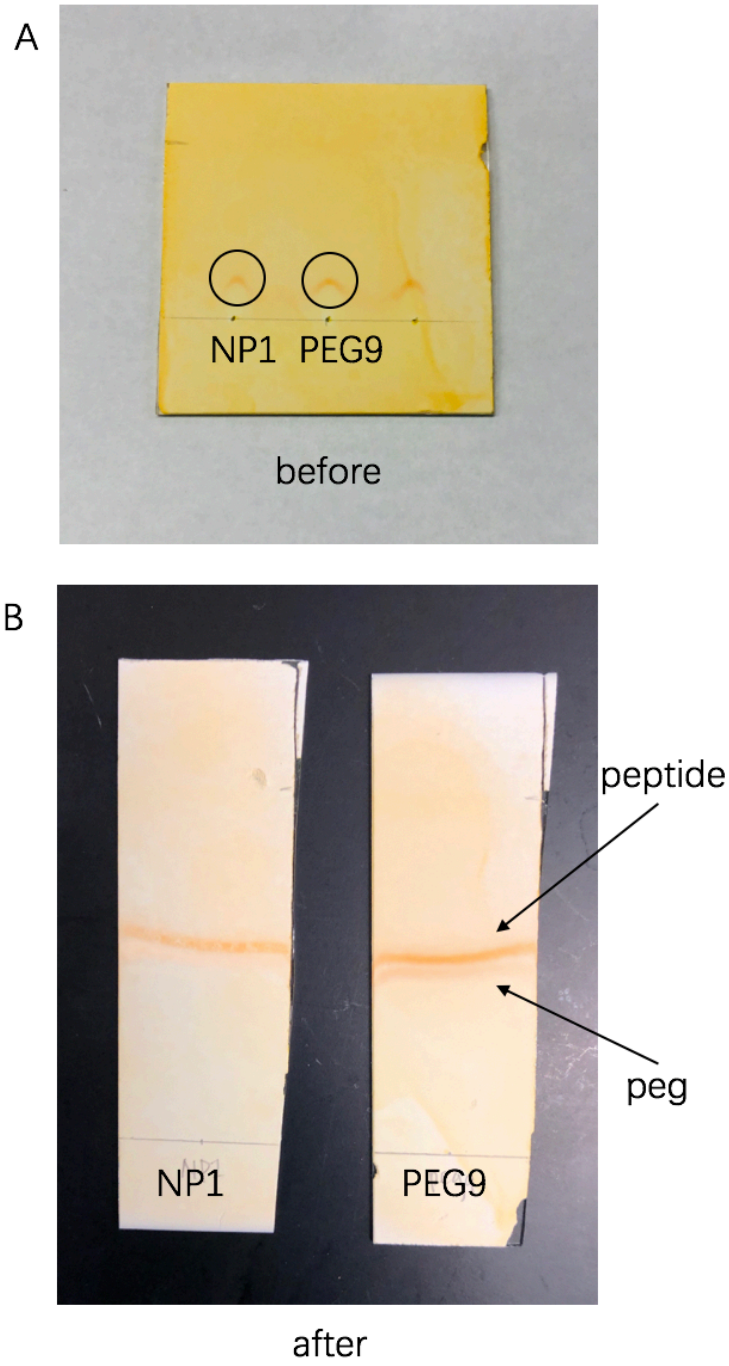


Figure 4.5 TLC results for MMP2 sensitive linker cleavage assay before adding MMP2 (A), and after incubation overnight at 37 °C with MMP2 (B).

HBS containing 10 mM CaCl2 at 37 °C overnight. In parallel, the unmodified NP1 was incubated in the same condition and later checked by TLC and visualized by Dragendroff's staining. The reaction mixture was analyzed by TLC (chloroform/methanol/acetone, 8:1.2:0.8, vol/vol) before adding MMP2 and after incubation (chloroform/methanol/acetone, 8:1.5:0.5, vol/vol)followed by Dragendroff reagent staining. In Fig. 4.4a, there is only one dot in both NP1 group and peg9 group before adding MMP2 which indicated that only one chemical was present in the solution. After being incubated with MMP2 overnight at 37 °C (Fig. 4.4b), there was only one line in the NP1 group and one clear line parallel with a faded line in peg9 group. Since the sensitive linker has been successfully cleaved by MMP2, there were 2 chemicals in the solution which were PEG and peptide on the contrary to NP1 group with only peptide due to the absence of the sensitive linker.

NOTE: the line shape in the after incubation results, which may due to the high solubility of peptide and PEG, should be 2 dots in peg9 group and 1 single dot in NP1 group. However, after adjusting the eluent composition for over 30 times, the results still stayed as a line shape or even disappeared from the whole plate in worse cases.

4.1.6 Cellular uptake

The efficiency of PEG modified peptides to deliver siRNA in 500 nM into A549 lung cancer cells in serum environment was evaluated using FACS. As shown in Figure 4.5, cellular uptake efficiency of siRNA was outstanding for lipofectamine group, which has the broadest

