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著者(和文)	HuangChih-Hao
Author(English)	Chihhao Huang
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Molecular Design and Biological Evaluation of siRNA-conjugated Polymer that Responds to Redox Potential

Huang Chih Hao

Supervisor: Professor Nobuhiro Nishiyama

Department of Environmental Chemistry and Engineering

Interdisciplinary Graduate School of Science and Engineering

Tokyo Institute of Technology

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Preface

Since the cancer became one of the leading causes of death during the modern centuries, many researchers pay great attention to various possibilities for fighting it and protecting the human for health life. Small interfering RNA (siRNA) has been developed and recognized as a potential medicine for cancer therapies. Recently, various constructions of siRNA have been investigated with improved therapeutic activities toward efficient treatment.

This dissertation is an attempt to develop a novel design of siRNA-polymer conjugate and optimized formulations for improved biological properties. In order to achieve this goal, an environment sensitive siRNA conjugated polymer system with redox-responsive chemical linkage has been created and prepared in this study, and its evaluations of physical and biological properties are described in details.

The study has been carried out at Tokyo Institute of Technology from 2013 to 2016, and the author strongly believes these efforts and the developed methodology would provide not only fundamental knowledge but also further application in the biomedical field.

Acknowledgement

Firstly, I would like to express my sincere gratitude to my advisor Prof. Nobuhiro Nishiyama for the continuous support of my Ph.D study and related research, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D study.

My sincere thanks also goes to my mentor, Assistant Professor Hiroyasu Takemoto, who guided me patiently into a new research field with the right direction. Without his precious support it would not be possible to conduct this research.

Thanks also to Assistant Professor Takahiro Nomoto, who provided lots of talented suggestions to the experiments. I also thank Assistant Professor Keishiro Tomoda, Assistant Professor Makoto Mtsui, who were also always patient with any questions I would ask them.

I thank my labmates, Noor Faizah, Naoto Yamada, Dou Xuebo, Ranneh Abdul-Hackam, and all members in Lab Nishiyama for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last three years. Also I am grateful to my lovely friends for their accompany throughout my research journey and life in Japan.

Last but not the least, I would like to thank my dearest family for supporting me spiritually throughout writing this doctoral dissertation and my life in general. Living as a foreigner in Japan is not easy, but I would not change my decision to study here.

Abbreviation

DNA : deoxyribonucleic acid

RNA : ribonucleic acid

siRNA : small interfering RNA

siScr : scrambled siRNA

ON : oligonucleotides

RNAi : RNA interference

dsRNA : double strand RNA

RISC : RNAi induced silencing complex

Ago2 : argonaute2 proteins

RNase : ribonuclease

mRNA : messenger RNA

PEG : poly(ethylene glycol)

DPC : dynamic polyconjugate system

LNP : lipid nanoparticles system

PVP : poly(vinylpyrolidone)

RES : reticuloendothelial system

EPR : enhanced permeability and retention

DOX : doxorubicin

MAA : maleic acid amide

GSH : glutathione

PIC : polyion complex

GST : glutathione-*S*-transferase

DBCO : dibenzocyclooctyl

SEC : size exclusion chromatography

HPLC : high performance liquid chromatography

RNase A : ribonuclease A

FCS : fluorescence correlation spectroscopy

DP : degree of polymerization

RLU : relative luminescence unit

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Chapter 1

General Introduction

1.1 Background of oligonucleotide therapy

1.1.1 Genetic information in biological system

Gene is the hereditary molecular unit consisting of a sequence of DNA, which represents the genetic information of living cells. It can be also materialized into some stretches of DNA and RNA that code for a polypeptide or for an RNA chain with the function in the organism.[1] There are three concepts describing the whole organism at the molecular level; genome, transcriptome, and proteome, which represent the classification of biological macromolecules necessary for each cell in living organism responsible for daily functions, and can be defined as all of genetic information of an organism, the series of all RNA molecules, and the series of all proteins expressed by an organism, respectively.[2] Genes specify all of proteins and functional RNA chains that living beings depend on. Genes also carry the information to build and maintain the cells and pass genetic features to their descendant. There are many genes in organisms, which correspond to various

biological traits, such as immediately visible features; eye color or skin color, and recessive features; blood type or the specific diseases.[3] The function of cell and the organism itself are dependent on the sequence of nucleic acids present in the genome.[2]

There are two major processes of conversion from gene into protein: First, the gene must be transcribed from DNA to messenger RNA (mRNA); and then, second, it must be translated from mRNA to protein (Figure 1-1).[4, 5] The process of producing a biologically functional molecule from gene to protein is described as gene expression. The genetic code is the series of chemical symbols by which a gene is translated into a functional protein. Each gene consists of a specific sequence of nucleotides encoded in a DNA (or sometimes RNA in some viruses) strand.

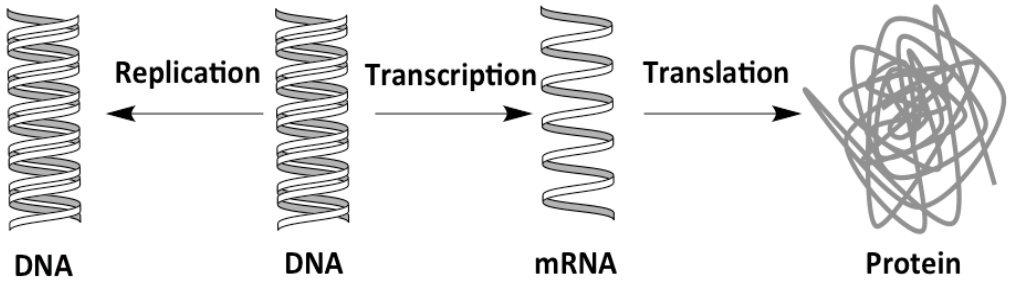


Figure 1-1. Flow of genetic information in biological system.

1.1.2 Mechanism of RNA interference

Based on the processes of DNA to RNA to proteins, the development of technologies employing synthetic oligonucleotides (ONs) attracted tremendous interests and generated various ONs therapeutics for manipulating the gene expression.[4, 6] ONs are chemically synthesized short nucleic acid sequences, usually

shorter than 30 nucleotides of DNA or RNA, which cause the selective inhibition of gene expression by interfering the flow of information in biological systems.[7] It was thought ON therapeutics would be a useful treatment for diseases including cancer.[8]

One of mechanism of ONs therapeutics, post-transcriptional gene silencing including RNA interference (RNAi), which is a natural process in eukaryotic cells, was concerned as a potential therapy.[9] The discovery of RNA interference (RNAi) in mammalian has been recognized as a key powerful therapeutics for cancer therapies. A short double strand RNA (dsRNA) composed of 21-23 base pairs, also called small interfering RNA (siRNA), has been demonstrated to have potential to regulate gene expression.[10-12]

RNA interference (RNAi) is a process that is used by cells for post-transcriptional silencing of gene expression, which begins with the entrance of a double-stranded RNA (dsRNA) into the cells that triggers the RNAi process.[10] The dsRNA is processed into small fragments by endonuclease Dicer that generates small interfering RNA (siRNAs) with around 21 nucleotides long, of which 19 nucleotides form a double helix and 2 nucleotides on each of the 3' ends are unpaired (Figure 1-2).

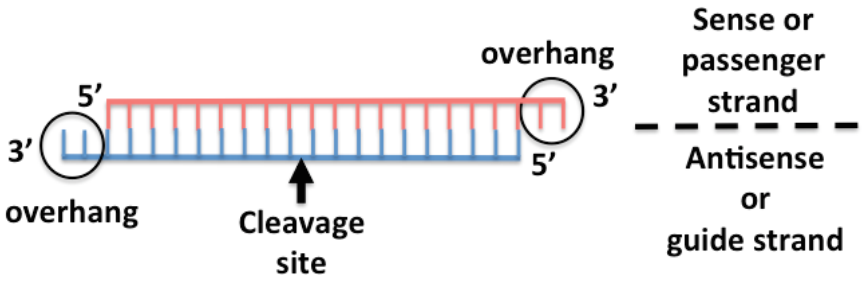


Figure 1-2. Structure of small interfering RNA.

RNAi induced silencing complex (RISC) is the actual effector of RNAi process, which is guided by asRNA in siRNA to the complementary target mRNA. As a result,

the targeted mRNA is cleaved at a specific site in the center of the duplex, around 10 nucleotides from the 5' end of the siRNA strand.[13] The component catalyzes the cleavage, which is identified as Argonaute2 proteins (Ago2). Ago2 contains a domain that resembles RNase H, a well-known protein having function to cleave the RNA component of a DNA/RNA duplex.[14, 15] The RISC binds the mRNA and gene expression can be silenced by suppressing translation of the mRNA molecule (Figure 1-3).[16]

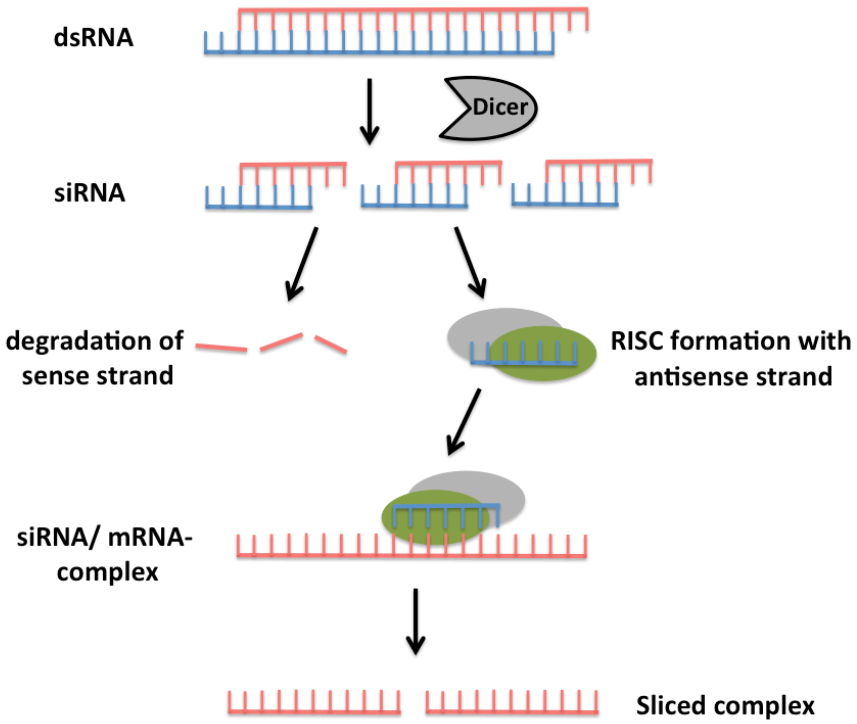


Figure 1-3. Mechanism of RNA interference triggered by siRNA.

1.1.3 Delivery approaches for siRNA therapeutics

A growing number of studies related to siRNA therapeutics have been reported for various diseases, including cancer and genetic disorders.[6] However, the application of siRNA in the clinical uses has still been a great challenge due to the

lack of efficient delivery system *in vivo* which is necessary for achieving treatment success.[17] The site of action of siRNA therapeutics is the cytosol. It is required that siRNA should be transferred to the interior of the target cells to activate the RNAi pathway.[18] The intravenously injected naked siRNA fails to accumulate in target organs or tissue because of the several physiological barriers during the delivery pathway, such as enzyme degradation in serum conditions and renal clearance.[19] In addition, siRNA carries a net negative charge on the sugar-phosphate backbone under normal physiological conditions, which does not readily cross the cell membrane that also carries negative charges on the surface.[20] Therefore, while siRNA is transferred to the interior of the target cells, the prolonged circulation time after injection, stability against enzyme degradation in serum conditions, evasion of the immune system, penetration of anionic cell membrane, and avoidance of interaction with non-specific cells should be considered as an ideal delivery system for siRNA therapeutics (Figure 1-4).[21]

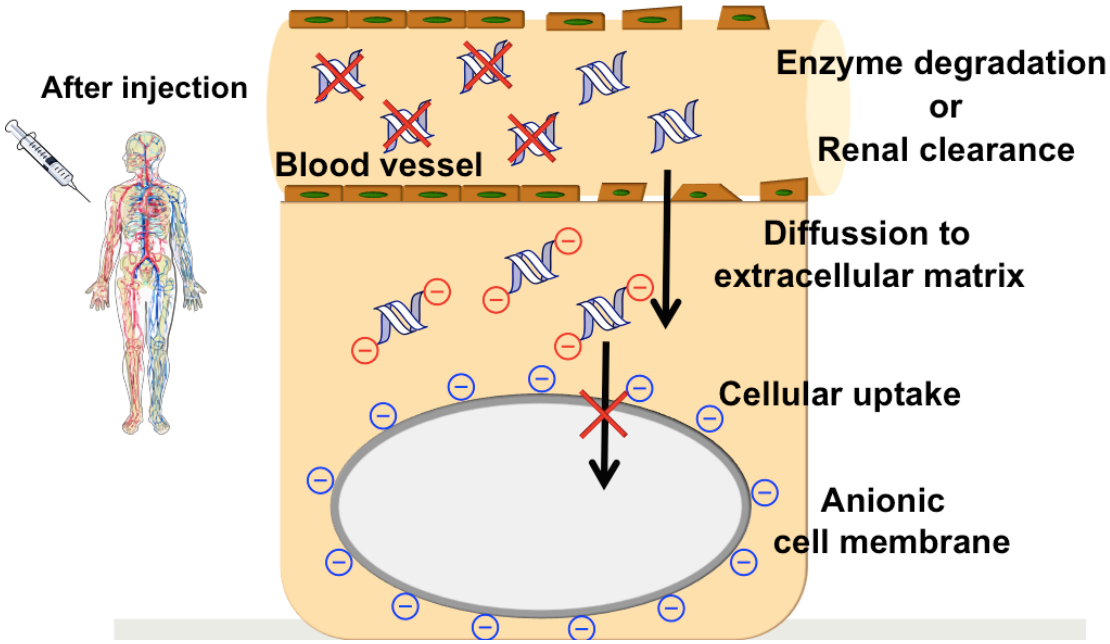


Figure 1-4. Obstacles for siRNA therapeutics.

To utilize siRNA in practical application, a variety of effective siRNA delivery strategies have been extensively developed to overcome the obstacles. Construction of siRNA carrier typically involves complexing siRNA with a positively charged vector, e.g., cationic polymers, dendrimers, and lipids for siRNA readily crossing anionic cell membrane, and cationic cell penetrating peptides for improved cellular internalization[22, 23]; conjugating with small molecules, e.g., cholesterol and bile acids for improved stability as well as cellular internalization[18, 24]; conjugating with antibodies for enhanced cell-specific active targeting[25, 26]; encapsulating siRNA into nanoparticulate formulations and conjugating with polymer, e.g., poly(ethylene glycol) (PEG) for enhanced stability against enzymatic degradation (Figure 1-5).[27, 28] Additionally, great amount of effort and capital have been invested for RNAi-based therapeutics, which have entered clinical trials, such as dynamic polyconjugate system (DPC), lipid nanoparticles system (LNP), and siRNA conjugate.[17, 29]

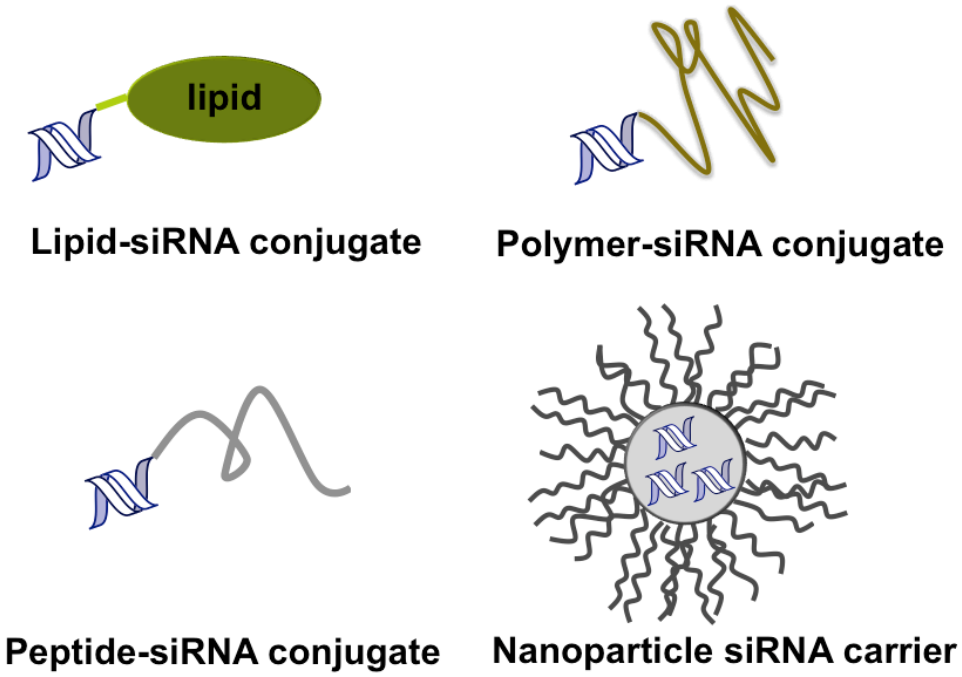


Figure 1-5. Various constructions for siRNA delivery.

1.2 Development of polymer based therapeutics for drug delivery system

1.2.1 Background of polymer based therapeutics in clinical applications

Since the forthcoming new concepts for the application of polymer conjugate as delivery carriers in the 1970s, polymer science has played a significant role in both the design of novel carriers for various disease treatments.[30, 31] The development of polymer based drug delivery systems has made a significant improvement and gained great attention for the clinical applications, due to the improved solubility and pharmacokinetics of therapeutic cargoes, the enhanced drug localization at the disease sites, as well as enhanced drug safety and efficacy.[32] Polymer is widely applied for the clinical uses for a long history. In the past twenty years, a plenty of polymer based therapeutics have been developed for the treatment of cancer, diabetes, pain, bacterial infections, etc. In the field of polymer based therapeutics, it is a term used to describe polymeric drugs, polymer conjugates of proteins, drugs, aptamers, together with those block copolymer micelles and multicomponent non-viral vectors which contain covalent linkages (Figure 1-6).[33]

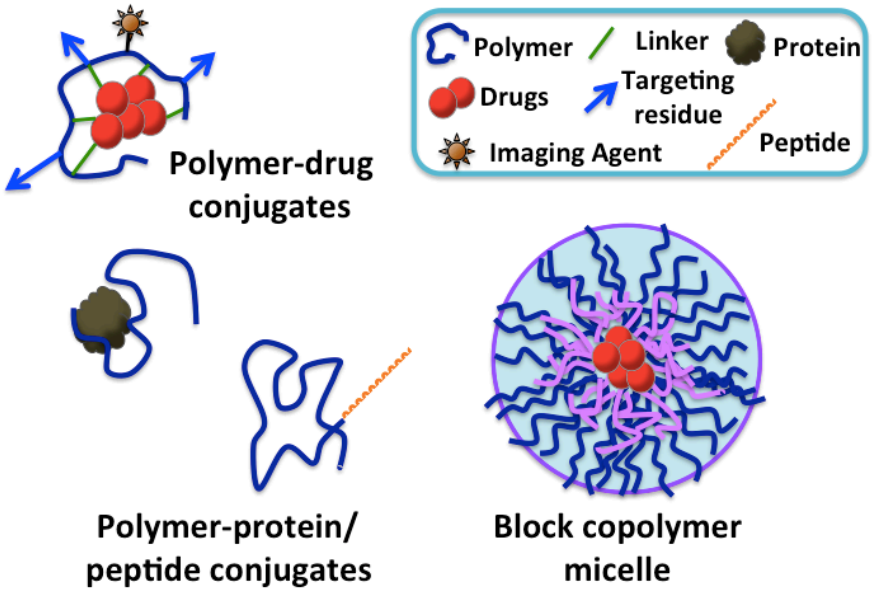


Figure 1-6. Polymer-based therapeutics for drug delivery system.

In the beginning, clinical polymers studied were mainly used for wound dressing and antiseptics, for example poly(vinylpyrolidone) (PVP) and dextran.[33] It is also worth to note that polymers have also been used as coatings. From the 1960s synthetic polymer-based drugs, polymer–drug conjugates began to develop, especially poly(ethylene glycol) (PEG)-protein conjugates.[33, 34] The interests in the lysosome type of polymer–drug conjugates, block copolymer micelles, and PEG-protein conjugates began to grow drastically due to the several pioneering works during the 1970s. Over the years, PEGylation of proteins and recently aptamers has produced an increasing number of important medical products.[35] The PEGylation is now widely applied due to the success of PEG-proteins was critical to the whole field of polymer therapeutics. In the 1980s/90s, the important contribution was the development of the first advanced block copolymer micelles to enter Phase I/II trials as novel anticancer agents.[33]

There are some examples of PEGylated therapeutics shown as follows. A number of formulations based on PEGylated polymeric nanoparticles are currently

undergoing clinical trials. In general, these drug-loaded nanoparticles exhibit prolonged systemic circulation time, sustained drug release kinetics, and better tumor accumulation as compared to small-molecule drugs.

The research group of Kataoka developed the polymer micelle carrier system for doxorubicin to the cancer therapy. A polymeric micelle carrier system for doxorubicin (DOX), named as NK911, consists of poly(ethylene glycol) (PEG) and conjugated doxorubicin-poly(aspartic acid).[36] It has hydrophobic inner core, and therefore can entrap the sufficient amount of DOX. In summary, NK911 is a small-size spherical micelle particle (~40 nm) that entraps doxorubicin, and circulates in blood for long-time because of evading the reticuloendothelial system (RES) uptake due to the hydrophilic poly(ethyleneglycol) (PEG) outer layer. It was effectively accumulated in tumor tissue by EPR effect. The incorporated DOX is released from the inner core by diffusion and expressed stronger activity than free DOX against all the tumor lines tested.

Polymer therapeutics offer an efficient and versatile platform for designing novel and more efficacious therapeutics. A common characteristic behind the various polymer-based therapeutics in clinical development is their stealth ability and biocompatibility that enables prolonged pharmacokinetics and improved biodistribution.[37]

As the polymer science developed and applied to cancer therapies, poly (ethylene glycol) (PEG) is one of the extensively used biocompatible polymers for *in vivo* use due to its high water solubility, safety, and easy chain modification.[38] Recently, PEG served as the standard polymer for cancer therapeutics with high stealth functionalization in applications, and optimization of PEG coating on polymeric nanoparticles has been extensively studied. PEG is a non-toxic polymer

and a polymer chain with a molecular weight of less than 30,000 can be cleared from the body through renal filtration.[39] Also due to their high surface hydrophilicity, half-life of blood circulation increases by decreasing interactions with blood protein and prevention of enzymatic degradation.[40] As their high flexibility and the large exclusion volume in water, PEG forms a shell that renders the micelles sterically stabilized, PEGylation can be conducted as a significant technique via conjugating to form a PEG derivatives or adsorbing PEG chains onto the surface of the nanoparticles. The effects of PEGylation are highly dependent on the PEG molecular weight, polymer chain architecture, and surface density of the PEG coating. Two general morphologies of PEGylation after coating different density of PEG onto the surface; mushroom conformation for the low PEG density coating and brush conformation for the high PEG density coating.[41]

1.2.2 Polymer based therapies for siRNA delivery

siRNA conjugation with polymers potentially allows installation of multiple functionalities because of a plenty of reaction sites in one polymer molecule, and thereby molecularly programmed tactics can be input into the structure of siRNA-polymer conjugate for effective siRNA therapeutics.[42, 43] For example, siRNA conjugated with poly(ethylene glycol) (PEG), which is a biocompatible, hydrophilic, and nonionic polymer, contributes to the enhanced stability against enzymatic degradation and induces great blood compatibility for intravenous administration, due to stealth functionality of PEG.[44] The PEG-siRNA conjugate itself exhibited much higher level of siRNA stability than the naked siRNA in the

presence of 50% serum, maintained its integrity up to 16 hours.[28] And then, the conjugation with PEG can be subsequently complexed with cationic polymer or peptides for improved cellular internalization.[45]

For development of application for RNA interference as a polymer based therapeutics, CALAA-01 comprised of cyclodextrin-containing polymer is a nanoformulation carrying siRNA for targeted RNAi therapy.[46] These nanoparticles are prepared via the self-assembly of cyclodextrin-containing polymers in the presence of nucleic acids, which yields colloidal particles approximately 70 nm in diameter. The particles are also stabilized with PEG and functionalized with a targeting ligand or transferrin. By providing a protective barrier that precludes siRNA from plasma degradation and renal clearance, CALAA-01 enables the nucleic acid-based therapeutic to be systemically administered. In addition, the targeting ligands are incorporated to promote the intracellular uptake of CALAA-01, and the nanocarriers readily undergo particle disassembly in acidic environment to promote endosomal escape.