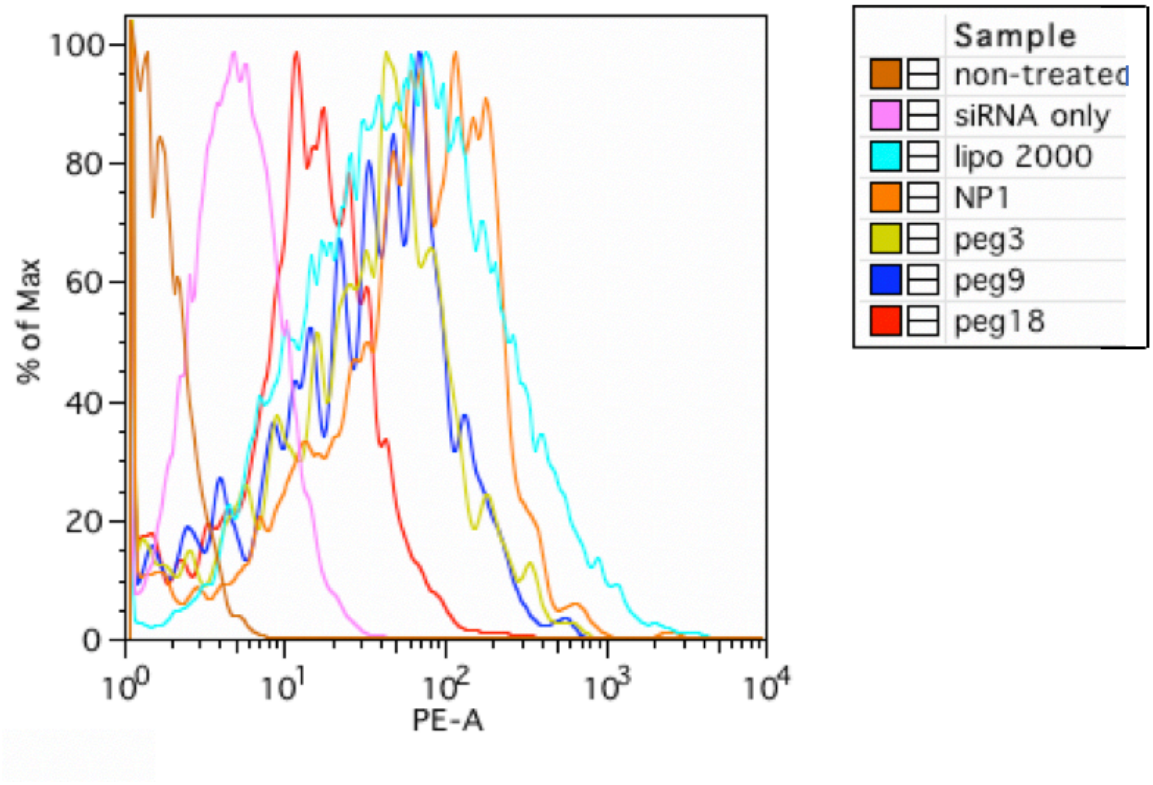


Figure 4.6 Flow cytometry results for Cy3-labeled siRNA 500 nM delivered by NP1, peg3, peg9 and peg18 at molar ratios of 1:60 in A549 cells. Lipofectamine 2000 served as positive control.

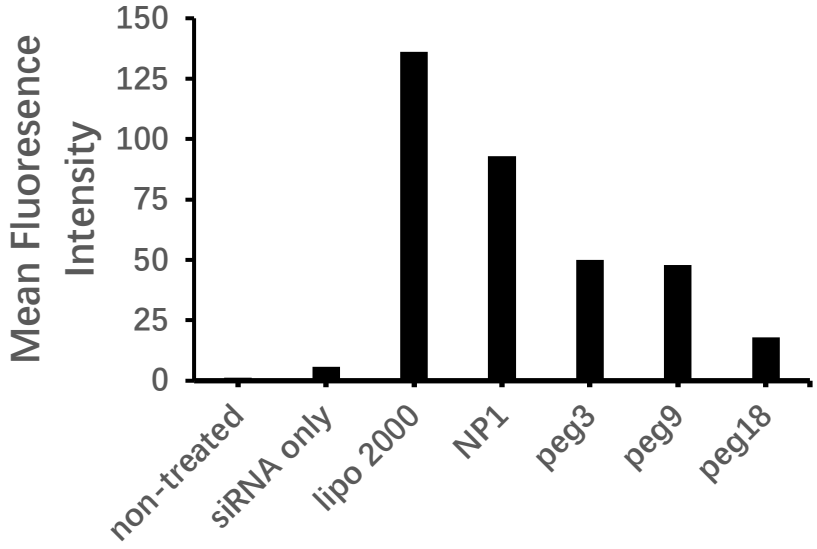


Figure 4.7 Mean fluorescence intensity of Cy3-siRNA (500nM) complexes formulated with NP1, peg3, peg9 and peg18 at 3 h after the treatment of A549 cells.

fluorescence peak, and followed by the NP1 group. However, the uptake efficiency for peg3/peg9 siRNA complex showed only one third of the mean fluorescence intensity (Fig.4.6) comparing with unmodified NP1, while the uptake for peg18 complex was even worse.

This unexpected results may due to the hinderance of the PEG in the outmost layer. Although it significantly shelter the particles from degradation, it prevents the oligoarginines, which can bind to the cell surface with high affinity [137], from exposure to the cell membrane.

4.1.7 Gene silencing

As GAPDH siRNA uptake for PEG modified peptide cannot be as effective as NP1 or lipofectamine group for A549 cells, the results for proving whether the delivered siRNA is able to perform RNAi and decrease the expression level of GAPDH mRNA is considerably important.

The knockdown efficiency was first tested by delivering 100 nM of GAPDH siRNA using modified peptide carrier to A549 cells in the environment without serum. In figure 4.7 of 2 times of transfection experiments, no obvious superiority can be found in the modified peptide group comparing with the unmodified NP1. Moreover, they seem even little weaker in gene silencing performance but relatively uniform in different times typically for peg3 and peg9 group.

The knockdown efficiency was then tested by delivering 100 nM, 300nM and 500 nM of GAPDH siRNA separately using the modified peptides into A549 cells in the environment with the presence of serum. The GAPDH siRNA in different concentrations were complexed and transfected with peg3, peg9, peg18 and NP1 into A549 cell in F-12K with 10% FBS medium using the protocol stated in chapter 3 section 4.

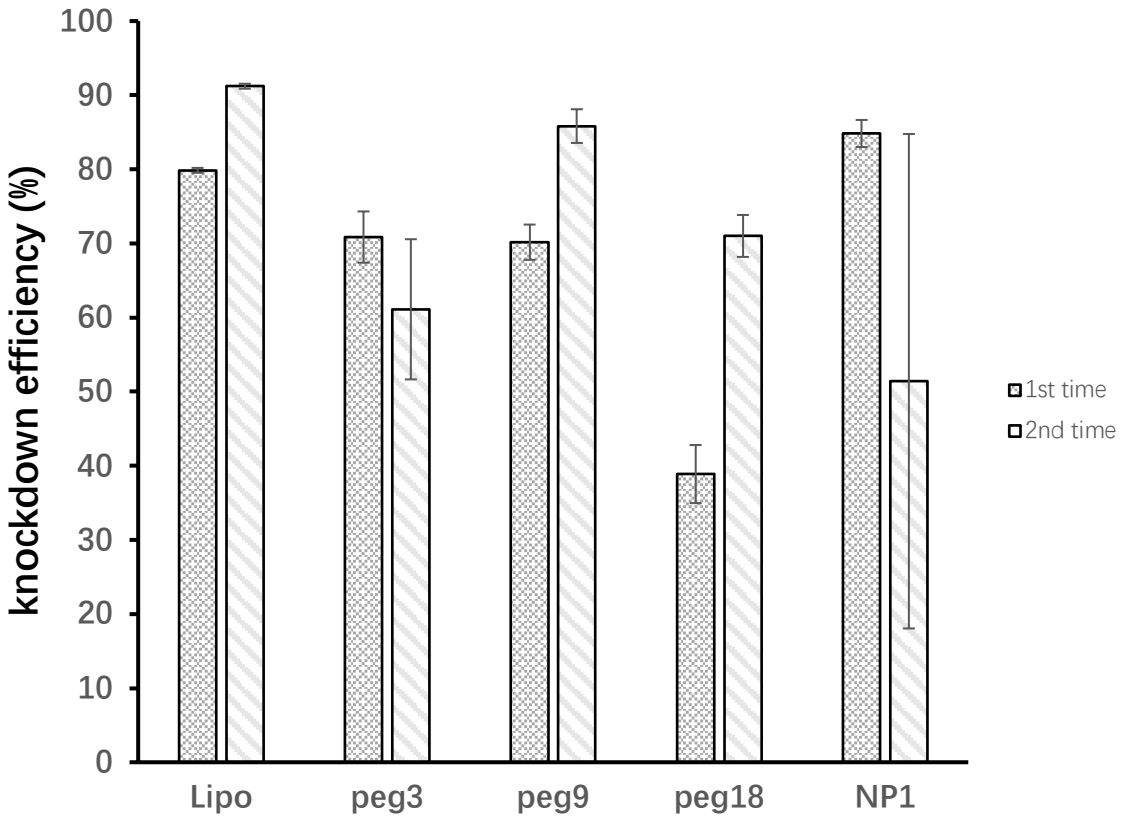


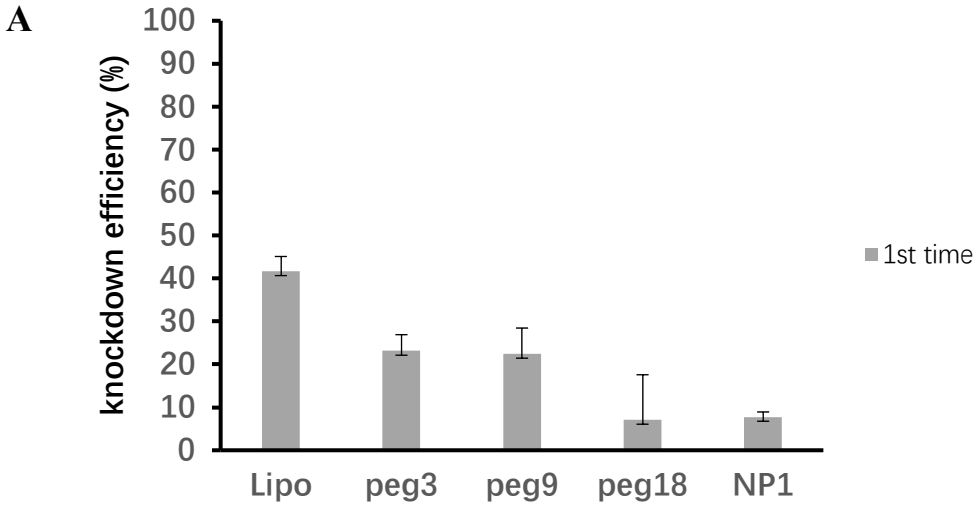
Figure 4.8 Gene silencing efficiency of PEG modified peptide/siRNA complexes and NP1 on A549 cells. Relative GAPDH mRNA level in A549 cells after transfected in OPTIMUM medium was measured by qRT-PCR method. All the data were normalized to another house-keeping gene cyclophilin.

In Figure 4.8, as the concentration of GAPDH siRNA increasing from 100 nM to 500 nM, smaller distinction in knockdown efficiency and larger difference in result consistency was noticed. Starting from the siRNA concentration of 100 nM, which was the same concentration

in the transfection experiment without serum, all the group showed unsatisfactory results due to the substantial severity of serum environment. The PEG modified groups only had roughly 10 percent higher in the knockdown result than the NP1 group.

As the siRNA concentration rising to 300 nM, the advantage in particle stability with PEG modification became more prominent. Although the knockdown efficiency was still not conspicuously overtop the NP1 group with only 20 percent higher in value, the uniformity of the results in 2 transfection experiments obviously precede than NP1 group.

This situation was noticed in the transfection experiments of 500 nM siRNA as well.