1.3 Biological stimuli-responsive system for siRNA therapeutics

1.3.1 General introduction of stimuli-responsive system

Stimuli-responsive systems have been widely studied and applied to drug

delivery systems, which can release the active cargoes in response to specific stimulus. The specific stimulus can be characterized as endogenous stimuli, such as changes in pH, redox potential, and enzyme concentration; exogenous stimuli, such as change of temperature, light, and magnetic field.[47] In the past few years, the advanced drug delivery system having intelligently stimuli-responsive systems, which were like switches, controlled the on-off activity after accumulating to the target sites. If the drug carried by delivery system and released before accumulating to the tumor site, the treatment efficacies could not be achieved. Consequently, this concept has been considered as important issues for designing the drug delivery systems.[47]

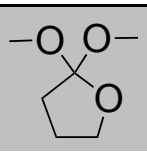
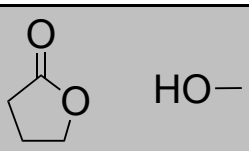
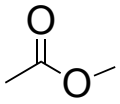
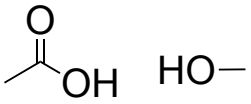
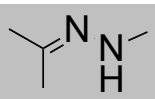
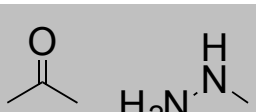
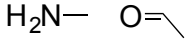
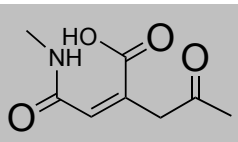
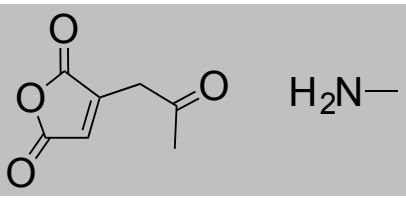
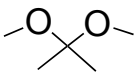
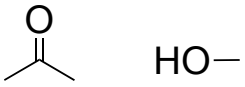
Recently, stimuli-responsive systems were also applied to the efficient constructions of siRNA carriers and attracted huge interests. Taking advantage of stimuli-responsive systems for siRNA delivery, the siRNA could be controlled and released in the right time and position for silencing ability.[20] Furthermore, there were several advantages provided by polymer for construction of siRNA carriers, such as siRNA-conjugated PEG for the improved stability.[27] However, it was reported that siRNA recognition was hindered by the steric hindrance effect of conjugated polymer for siRNA recruiting into RNAi pathway, which led to the compromised gene silencing activity.[48] Via utilization of stimuli-responsive systems for siRNA conjugated polymer systems, the steric hindrance effect of conjugated polymer could be decreased, leading to the efficient gene silencing efficacies.

1.3.2 pH-sensitive system

The pH-gradient is one of the exploited stimuli for the design of drug delivery system.[20] There are various pH gradients in physiological and pathological processes.[49] The extracellular pH value in solid tumor microenvironment has been reported to be slightly acidic, compared to normal tissues (pH ~ 7.4), with the value of pH ~6.5-6.8.[50] Solid tumors have an acidic environment caused by increased levels of metabolites, such as lactic acid. It is also well known that the more acidic pH values are found in endosome (pH ~5.5-6.0) and lysosome (pH ~4.5-5.5).[47, 49] On the basis of difference of pH, various pH-sensitive systems were developed for drug delivery systems. Taking the advantage of difference of pH values, one strategy is developed by the use of polymers (polyacids or polybases) with ionizable groups that undergo conformational changes in response to environmental pH variation.[50] When the pH and the ionic composition of the aqueous medium changes, pH-sensitive polymers are ionized and drastically change their conformation, resulting in exposure of cargoes.

Another strategy is to use acid-labile chemical bonds to covalently attach drug molecules onto the surface of carriers, or to construct new carriers.[50, 51] The acid-labile chemical bonds are stable at neutral pH but are degraded or hydrolyzed in acid media, which enable the release of drug molecules anchored at the polymer backbone. Based on the advantage of difference of the pH variation, there are several widely used acid-labile chemical bonds, such as hydrazone, ester, and acetal, illustrated in Table 1-1 with their chemical structures and degraded products.[51]

Table 1-1. Examples of widely used pH-sensitive linkages

Name	Acidic-labile chemical bonds	Degradation products
Orthoester		
Ester		
Hydrazone		
Imine		
***Cis*-aconityl**		
Acetal/ketal		

Examples of drug delivery carriers containing pH-sensitive systems are described as follows. The polymer micelle with pH-sensitive linkage was developed in 2005, which were prepared by self-assembling block copolymers, poly(ethylene glycol)-*b*-poly(aspartate hydrazone adriamycin).[52] The anticancer drug of adriamycin was conjugated to the hydrophobic segment of poly (aspartate) via acid-sensitive hydrazone linkage. The designed micelle could maintain its structure stably and protect the drug under physiological conditions (pH 7.4), then after cellular uptake, selectively release the drug in endosomes and lysosomes at acidic pH value (pH 5-6). Also, *in vitro* and *in vivo*, the results showed that the micelles with pH-responsive linkages were high drug releasing capability, tumor-infiltrating permeability, effective antitumor efficacies, and extremely low toxicity, which thought to be a

promising development of polymeric micelle drug delivery system containing pH-sensitive linkages.

In 2012, E. Koren *et al.* reported pH-sensitive PEGylated long-circulating liposomes. The PEGylated liposomes, which were not readily taken by the macrophages of the reticuloendothelial system and maintained a long time of circulation, which could enable these carriers passively accumulate in tumor site, via the enhanced permeation and retention (EPR) effect. They used pH-sensitive hydrazone bond between a long shielding PEG chains and surface of liposomes. In neutral pH, long PEG chains hide cell-penetrating TAT peptide that was on the surface of liposomes. On the other hand, in acidic pH, hydrazone bond will be cleaved, and TAT peptide will be exposed. Therefore, cell-penetrating function of TAT peptide will revive.[53]

The pH-sensitive systems were also applied to the siRNA-conjugated polymer system. In 2013, Takemoto *et al.* successfully incorporated the pH-sensitive linkage, maleic acid amide (MAA), into siRNA conjugate to fulfill the efficient siRNA release.[44] It is known that maleic acid amide (MAA) group is relatively stable at the extracellular neutral pH, while rapidly hydrolyzed at the endosomal acidic pH.[54, 55] The utilized siRNA conjugate with maleic acid amide (MAA) linkage between siRNA and polymer backbone, the free siRNA could be released via cleavage of maleic acid amide (MAA) group under acidic pH environment to achieve silencing ability in the cell (Figure 1-7).

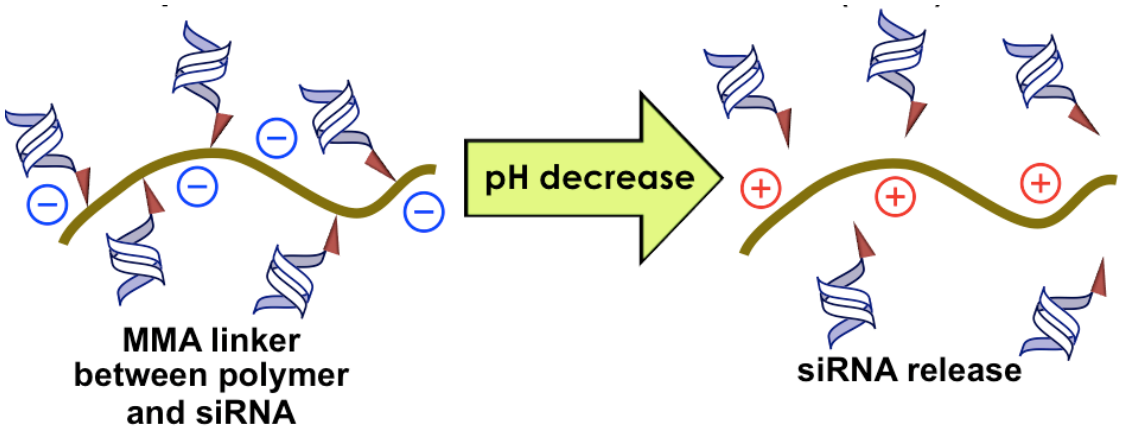


Figure 1-7. Acidic pH-responsive siRNA conjugate containing maleic acid amide (MAA) group.

1.3.3 Redox-sensitive system

The marked difference in the redox potential between extracellular and intracellular environments is another exploited stimulus for the design of drug delivery system.[56] The glutathione (GSH) is the most abundant low molecular weight thiol for regulating the redox potential in the animal cells.[57] A plenty of reduced GSH molecules are found in intracellular environment (~2-10 mM), which are ~1000 times higher than in extracellular environment (~2-20 μM).[57] Moreover, the cytosolic GSH level in some tumor cells has been found to be at least four times higher than in normal cells.[58] On the basis of difference in GSH levels between intracellular and extracellular environments, redox-sensitive systems provide the possibility for the design of drug delivery systems. The most conventional use of redox-sensitive system is disulfide group in response to change of redox potential across the cell membrane, which is widely applied for the drug delivery systems.[59] Via incorporating disulfide group to delivery system, the desired release of drug molecule can be triggered by the huge difference of concentration of GSH (from

~2-20 μM of GSH outside the cells to ~2-10 mM of GSH in the cytosol).[60-64]

In 2009, Matsumoto *et al.* reported a core-shell type polyion complex (PIC) system with a redox-sensitive disulfide linkage via assembly of iminothiolane-modified poly (ethylene glycol)-*block*-poly (L-lysine) (PEG-*b*-PLL-IM) and siRNA.[65] The PIC system exhibited spherical shape (~60 nm) with a narrow distribution. The structure of PIC was maintained under physiological environment, but was disrupted under reductive conditions, due to incorporation of disulfide linkages. Via the cleavable disulfide linkage, the desirable siRNA release from the PIC system in the intracellular reductive environment. It is worthy to note that ~100 fold higher silencing efficacies were achieved with the redox-sensitive disulfide linkage system, compared with PIC system prepared with the non-cleavable linkage of PEG-*block*-poly (L-lysine), which was unstable under physiological environment.

Further, redox-sensitive systems were also applied to the siRNA-conjugated polymer system. In 2010, Jung *et al.* successfully proposed a siRNA-polymer conjugates with cleavable disulfide linkage for achieving gene silencing efficacies.[27] In their work, they conjugated siRNA with PEG via cleavable disulfide and non-cleavable thioether linkages to different terminal ends of siRNA (sense 3', sense 5', antisense 3', and antisense 5'). It is worth to note that cleavable siRNA-PEG conjugates showed comparable gene silencing abilities to naked siRNA (without any modification), and exhibited sequence specific degradation of a target mRNA. The results indicated that the cleavage via incorporating with disulfide linkage to siRNA-polymer conjugates was required for eliciting free siRNA in cytosol to achieve gene silencing purposes.