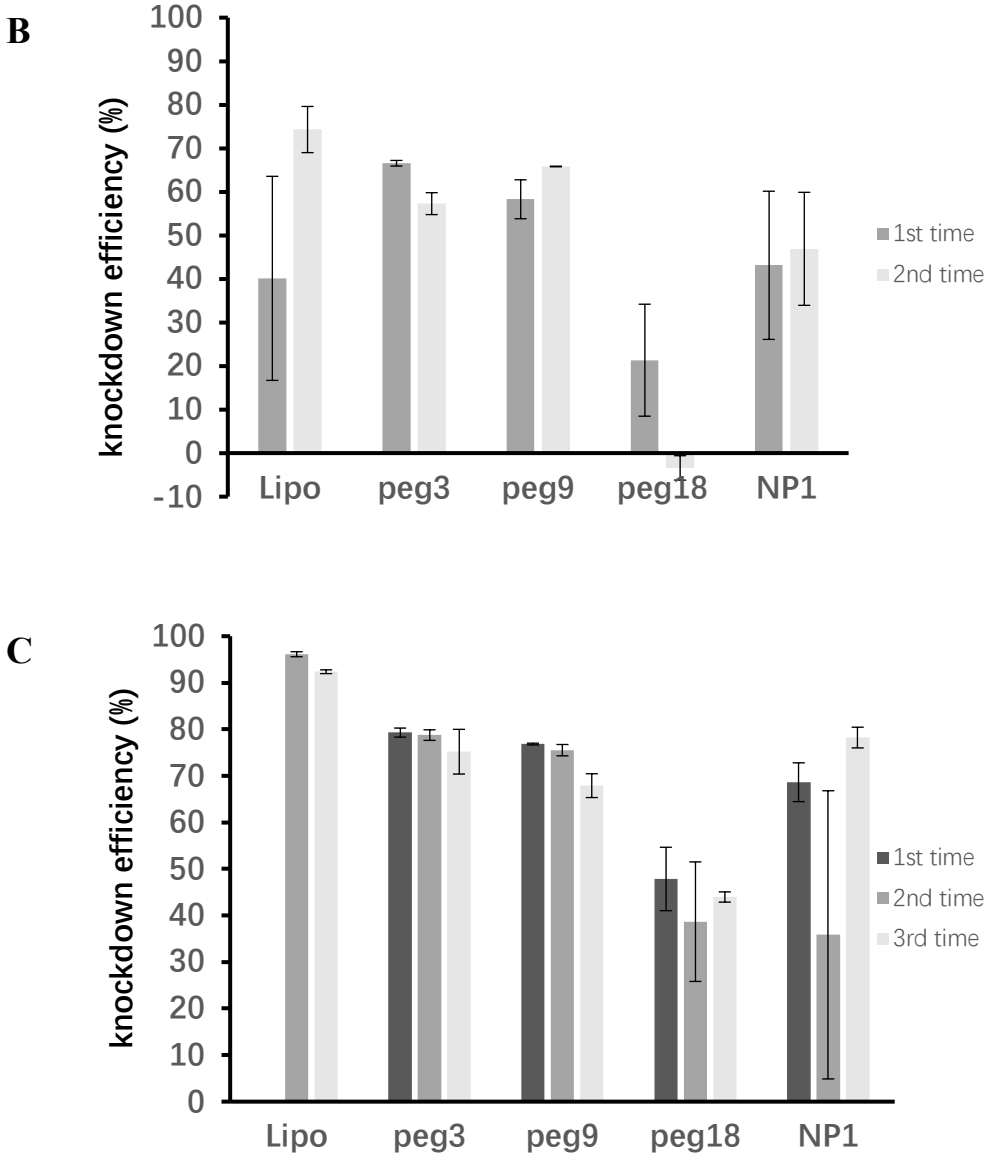


Figure 4.9 Gene silencing efficiency of 100 nM (A), 300 nM (B) and 500 nM (C) GAPDH siRNA delivered by PEG modified peptides and NP1 on A549 cells. Relative GAPDH mRNA level in A549 cells after transfected in F-12K with 10% FBS medium was measured by qRT-PCR method. All the data were normalized to another house-keeping gene cyclophilin.

In the combination of cellular uptake results in previous section, the PEG modified group, especially peg3 and peg9, was shown to achieve the same knockdown efficiency but in a more stable pattern among 3 times of transfection experiments with only one third of the cellular uptake compared to the NP1 group. This result strongly indicated the superiority in gene silencing of PEG and the smart linker modification towards the original unmodified NP1.

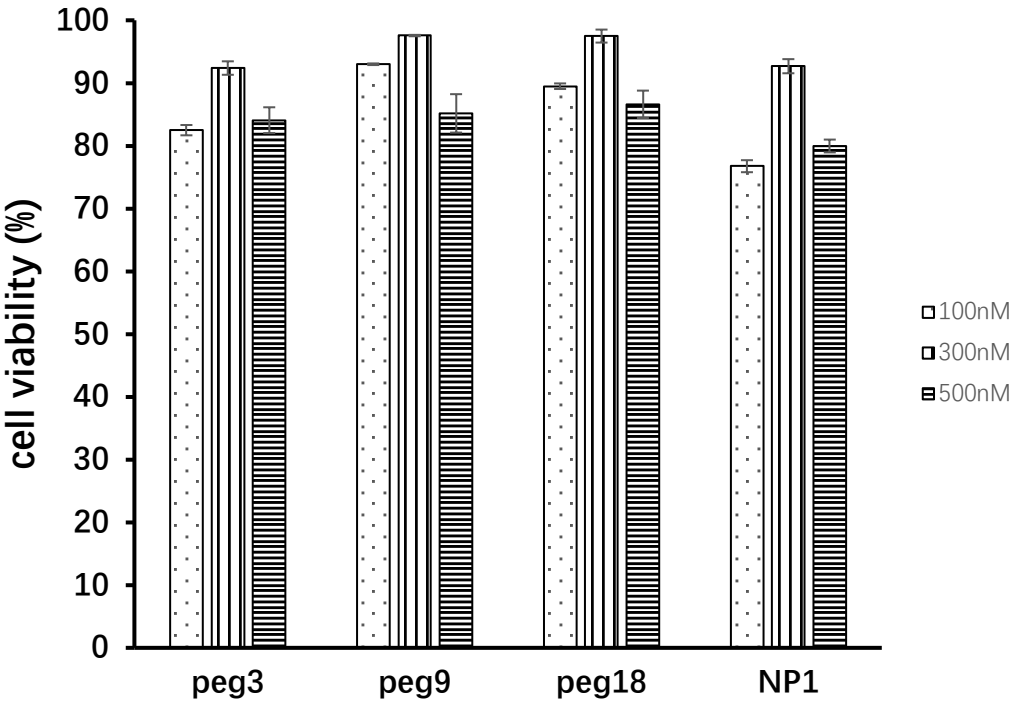
4.1.8 Cytotoxicity

CCK8 assay was performed to evaluate the cytotoxicity of peg3, peg9 and peg18 siRNA complexes comparing with NP1/siRNA complexes at various siRNA concentrations at 100 nM, 300 nM and 500 nM. As shown in Figure 4.9A, cell survival was not significantly impacted by the treatments of every group after 3 hours incubation. All of the experiment group showed more than 80% cell viability. There is no clear trend neither between the siRNA concentration to the cell viability nor the difference in molecular weight of PEG modification to the cell survival percentage in the result.