1.4 Significance of current study

In this study, a new molecular design of siRNA-conjugated polymer system in response to redox potential was developed with enhanced extracellular stability and improved biological activities (Figure 1-8). Firstly, I proposed a new redox-sensitive chemical linkage, 2-nitrobenzenesulfonmaide group, which exhibited the potent extracellular stability and specific cleavability in response to glutathione (GSH) and glutathione-*S*-transferase (GST) inside the cell for construction of siRNA-conjugated polymer system. 2-Nitrobenzenesulfonamide group also referred to nosyl group. Nosyl group can be acted as protecting group for primary or secondary amine, as well as resist strong basic or acidic environments.[66] The siRNA-conjugated polymer system was fabricated from poly(ethylene glycol) (PEG) conjugated to siRNA via copper free click reaction with redox-sensitive 2-nitrobenzenesulfonamide linkage between PEG and siRNA. The synthesized PEG-siRNA conjugate containing 2-nitrobenzenesulfonamide group exhibited strong stability under the extracellular environment, as well as cleavability in the cytosol with high specificity, due to the fact that 2-nitrobenzenesulfonamide group resists strong basic or acidic conditions, but can be cleaved selectively by thiol moiety via Meisenheimer complex formation.[67] Of note, the presence of GSH and GST, which is overexpressed in cancer cells, potentially elicits the highly selective cleavage of 2-nitrobenzenesulfonamide group in cancer cells.[68] Moreover, conventional methodology for siRNA-conjugated polymer system is limited in the already developed chemical processes that are designed for other drugs, but not optimized for siRNA-conjugated polymer system, resulting in unexpected results, such as

unacceptable extracellular stability and undesired siRNA release outside the cell. In this regard, a new optimized design of siRNA-conjugated polymer system is required toward the success in siRNA-polymer conjugates-based therapeutics. From this molecular design with redox-sensitive linkage and optimized formulation, the designed siRNA-polymer conjugate would provide the promising methodology and contribute to the development of siRNA-based therapeutics toward a realization of next generation of medicine.

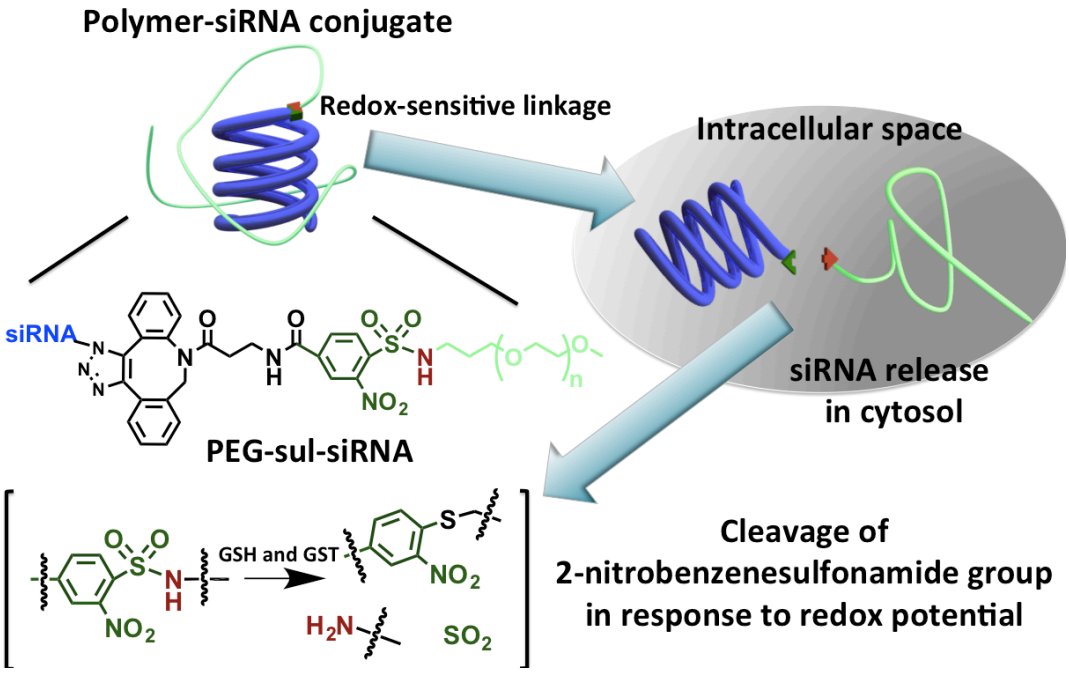


Figure 1-8. Illustration of PEG-siRNA conjugate containing redox-sensitive linkage, which responds to high redox potential in the intracellular space for achieving siRNA release.

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Chapter 2

Synthesis, preparation, and characterization of PEG-siRNA conjugates

2.1 Introduction

The PEG-siRNA conjugates were synthesized with three different linkages, i.e., 2-nitrobenzenesulfonamide, carboxylic amide, and disulfide group. The design of PEG-siRNA conjugate containing 2-nitrobenzenesulfonamide group will serve as a candidate for the construction of siRNA derivative, with potent stability under the extracellular environment as well as cleavability in response to exclusive presence of GSH and GST in the intracellular milieu. In contrast, PEG-siRNA conjugate containing disulfide group, which is conventionally used, and PEG-siRNA conjugate containing carboxylic amide group, which is the non-cleavable, act as control groups for the following evaluations, e.g. stability and cleavability in the mimicked cellular conditions, biological evaluations. Firstly, methyl 4-(chlorosulfonyl)-3-nitrobenzoate was synthesized as a chemical

linkage precursor containing 2-nitrobenzenesulfonamide group.[1, 2] An electrophilic center α carbon of the sulfonyl group in response to glutathione (GSH) is required for the cleavage, which is catalyzed and facilitated in the presence of glutathione-*S*-transferase (GST).[3, 4] Further, poly(ethylene glycol) (PEG) was conjugated to methyl 4-(chlorosulfonyl)-3-nitrobenzoate group, for the subsequent construction of PEG-2-nitrobenzenesulfonamide linker conjugates by the condensation reaction of carboxyl (–COOH) and amine ($–NH_2$) group. Poly (ethylene glycol) (PEG) was selected due to several advantages, e.g. the excellent biocompatibility, water solubility, and safety.[5] Recently, PEG served as a standard polymer for cancer therapeutics with excellent stealth ability for improving circulation half-life by decreasing the interactions with blood proteins in the *in vivo* application.[6] Finally, for the conjugation of siRNA to the poly (ethylene glycol) (PEG) to form PEG-siRNA conjugates, the copper free click chemistry between dibenzocyclooctyl (–DBCO) and azide ($–N_3$) group was selected. DBCO was introduced at the terminus of poly (ethylene glycol) (PEG) and azide ($–N_3$) group was introduced at the end of siRNA.[7] Recently, the copper free click chemistry was frequently utilized for the biomolecule modification, due to its less toxic metal-free conditions and the high reaction rate.[8]

The PEG-siRNA conjugate with non-cleavable linkage, carboxylic amide group, was synthesized as the control with the similar procedure via the copper free click chemistry. In addition, the PEG-siRNA conjugate with conventional redox-sensitive linkage, disulfide group, was synthesized via condensation reaction of carboxyl (–COOH) of 3,3'-dithiodipropionic acid and amine ($–NH_2$) group of methoxy-PEG-NH_2 to form PEG-disulfide-COOH.[9] Further, the similar procedure of the copper free click chemistry was selected as the same manner for

forming the PEG-disulfide-siRNA conjugate.[8]

All of the synthesized compounds and derivatives of poly (ethylene glycol) (PEG) were carefully characterized by ^{1}H NMR. The final products of PEG-siRNA conjugates were purified by the indicated processes in the following paragraph and determined by electrophoresis and size exclusion chromatography (SEC) for the purity of final products.

2.2 Materials and equipment

2.2.1 Materials

Glycylglycine, *N*,*N*-dimethylformamide, thioacetic acid, cesium carbonate, ethyl acetate, hydrochloric acid solution (5 M), sodium hydrate solution (5 M), sodium sulfate, acetonitrile, dichloromethane, 1,4-dioxane, chloroform-d (1 v/v% TMS), pyridine, methanol, tetrahydrofuran, triethylamine, and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methyl 4-choloro-3-nitrobenzoate and *N*-chlorosuccinimide were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). αeMethoxy-e-amino-poly(ethylene glycol) (Methoxy-PEG-NH_2) was purchased from NOF Co., Ltd. (Tokyo, Japan). Dibenzocyclooctyne amine (DBCO-amine) and Dibenzocyclooctyne-*N*-hydroxysuccinimidyl ester (DBCO-NHS ester) were purchased from Click Chemistry Tools, Ltd. (Scottsdale, AZ). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (1 M, pH 7.3) was purchased from AMRESCO Inc. (Solon, OH). Series of siRNAs were

synthesized by Hokkaido System Science Co. Ltd. (Hokkaido, Japan). The sequences are as follows:

(1) siGL3 (5'-azide-modified) :

5'-(N_3)-CUU ACG CUG AGU UCG AdTdT-3' (sense strand),

5'-UCG AAG UAG UCA GCG UAA GdTdT-3' (antisense strand)

(2) siScramble (5'-azide-modified) :

5'-(N_3)-UUC UCC GAA CGU GUC ACG UdTdT-3' (sense strand),

5'-ACG UGA CAC GUU CGG AGA AdTdT-3' (antisense strand)

*small letter indicates the 2'-O-methyl modification

2.2.2 Equipment

^{1}H-NMR (nuclear magnetic resonance) spectra were recorded on NMR400MHz (JEOL, Tokyo, Japan). Water phase high performance liquid chromatography (HPLC) were performed LC-Net II AS2055 system (JASCO, Tokyo, Japan) equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260 nm, and 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride as an eluent. Images of electrophoresis results were determined by ChemiDoc XRS Plus image Lab system (BIORAD, California, US).

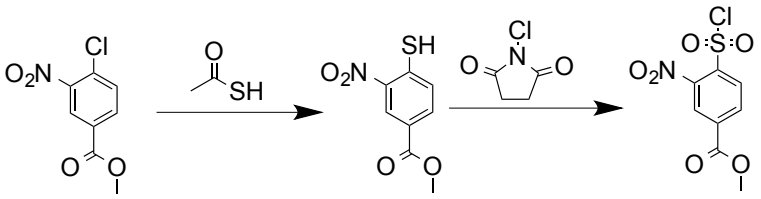
2.3 Experimental procedures

2.3.1 Synthesis of PEG-siRNA conjugates (2-nitrobenzenesulfonamide

series)

Methyl 4-(chlorosulfonyl)-3-nitrobenzoate was synthesized according to the following procedure (Scheme 2-1). 0.13 g of glycylglycine (0.001 mol) was dissolved in 20 mL of *N*,*N*-dimethylformamide. 0.81 mL of thioacetic acid (0.011 mol) and 7.3 g of cesium carbonate (0.022 mol) were added to the solution of glycylglycine at 0 °C, followed by a stirring for 10 minutes at 0 °C. 1.61 g of methyl 4-chloro-3-nitrobenzoate (0.0075 mol) was added to the reaction solution and was further stirred overnight on ice in a dark.

Then, product was extracted using ethyl acetate and 1 M hydrochloric acid solution, followed by washing with de-ionized water and saturated sodium chloride solution. The organic layer was dried over sodium sulfate, and the solvent was removed using a rotary evaporator to produce colorless powder. Next, the obtained colorless powder was dissolved in 10 mL of acetonitrile, and then, the solution was poured into the mixture of 20 mL of acetonitrile and 4 mL of 2 M hydrochloric acid solution containing 3.9 g of *N*-chlorosuccinimide (0.029 mol). The obtained reaction solution was stirred for 1 hour at 0 °C. The same extraction process was performed and the organic layer was removed using a rotary evaporator to obtain the crude product. The crude product was purified by silica gel chromatography (dichloromethane), and then, lyophilized using 1,4-dioxane to obtain methyl 4-(cholorosulfonyl)-3-nitrobenzoate as yellowish powder.



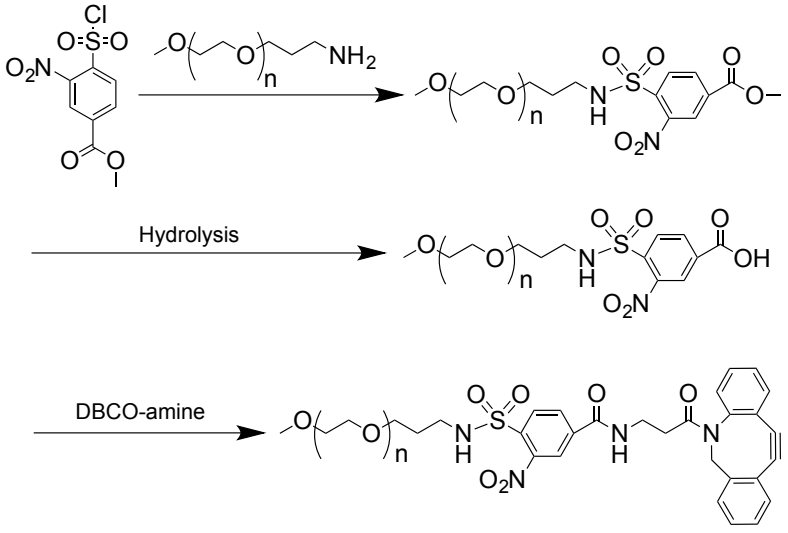
Scheme 2-1. Synthetic route of methyl 4-(chlorosulfonyl)-3-nitrobenzoate

PEG-sul-DBCO containing 2-nitrobenzenesulfonamide linkage was synthesized according to the following procedure (Scheme 2-2). Firstly, methyl 4-(chlorosulfonyl)-3-nitrobenzoate was introduced into the terminus of PEG via sulfonamide linkage. 200 mg of methoxy-PEG-NH_2 (molecular weight: 40,000 Da, 0.005 mmol) was dissolved in 3 mL of pyridine and stirred for 10 minutes at 0 °C. 14.0 mg of methyl 4-(chlorosulfonyl)-3-nitrobenzoate (0.025 mmol) was dissolved in 2 mL of pyridine at 0 °C and added to the solution of methoxy-PEG-NH_2, followed by stirring overnight at 0 °C. Then, the reaction solution was dialyzed against de-ionized water for removing excess reactant and pyridine (molecular weight cut off: 3,500 Da). The dialyzed solution was lyophilized to obtain PEG derivative containing methyl 3-nitrobenzoate at the terminus via sulfonamide linkage.

The methyl ester at the terminus of the obtained PEG derivative was hydrolyzed for further modification. 180 mg of PEG derivative containing methyl 3-nitrobenzoate at the terminus via sulfonamide linkage (0.0045 mmol) was dissolved in 18 mL of the mixture of water/methanol/5 M sodium hydrate solution (1/8/0.05). The reaction solution was stirred overnight at room temperature. Then, the reaction solution was dialyzed against de-ionized water (molecular weight cut off: 3,500 Da), and the dialyzed solution was lyophilized to

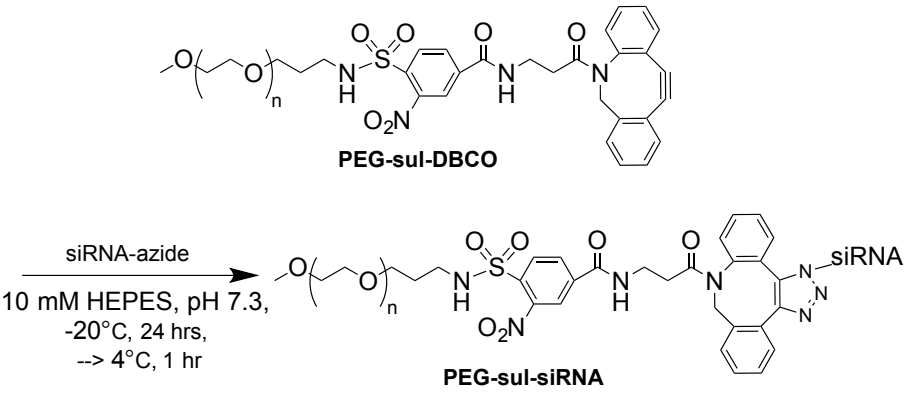
obtain methoxy-PEG derivative containing 3-nitrobezoic acid at the terminus via sulfonamide linkage.

Finally, dibenzocyclooctyne (DBCO) moiety was introduced to obtain methoxy-PEG-sulfonamide-DBCO (PEG-sul-DBCO). 138 mg of PEG derivative containing 3-nitrobezoic acid at the terminus via sulfonamide linkage (0.0034 mmol) was dissolved in 7 mL of tetrahydrofuran. 9.6 mg of dibenzocyclooctyne amine (0.034 mmol) was dissolved in 3 mL of tetrahydrofuran and added to the solution of the PEG derivative. To this mixture, the suspension of 9.6 mg of DMTMM (0.034 mmol) in 3 mL of tetrahydrofuran was added, followed by a stirring overnight at room temperature. Then, the reaction solution was dialyzed against de-ionized water (molecular weight cut off: 3,500 Da) and filtered for removing unreacted dibenzocyclooctyne amine. The solution was lyophilized to obtain final desired product PEG-2-nitrobenzenesulfonamide-DBCO, which was noted as PEG-sul-DBCO.



Scheme 2-2. Synthetic route of PEG-sul-DBCO

The PEG-sul-5'-siRNA conjugates were synthesized via click conjugation between dibenzocyclooctyne and azide (Scheme 2-3).[8] 6 mg of PEG-sul-DBCO conjugate (0.00015 mmol) was dissolved in 500 μL of HEPES (10mM; pH7.3). The 65 μL of siRNA (3 mg/mL) was added to the solution of PEG-sul-DBCO conjugate and followed by adding 335 μL of HEPES (10 mM; pH 7.3). The solutions were frozen at -20 °C for overnight and then thawed at 4 °C for 1 hour. The solutions were subsequently characterized and purified via HPLC, where LC-Net II AS2055 system (JASCO, Tokyo, Japan) was equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260 nm, and 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride as eluents.

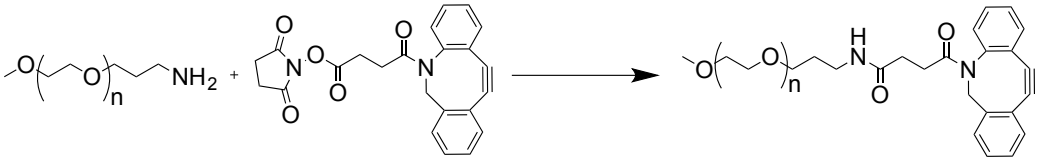


Scheme 2-3. Synthetic route of PEG-sul-siRNA conjugate.

2.3.2 Synthesis of PEG-siRNA conjugates (carboxylic amide series)

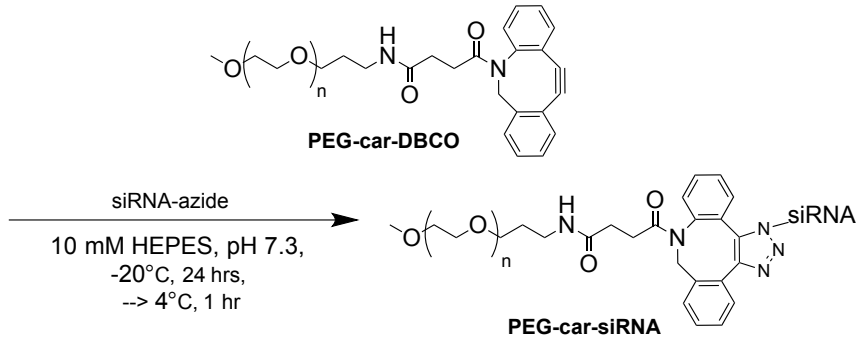
PEG-car-DBCO containing carboxylic amide linkage was synthesized according to the following procedure (Scheme 2-4). 100 mg of methoxy-PEG-amine (0.0025 mmol) was dissolved in 4 mL of dichloromethane and 16.2 mg of DBCO-NHS ester (0.025 mmol) was added to the solution of

methoxy-PEG-amine in dichloromethane, followed by a stirring overnight at room temperature. The 2 drops of triethylamine was added for activating the reaction. Then, the reaction solution was dialyzed against methanol and de-ionized water (molecular weight cut off: 3,500 Da). The obtained solution was lyophilized to obtain PEG-car-DBCO and was confirmed by ^{1}H NMR analysis.



Scheme 2-4. Synthetic route of PEG-car-DBCO.

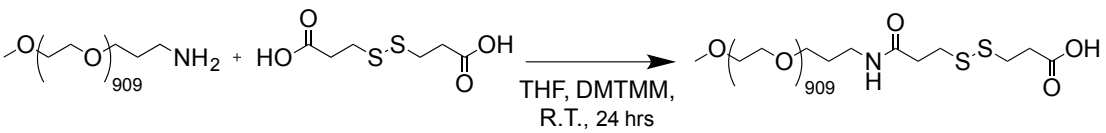
The PEG-car-5'-siRNA conjugations were synthesized via click conjugation between dibenzocyclooctyne and azide (Scheme 2-5). 6 mg of PEG-car-DBCO conjugate (0.00015 mmol) was dissolved in 500 μL of HEPES (10 mM; pH 7.3). The 65 μL of siRNA (3 mg/mL) was added to the solution of PEG-car-DBCO conjugate and followed by adding 335 μL of HEPES (10 mM; pH 7.3). The solutions were frozen at -20 °C for overnight and then thawed at 4 °C for 1 hour. The solutions were subsequently characterized and purified via HPLC, where LC-Net II AS2055 system (JASCO, Tokyo, Japan) was equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260 nm, and 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride as eluents.



Scheme 2-5. Synthetic route of PEG-car-siRNA conjugate.

2.3.3 Synthesis of PEG-siRNA conjugates (disulfide series)

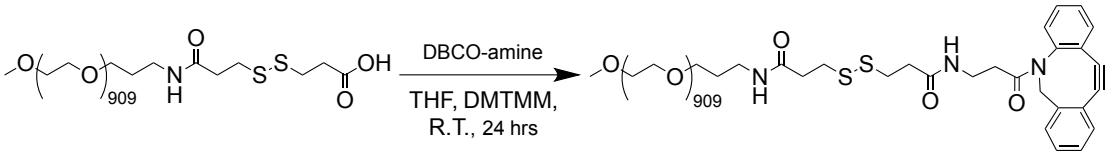
PEG-disulfide-DBCO was synthesized according to the following procedure (Scheme 2-6). Firstly, 3,3'-dithiodipropionic acid was introduced into the terminus of PEG. 100 mg of methoxy-PEG-NH_2 (molecular weight: 40,000 Da, 0.0025 mmol) was dissolved in 4.0 mL of tetrahydrofuran. 5.3 mg of 3,3'-dithiodipropionic acid (0.025 mmol) was dissolved in 1 mL of tetrahydrofuran and added to the solution of methoxy-PEG-NH_2. To this mixture, the suspension of 6.9 mg of DMTMM (0.025 mmol) in 2 mL of tetrahydrofuran was added, followed by a stirring overnight at room temperature. Then, the reaction solution was dialyzed against de-ionized water (molecular weight cut off: 3,500 Da) and filtered for removing unreacted 3,3'-dithiodipropionic acid. The obtained solution was lyophilized to obtain PEG-disulfide-COOH.



Scheme 2-6. Synthetic route of PEG-disulfide-COOH.