However, observing from the cell viability results after 24 hours incubation, the cytotoxicity level in PEG modified groups were apparently lower than NP1 group , especially

in higher siRNA concentration of 300 and 500 nM. The average lead in cell viability from peg3 or peg9 was around 70 % over the NP1 group.

A



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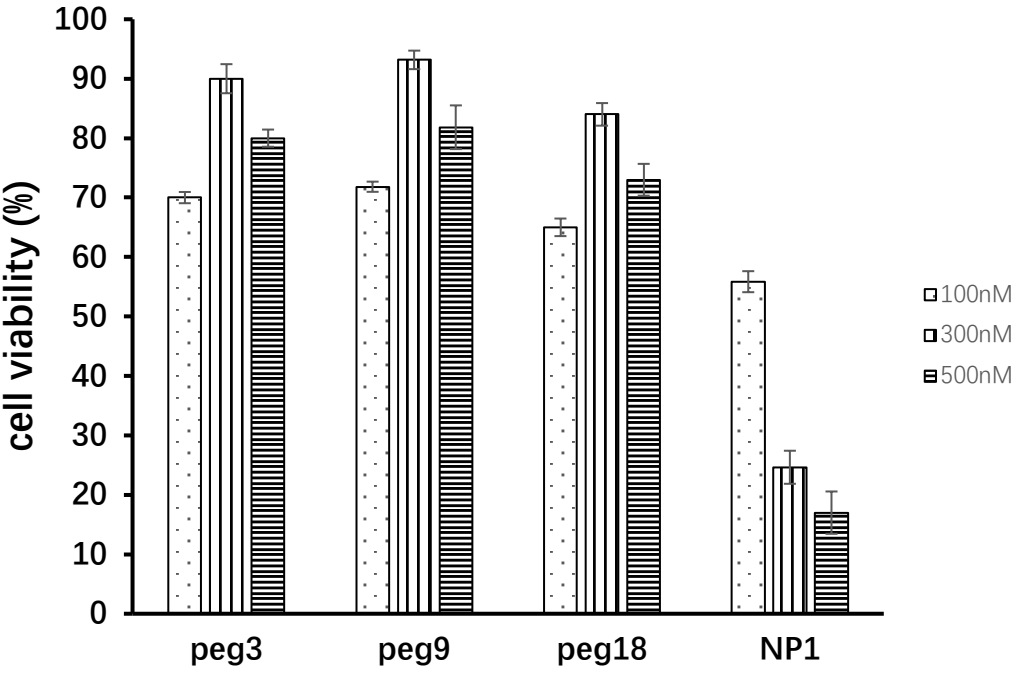


Figure 4.10 Cytotoxicity assay of peg3, peg9, peg18 and NP1 complexed with GAPDH siRNA was conducted in A549 cells in different siRNA concentrations after 3 hours (A) and 24 hours (B). Results correspond to the average of three separate experiments and normalized to untreated cells cultured in the same condition.

4.1.9 Heparin destruction

Heparin competition assay was used to examine the stability of the formed complex in the presence of heparin. Peptide can interact with siRNA through noncovalent interactions such as hydrogen bonding and Coulombic forces. In particular, the peptide riches in arginine and histidine carries positive charges and can interact with the negatively charged phosphate groups on the siRNA sugar rings through electrostatic interactions. When the negatively charged heparin introduced to the environment, it will compete with siRNA to bind with the positively charged peptide eventually result in the complex disassociation. The freed siRNA molecules could move toward the positive electrode when the voltage is applied, whereas the inability of peptide/siRNA complexes to be seen in agarose gel suggests the formation of stable complex with no free siRNAs to be shown under imaging.

The stability of peg3/siRNA complexes at different molar ratios in the presence of heparin was analyzed by agarose gel electrophoresis. As shown in Figure 4.10, peg3/siRNA complexes were stable in the absence and 0.5 μg heparin per 10 μL samples (first and second well from

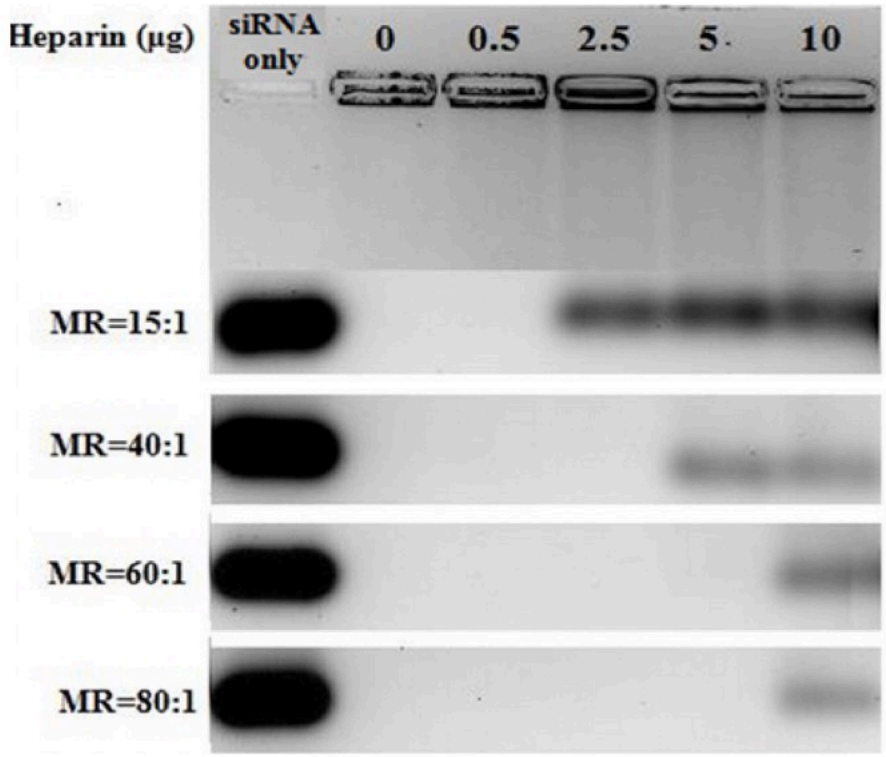


Figure 4.11 Stability of peg3/siRNA complex demonstrated by heparin competition assay. Different amounts of heparin corresponding to final concentrations of 0 to 10 μg heparin per 10 μL of complex were added to peg3/siRNA complexes solution at different molar ratios. The stability of complexes was analyzed by electrophoresis on agarose gel (1.8 wt %/vol) stained with gel red. For better comparison, only the desired siRNA bands from four independent gels were put in the same image.