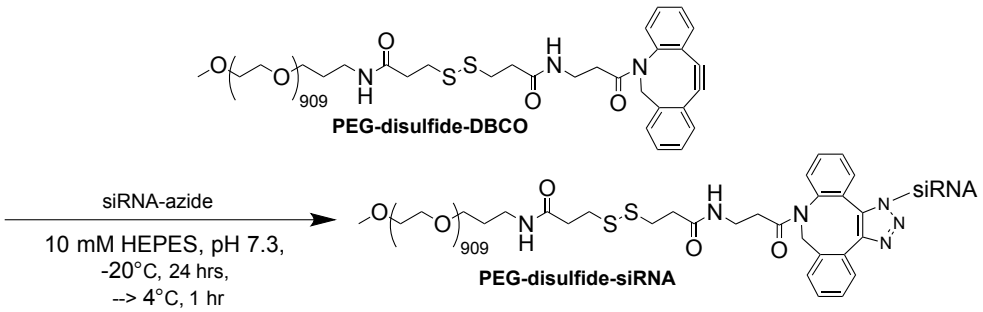
Next, dibenzocyclooctyne (DBCO) moiety was introduced to obtain

PEG-disulfide-COOH (Scheme 2-7). 60 mg of PEG derivative having disulfide linkage at the terminus (0.0015 mmol) was dissolved in 3 mL of tetrahydrofuran. 4.1 mg of dibenzocyclooctyl amine (0.015 mmol) was dissolved in 1 mL of tetrahydrofuran and added to the solution of the PEG derivative. To this mixture, the suspension of 4.2 mg of DMTMM (0.015 mmol) in 2 mL of tetrahydrofuran was added, followed by a stirring overnight at room temperature. Then, the reaction solution was dialyzed against de-ionized water (molecular weight cut off: 3,500 Da) and filtered for removing unreacted dibenzocyclooctyl amine. The obtained solution was lyophilized to obtain PEG-disulfide-DBCO.



Scheme 2-7. Synthetic route of PEG-disulfide-DBCO.

PEG-disulfide-siRNA was synthesized via click reaction between dibenzocyclooctyne group at the terminus of PEG-disulfide-DBCO and azide group at the terminus of azide-siRNA (Scheme 2-8). 6 mg of PEG-disulfide-DBCO (0.00015 mmol) was dissolved in 500 μL of HEPES buffer (10 mM, pH 7.3). The 65 μL of siRNA (3 mg/mL) was added to the solution of PEG-disulfide-DBCO and followed by adding 335 μL of HEPES buffer (10 mM, pH 7.3). The solution was frozen at -20 °C for overnight and then thawed at 4 °C for 1 hour. The solution was subsequently characterized and purified by HPLC [column: Mono Q^{TM} 5/50GL column (GE Healthcare), detection: UV detection at 260 nm, eluent: 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride for gradient elution].



Scheme 2-8. Synthetic route of PEG-disulfide-siRNA conjugate

2.3.4 Agarose gel electrophoresis

All of PEG-siRNA conjugates, which include 2-nitrobenzenesulfonamide, carboxylic amide, and disulfide linkages as linkers, were purified by HPLC and subsequent desalting by ultrafiltration for removing excess buffer and salts, which were contained in buffer during HPLC purification process. The obtained PEG-siRNA conjugates were further analyzed by agarose gel electrophoresis. Unconjugated siRNA and PEG-siRNA containing different linkages (100 ng of siRNA for each sample) were loaded onto agarose gel (2.5% agarose, 1 × TBE) including SYBR safe (Thermo Fisher Scientific Inc., Waltham, MA, USA) and treated at 100 V for 70 min. Next, the band from siRNA and its polymer conjugates were visualized using ChemiDoc XRS Plus Image Lab System (BIO-RAD, California, USA).

2.4 Results and discussion

2.4.1 Synthesis of PEG-2-nitrobenzenesulfonamide-siRNA conjugates (PEG-sul-siRNA)

The synthesized methyl 4-(cholorosulfonyl)-3-nitrobenzoate (1.5368 g, yield 70.8%) was characterized from ^{1}H NMR spectrum in $CDCl_3$, which exhibited the peak of 8.48-8.44 contributed by 1H (Ar); 8.35-8.33 contributed by 2H (Ar); 4.04 contributed by 3H (Methyl). The result indicated the methyl 4-(chlorosulfonyl)-3-nitrobenzoate was successfully synthesized (Figure 2-1).

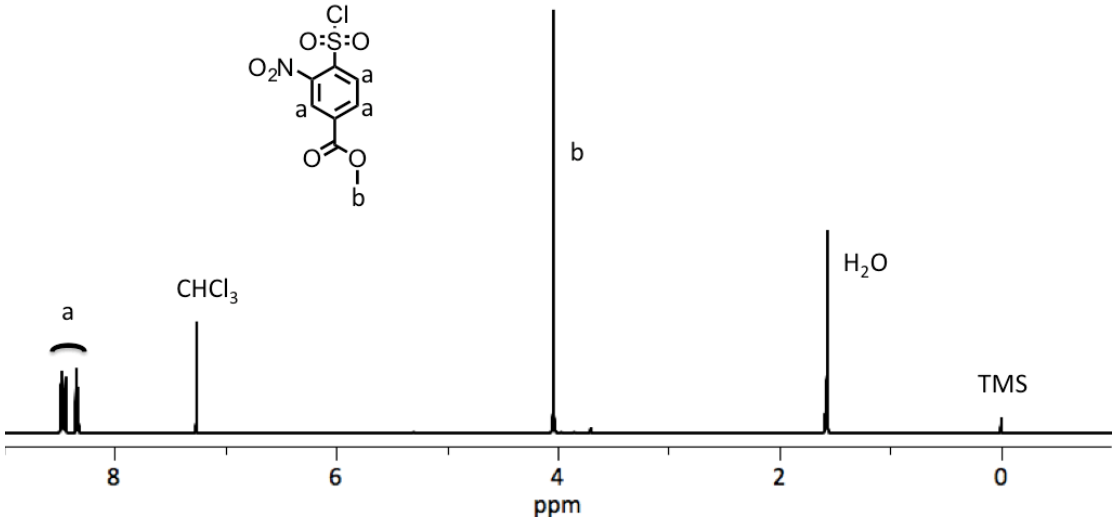


Figure 2-1. ^{1}H-NMR spectrum of methyl 4-(chlorosulfonyl)-3-nitrobenzoate.

Further, the PEG derivative containing methyl 3-nitrobenzoate at the terminus via sulfonamide linkage was obtained as whiter powder (190.3 mg, 88.9%). The successful modification was confirmed by ^{1}H NMR analysis, as suggested by methyl peak (peak b in Figure 2-2) appearance at 3.2 ppm (Figure 2-2). Then, the further hydrolysis of methyl ester at the terminus of the obtained PEG derivatives to methoxy-PEG derivatives containing 3-nitrobezoic acid at the terminus via sulfonamide linkage was obtained as white powder (156.8 mg,

87.1%). The complete hydrolysis of methyl benzoate was confirmed by the disappearance of the proton signal of methyl protons at 4.0 ppm in ^{1}H NMR spectrum (Figure 2-3). The dibenzocyclooctyne (DBCO) moiety was introduced to obtain methoxy-PEG-sulfonamide-DBCO (PEG-sul-DBCO) as white powder (117.4 mg, 79.6%). Also, the successful DBCO introduction was confirmed by ^{1}H NMR analysis, as suggested by the peak appearance at 7.3-7.7 ppm (Figure 2-4).

Finally, via the reaction of copper free click chemistry between dibenzocyclooctyne and azide, the desired product of PEG-sul-5'-siRNA conjugate was successfully purified and collected by the HPLC system (Figure 2-5).

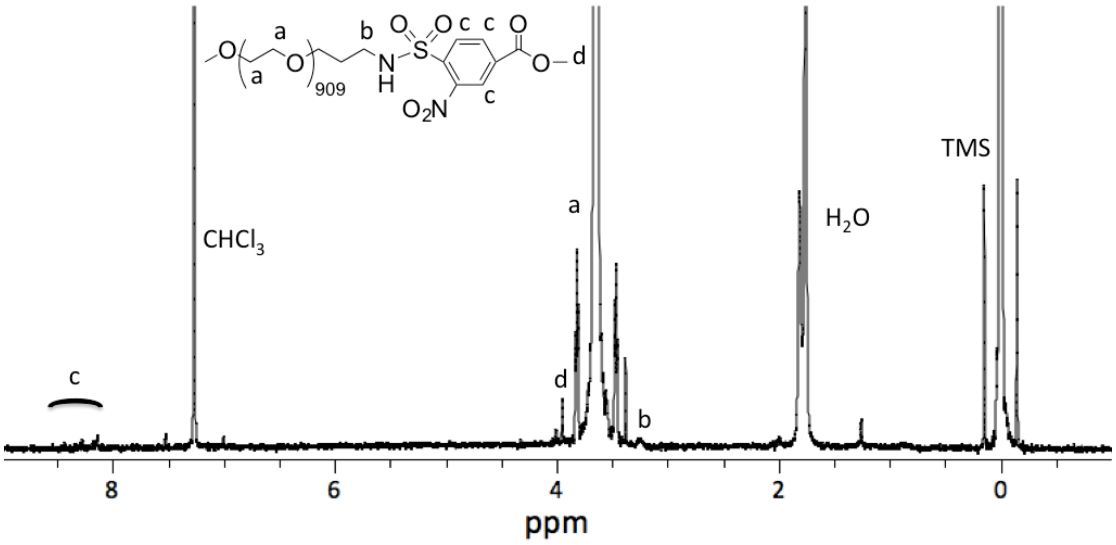


Figure 2-2. ^{1}H NMR spectrum of PEG (40k) derivative containing methyl 3-nitrobenzoate at the terminus.

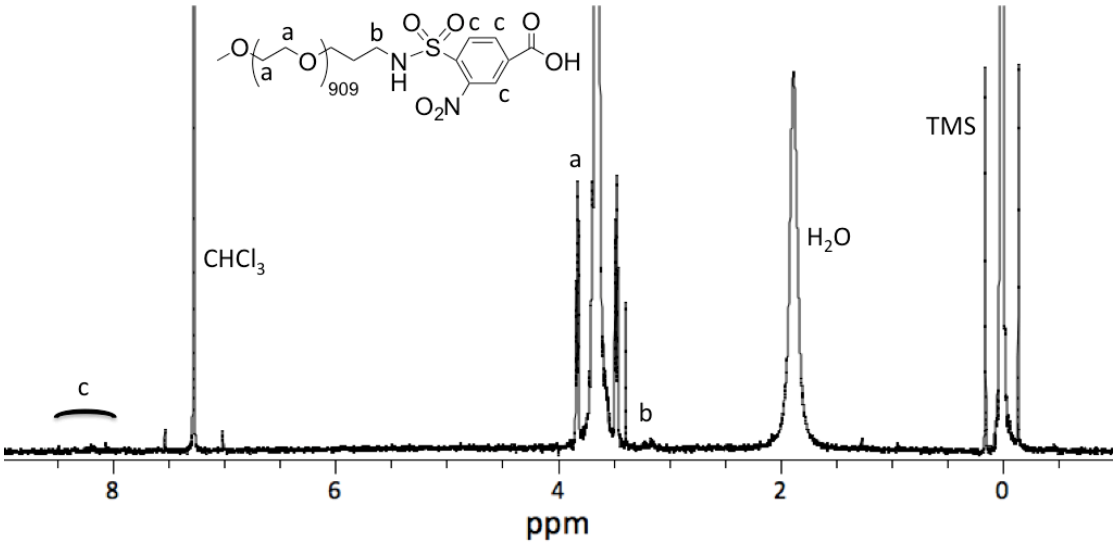


Figure 2-3. ^{1}H NMR spectrum of PEG (40k) derivative after hydrolysis of methyl ester.

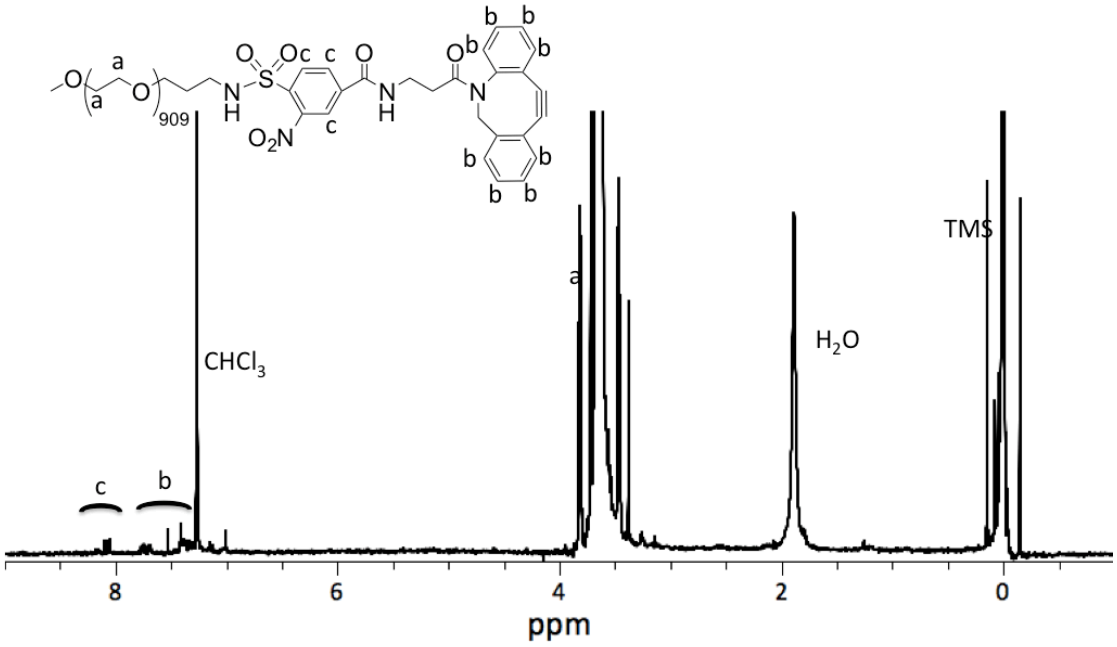


Figure 2-4. ^{1}H NMR spectrum of PEG-sul-DBCO.

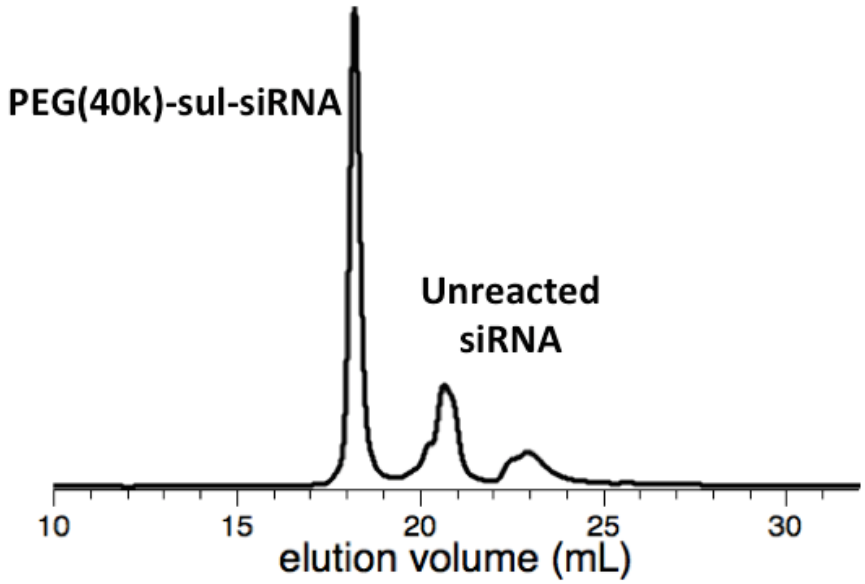


Figure 2-5. HPLC chart of the reaction solution of PEG-sul-DBCO and azide-siRNA.

2.4.2 Synthesis of PEG-carboxylic amide-siRNA conjugates (PEG-car-siRNA)

The PEG-car-DBCO was obtained as white powder (80.8 mg, 69.5%) and successful DBCO introduction was confirmed by ^{1}H NMR analysis, as indicated by the presence of the peaks at 7.2, 7.4 and 7.5 ppm (Figure 2-6). Then, via the same reaction of copper free click chemistry between dibenzocyclooctyne and azide, the desired product of PEG-car-5'-siRNA conjugate was successfully purified and collected by the HPLC system (Figure 2-7).

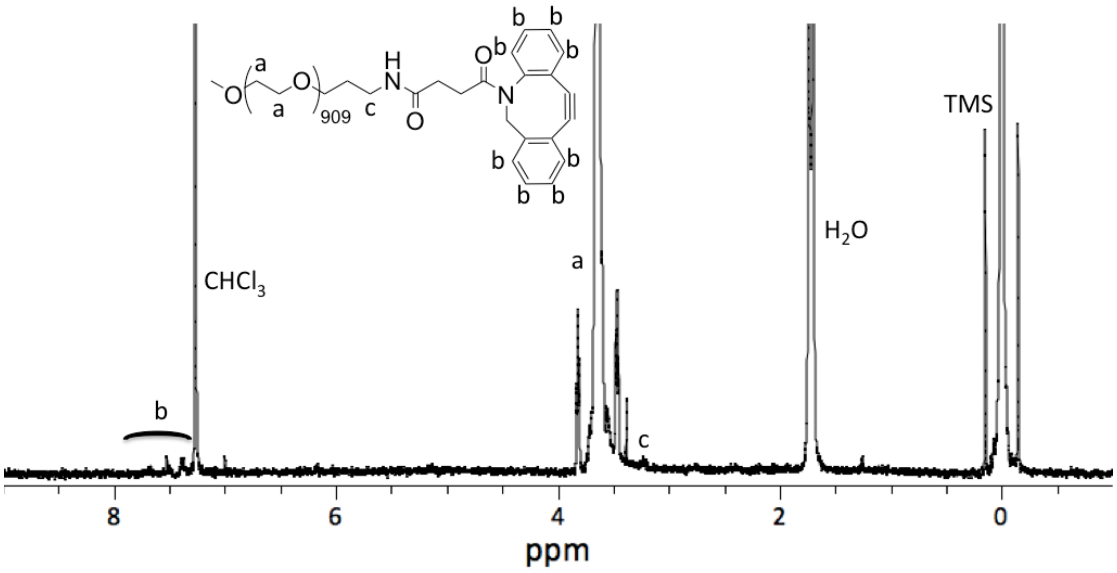


Figure 2-6. ^{1}H NMR spectrum of PEG-car-DBCO.

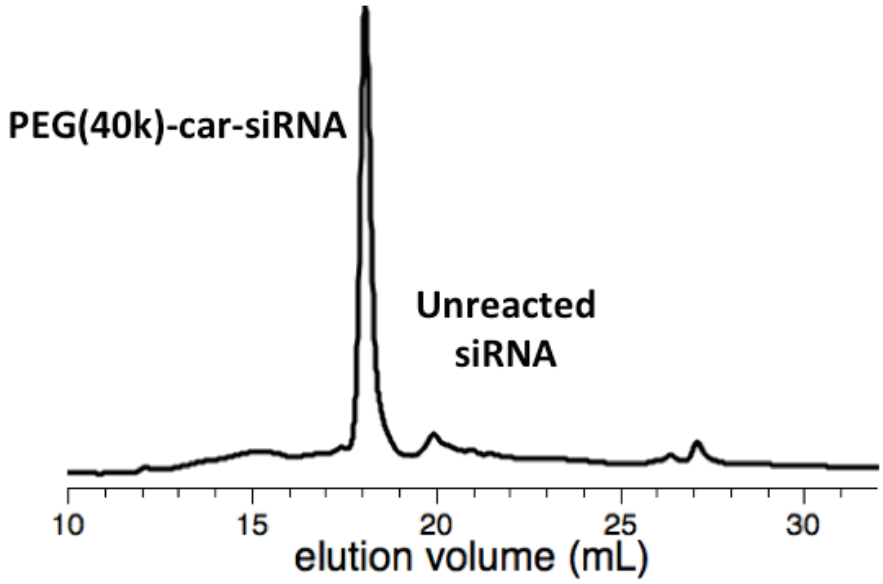


Figure 2-7. HPLC chart of the reaction solution of PEG-car-DBCO and azide-siRNA.

2.4.3 Synthesis of PEG-disulfide-siRNA conjugates (PEG-disulfide-siRNA)

The lyophilized PEG-disulfide-COOH was obtained as white powder (87.5 mg, 83.1%) and the successful introduction was confirmed by ^{1}H NMR analysis,

as suggested by the peak appearance at 2.6 - 3.0 ppm (Figure 2-8). Further, the PEG-disulfide-DBCO was obtained as white powder (45.5 mg, 70.9%). Also, the successful DBCO introduction was confirmed by ^{1}H NMR analysis, as suggested by the peak appearance at 7.3 – 7.7 ppm (Figure 2-9). Finally, the desired product of PEG-disulfide-siRNA conjugate was successfully purified and collected as the same manner by the HPLC system (Figure 2-10).

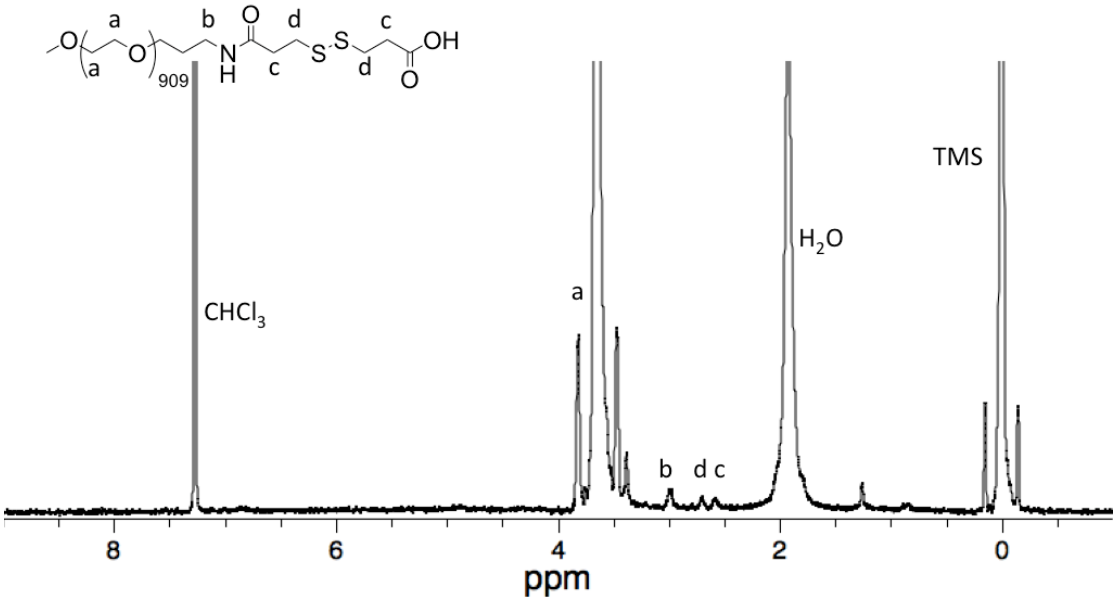


Figure 2-8. ^{1}H NMR spectrum of PEG-disulfide-COOH.

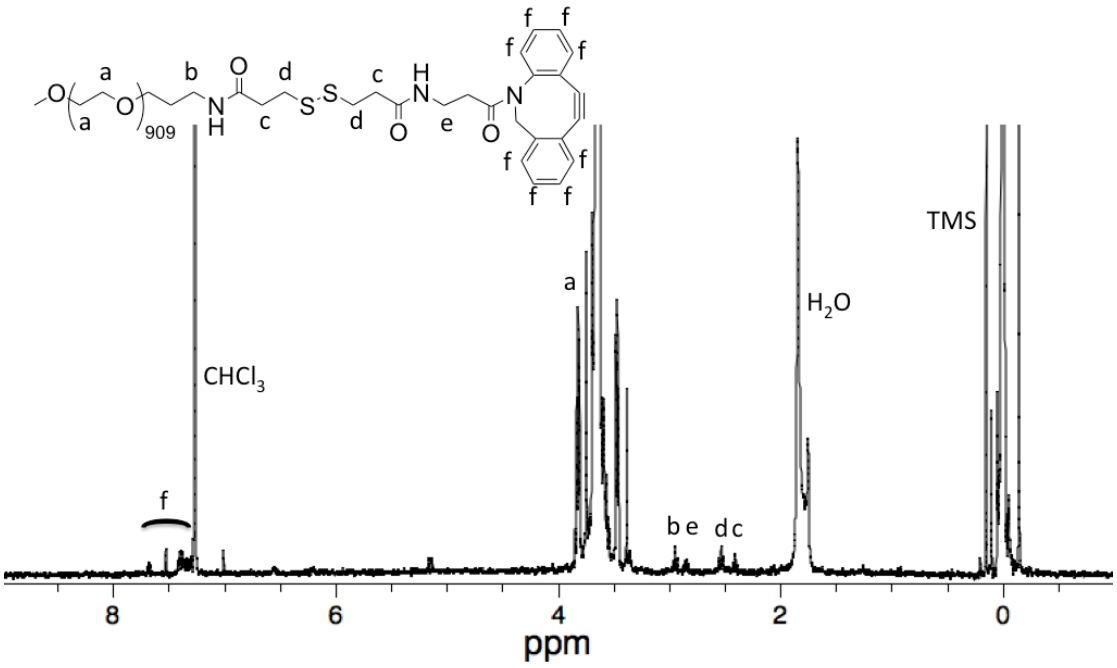


Figure 2-9. ^{1}H NMR spectrum of PEG-disulfide-DBCO.