left), and no free siRNA was shown in siRNA bands at all molar ratios (MRs). As the MR increased from 15:1 to 40:1, it was clear that 2.5 μg heparin per 10 μL could not destruct the complex any more since no siRNA band was seen, and the band showed up when the heparin concentration raised to 5 μg per 10 μL of sample. However, as the MR reached 60:1 and even higher to 80:1, the complex both remained to be completely stable under the heparin concentration of 5 μg per 10 μL of sample and dissociated at higher heparin concentration (10 μg in 10 μL of loaded sample) which demonstrated that the highest heparin concentration of 10 μg in 10 μL of sample wass already saturated for particles to deconstruct in both MRs. Thus, 10 μg of heparin in 10 μL of sample, which had a concentration of 1μg/μL in the final solution, were determined and applied for further particle destruction experiments.

4.1.10 Particle stability in serum/RNase (qualitative assessment)

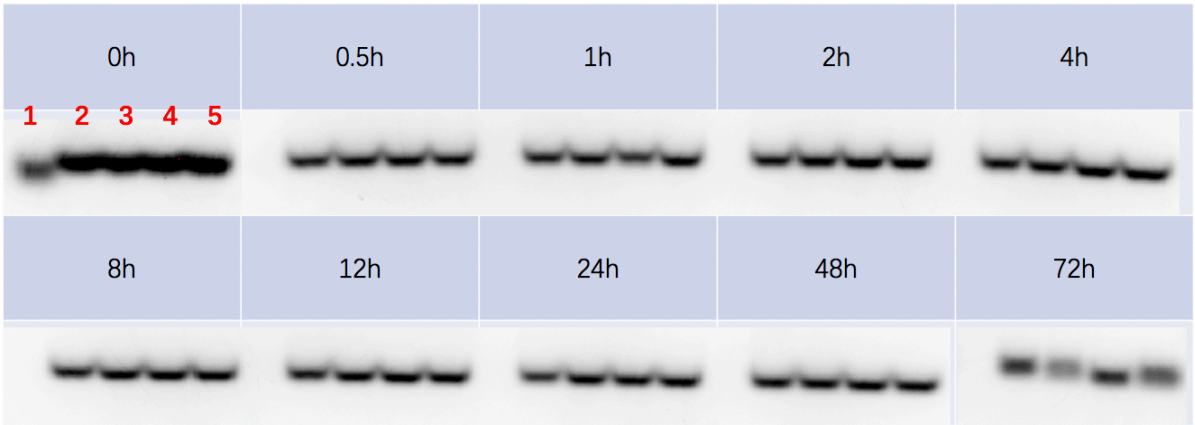
The 3 different PEG modified peptide and NP1 were complexed with siRNA at molar ratio 60:1 for particle stability assay by gel electrophoresis. Naked siRNA group and NP1 group were served as comparison. The color unevenness of siRNA bands shown in Figure 4.11

indicated the condition of siRNA protection by peptides — the lighter the color indicates the less of the remaining siRNA.

All the groups with peptide protection showed significant siRNA existence among 72 hours incubation in opposite to the rapid degradation of naked siRNA group under the environment containing 20% of human serum, yet there was no clear intra-group difference between modified and unmodified peptide with PEG. (Figure 4.11B)

In the degradation experiments by 20% FBS or RNase, siRNA bands all showed various degrees of fadedness after 24 hours, especially in those that were incubated with RNase. The naked siRNA degraded at the contact with RNase immediately, while the siRNA showed approximately half remaining after 72 hours incubation with the peptide protection and even better with the protection from the peptides modified with PEG.

A



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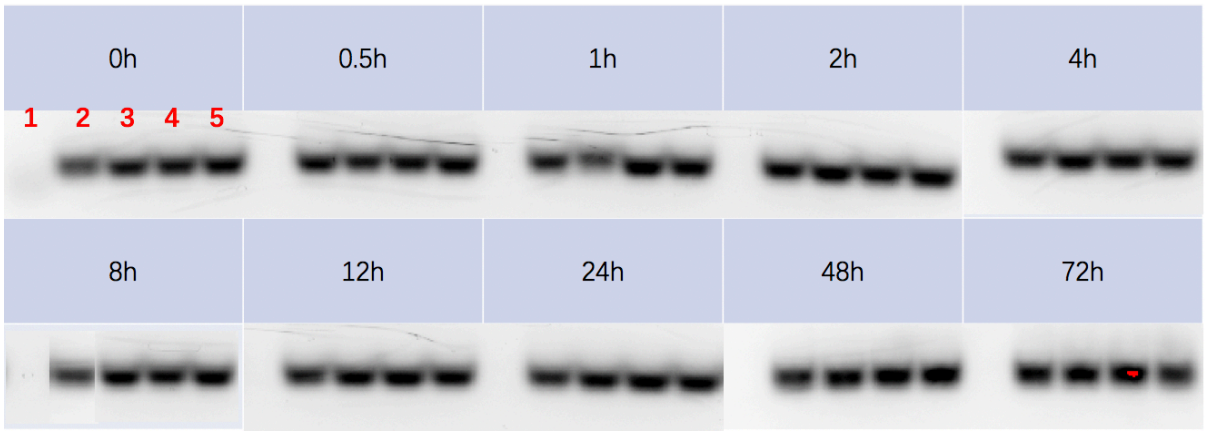


C

Figure 4.12 Qualitative assessment of the stability of siRNA in FBS (A), human serum (B) and RNase (C) with and without the protection of peptide by agarose gel retardation assay. The samples from left to right were naked siRNA, NP1, peg3, peg9 and peg18 consecutively.

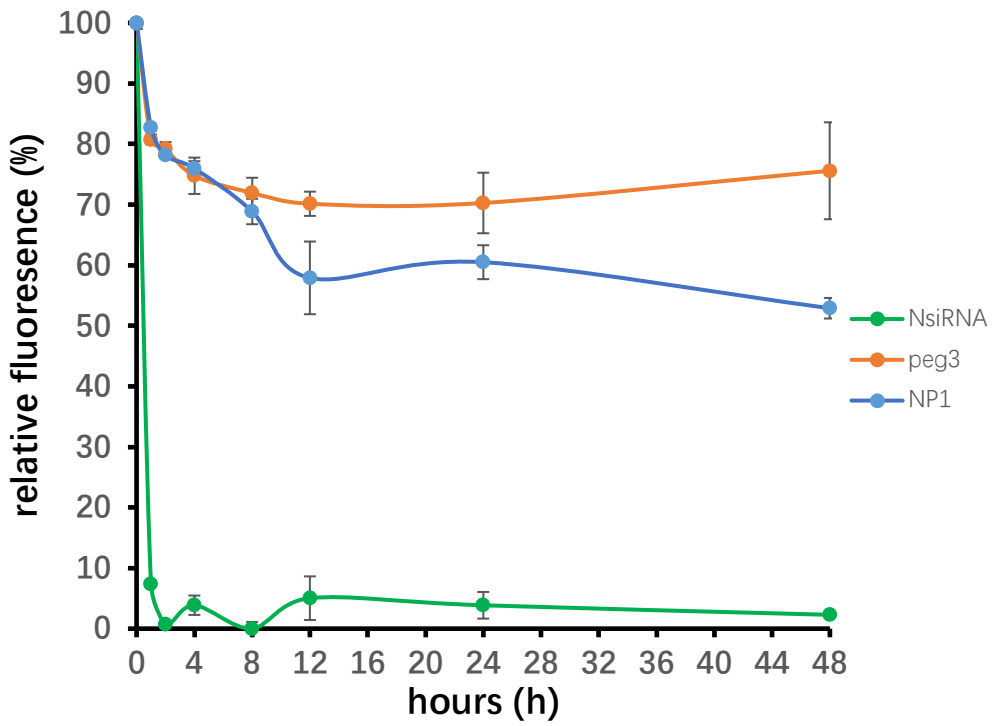
Overall, although there was a slightly difference between peg modified groups and the NP1 group, they can both protect siRNA from degradation in a certain degree after 72 hours.

4.1.11 Particle stability in RNase (quantitative assessment)

Particle stability can be quantified by measuring the fluorescence intensity of SYBR Green labelled siRNA after RNase degradation. The higher intensity, the more remaining siRNA, which directly indicated a better protection provided from the peptide. The relative fluorescence of each sample was calculated by the fluorescence intensity of each time divided by the initial fluorescence intensity at time 0. NP1 and NsiRNA (naked siRNA) were served as comparison. The expected trend for all samples should be a smooth gradual decrease line, however the bumpy lines shown in Figure 4.12 may due to the experimental errors in each sample caused by the following process. A total volume of 200 μL different complexes were prepared individually and aliquoted into 20 μL for each time measurement, the complexes may not have been homogenously distributed in the stock solution which may have been attributed to the slightly quantity difference of complexes in each small portion. Moreover, the percentage of siRNA that was released from the peptide after adding heparin was also slightly different in each sample, in spite of the fact that heparin quantity has been proven to be excessed by heparin destruction assay. However, despites of these uncertainties, there is a clear trend in the peg3, peg9 and peg18 group compared to the NP1 group. The complexes with PEG protection

showed a steady relative fluorescence percentage which was around 75% after 48 hour incubation with RNase in the contrast to a decrease trend in NP1 group without PEG modification. As expected, the naked siRNA was rapid degraded in the presence of RNase.

A



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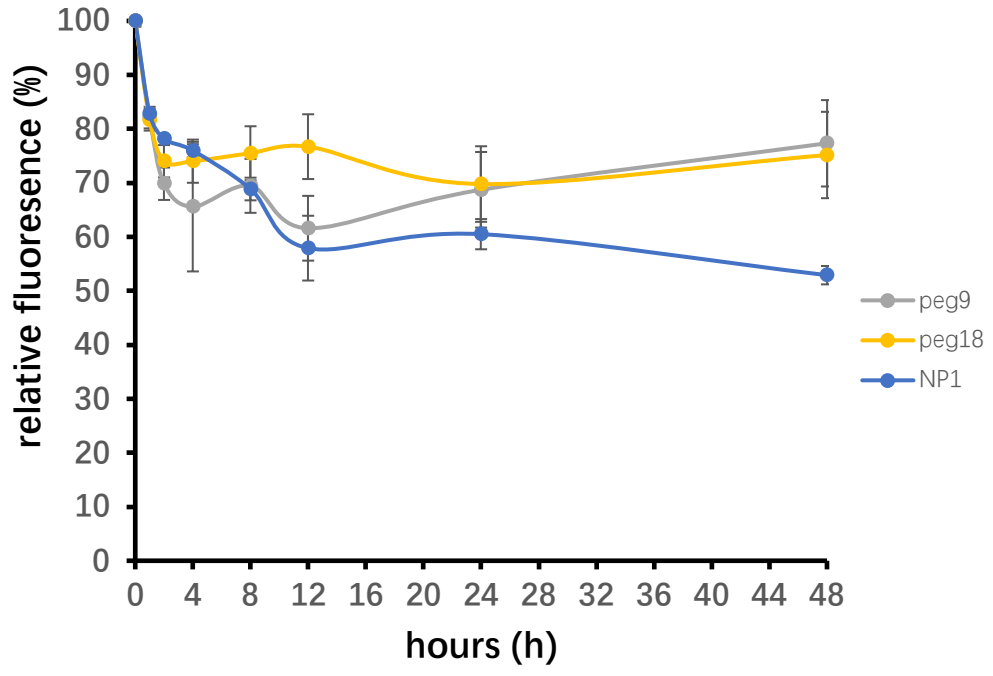

Figure 4.13 RNase resistance comparison of (A) naked siRNA, PEG3 and NP1 with siRNA complexes, (B) peg9, peg18 and NP1 with siRNA complexes. The complexes were prepared by incubation at room temperature for 30 min. All the data point stated were the average of 3 times repeat experiments.

4.2 Discussion and Conclusion

In this study, matrix metalloproteinase 2 sensitive amino acid linker has been utilized in cell-penetrating peptide modification in the purpose of improving the particle stability while maintaining the transfection efficacy of the cargo in the present of serum. The modification was based on the original structure of peptide called NP1 which has been previously developed in our group for years. In the opposite to the hydrophobic end modified with stearyl acid in NP1, the 8 specific amino acids in the sequence of GPLGIAGQC and PEG were consecutively coupled to the existing structure. PEG can prevent the peptide/siRNA complexes from serum degradation and enhance passive tumor targeting before entering the cells, also de-shield the complex upon MMP2 contacting before linker cleavage. Due to relatively higher MMP2

secretion and accumulation in tumor site, the design was realized in A549 lung cancer cells in this work.

As for the physical property of the complex, including the particle size and zeta potential, the PEG modified peptide were adequate for siRNA delivery comparing with NP1 with an average overall size of 150 nm-300nm and positive charged surface. The size was qualified for the complexes undergo EPR effect spontaneously and the cationic surface facilitated the penetration across the cellular membrane.

The MMP2 cleavage essay through TLC examination has indicated the success of PEG detachment from the peptide backbone as expected, however, the actual situation of linker breakage in the transfection process cannot be monitored. Thus, whether all the linker were fully cleavage by MMP2 and let all peptide/siRNA complexes exposed to the cell membrane remained unknown. The un-cleaved particles which may not successfully release siRNA since the hinderance from PEG or even haven not been endocytosed by the cells could lead to an incomplete transfection efficiency.

Taking consideration of the concern, FACS has been performed in order to acquire the

particle uptake situation. Based on the observations of the results, there was a considerable difference between the peptide with PEG modification and NP1 complexes. At the siRNA concentration of 500 nM together with a peptide to siRNA molar ratio of 60:1, the mean fluorescence intensity, which indicated the siRNA quantity that has been uptake by the cells, of NP1 group was as twice as higher than peg3 or peg9 group and the result from peg18 was even worse. The relative low uptake from PEG modification group may be due to the effect on PEG masking and size disadvantages. Although final size stabilized at an ideal size range of 100 to 300 nm, the complexes with PEG modification still showed 100 to 150 nm larger than NP1 complexes, which was unfavored for being endocytosed by cell. Meanwhile, oligoarginines in the peptide sequence can bind to the cell surface with high affinity and facilitate the uptake, but PEG protection masked the interaction between oligoarginines and cell membrane thus resulted in another disadvantage.

Nevertheless, surprisingly noticed from the results of gene silencing assay, the knockdown efficiency from peg3 and peg9 groups were equal to the result collected from NP1 at the same concentration in FACS experiment, which may contributed from the reason that endosomal escape of siRNA delivered by PEG modified peptide was easier than the siRNA carried by

NP1. Since the negatively charged siRNA and cationic peptide were complexed through electrostatic interaction, the smaller in size means the stronger interaction, which could potentially cause difficulty in siRNA release after entering the cells. However, with the outmost PEG shell, free siRNA molecule could be locked in the core area by the polymer crosslink chain and a relatively weaker ionic interaction with the peptide, ultimately leading to an easier releasing of siRNA contrasted to NP1 group. Moreover, in both siRNA concentration of 300 nM and 500 nM, the gene silencing results from peg3 and peg9 groups showed higher consistency as well.

The superiority of PEG modification was more embodied in the results of cell viability. Those groups with the surrounding of PEGylation showed a cell survival percentage above 80% after 3 hours incubation, which is also the transfection time, and almost no decrease in the next 21 hours no matter for low siRNA concentration of 100 nM or high concentration of 300 or 500 nM. On the contrary, the majority of cells died in 24 hours in NP1 group at high concentration, also incomparable with peg3 or peg9 group in the low concentration of 100 nM. The toxic behavior of NPs varies with their size, shape and surface charge etc. In this case, the difference in toxicity with or without PEG may due to the size distinction and blocking effect

from PEGylation upon membrane contact. The NP1 groups showed an average size of 150 nm , about 200 nm smaller than the complexes with PEG, thus caused higher toxicity in the environment since toxicities are normally inversely proportional to the size of the NPs[138–140]. Meanwhile, the cationic surface of NP1/siRNA complexes can be directly attracted to the negatively charged prokaryotic membrane and induced disintegration of the membrane leading to a subsequent collapse of electrochemical gradient[141], eventually caused the death of the cells.

The RNase resistance assay demonstrated the siRNA remaining level after RNase degradation straightforwardly. It was distinct from the results that the relative fluorescence intensity, which reflected the relative quantity of siRNA in the system, still maintain in a high level with almost no sign of decreasing with the PEG protection. Nevertheless, there was a gradually descending trend of the fluorescence intensity in NP1 group in the 48-hours-period incubation.

Conclude from this work, the modified peptide peg3 and peg9 which has a matrix-metalloproteinase-2 sensitive linker coupled with various molecular weight of PEG have been proved better performance in delivering GAPDH siRNA into A549 cancer cells than unmodified peptide holistically. The results demonstrated an equal and consistent transfection

efficacy from peg3 especially peg9 under the premise of lower cellular uptake comparing to NP1, along with a much higher cell viability at the same siRNA concentration with serum presence. Meanwhile, benefit from the usefulness of PEGylation, particle stability was also been improved in the serum environment. However, the quantity of MMP2 secretion by the cells and the sensitive linker cleavage efficiency are still the key factors that could potentially dominate the overall performance of the PEG modified particles.

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