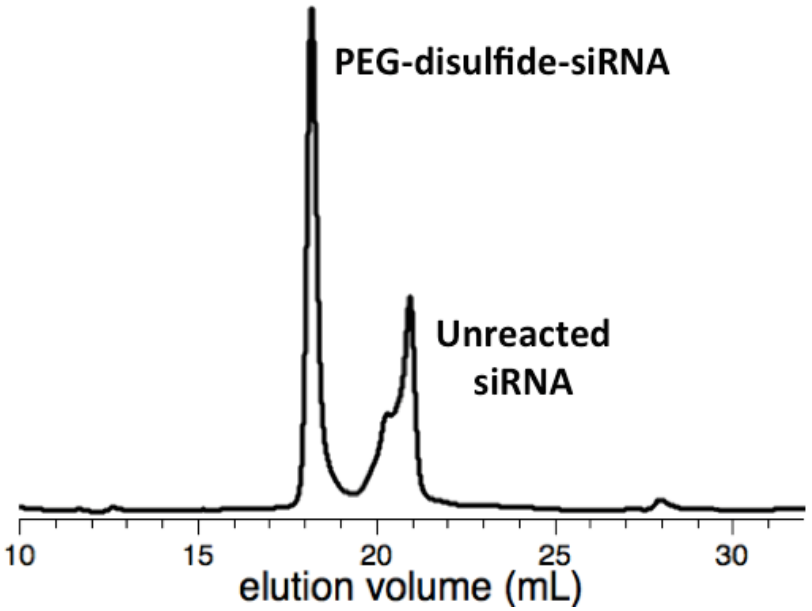


Figure 2-10. HPLC chart of the reaction solution of PEG-disulfide-DBCO and azide-siRNA.

2.4.4 Agarose gel electrophoresis

The conjugates were analyzed by agarose gel electrophoresis, and the successful synthesis of PEG-siRNA conjugates with different linkages were confirmed. The PEG-siRNA conjugates showed retarded migration profiles due to the increased molecular weight (M.W.~53000) and hydrodynamic radius by PEG, whereas unconjugated siRNA showed longer migration profile according to its net negative charges and lower molecule weight (M.W.~13000). Furthermore, there was no difference of migration profile among the PEG-siRNA conjugates regardless of the linkages. The result indicates that PEG-siRNA conjugates with different linkages were synthesized and purified successfully via previously described procedures (Figure 2-11). In addition, the purified PEG-siRNA conjugates were also characterized by size exclusion chromatography [column: Superdex 75 (GE Healthcare), detection: UV detection at 260 nm, eluent: 10 mM

HEPES (pH 7.4) with 150 mM of sodium chloride], and the obtained result also indicated the successful synthesis and purified procedures of PEG-siRNA conjugates (Figures 2-12, 13, and 14).

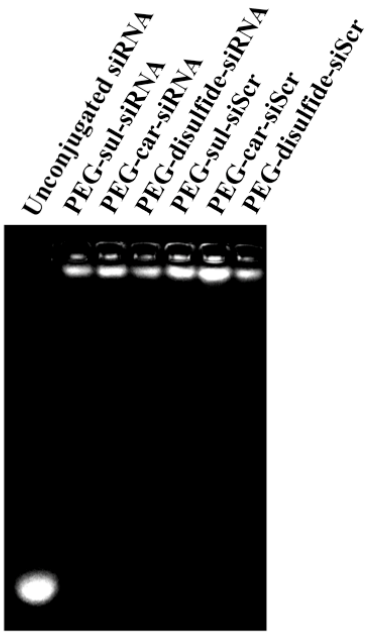


Figure 2-11. Agarose gel electrophoresis of PEG-siRNA conjugates.

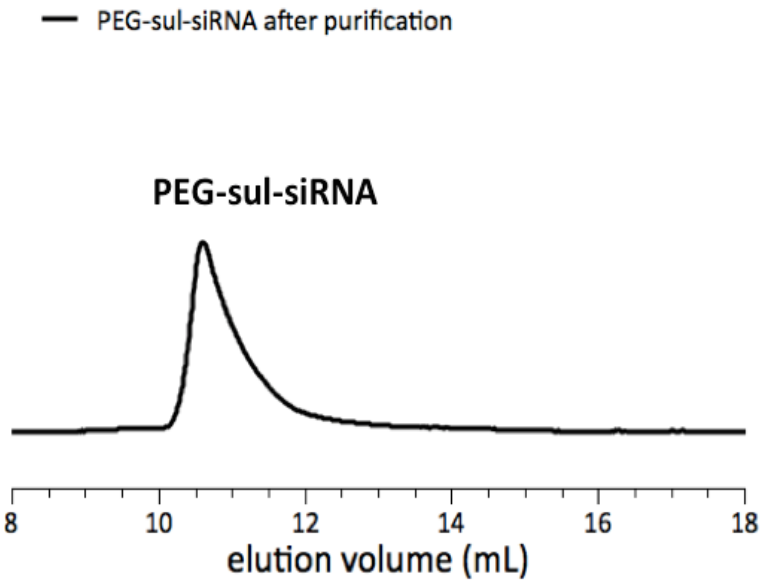


Figure 2-12. SEC chart (UV detection at 260 nm) of PEG-sul-siRNA after purification process.

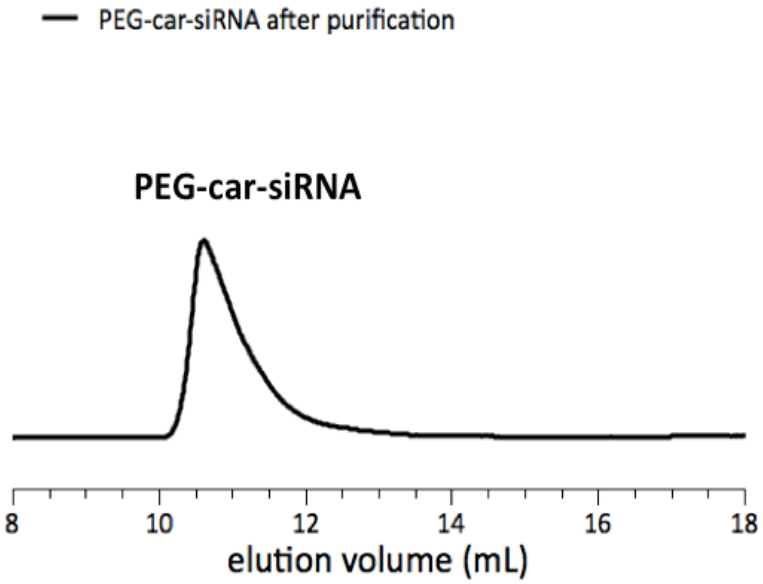


Figure 2-13. SEC chart (UV detection at 260 nm) of PEG-car-siRNA after purification process.

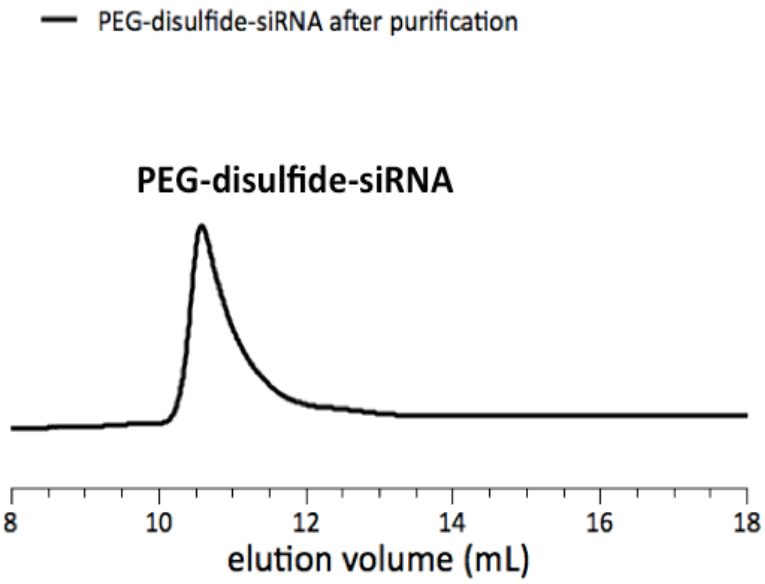


Figure 2-14. SEC chart (UV detection at 260 nm) of PEG-disulfide-siRNA after purification process.

2.5 Conclusion

In this chapter, the PEG-DBCO derivatives, having

2-nitrobenzenesulfonamide, carboxylic amide, and disulfide group, were successfully synthesized and carefully characterized by ^{1}H NMR. Further, the PEG-siRNA conjugates with linkages of 2-nitrobenzenesulfonamide, carboxylic amide, and disulfide groups, were successfully synthesized, purified by ion-exchange HPLC system, and analyzed via agarose gel electrophoresis and size exclusion chromatography. The results suggested the good purity of synthesized PEG-siRNA conjugates which would have no influence on the further evaluations. PEG-car-siRNA and PEG-disulfide-siRNA were selected as the controls, due to the non-cleavability of carboxylic amide group and conventional redox-sensitive linkage of disulfide group, respectively. Thereby, the role of 2-nitrobenzenesulfonamide group in response to GSH and GST, which was incorporated into polymer-siRNA conjugate, will be evaluated to serve as an alternative candidate of reductive linkage for polymer-siRNA conjugates.

Next, the cleavage assay of PEG-siRNA conjugates in the mimicked cellular environments, the physical properties of PEG-siRNA conjugates, and biological evaluations will be described in the following chapters.

2.6 References

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Chapter 3

Evaluation of PEG-siRNA conjugate containing different linkages

3.1 Introduction

In the design of PEG-siRNA conjugates containing 2-nitrobenzenesulfonamide group, glutathione (GSH) and glutathione *S*-transferase (GST) play important roles for the cleavage of 2-nitrobenzenesulfonamide group to release siRNA from the conjugates to work its function. Glutathione (GSH) is the most abundant low molecular weight thiol for regulating the redox potential in the animal cells, which is naturally synthesized from its constituent amino acids: glutamic acid, cysteine, and glycine, in the intracellular environment.[1] Several different functions and vital physiological roles were maintained by the abundance of GSH in the cell, which adjust the balance between oxidation and antioxidation.[2] Also, GSH is crucial for

the cellular detoxification, which is required in many aspects of the immune responses (Table 3-1).[3]

Table 3-1. Roles of glutathione in animals

Roles of glutathione (GSH) in animals
Antioxidant defense
Scavenging free radicals and other reactive species
Removing hydrogen and lipid peroxides
Preventing oxidation of biomolecules
Metabolism
Synthesis of leukotrienes and prostaglandins
Conversion of formaldehyde to formate
Production of D-lactate from methylglyoxal
Formation of mercapturates from electrophiles
Formation of glutathione-NO adduct
Storage and transport of cysteine
Regulation
Intracellular redox status
Signal transduction and gene expression
DNA and protein synthesis, and proteolysis
Cell proliferation and apoptosis
Cytokine production and immune response
Protein glutathionylation
Mitochondrial function and integrity

Glutathione transferases (EC 2.5.1.18) have historically been called glutathione *S*-transferase (GST), which are phase II detoxification enzymes that catalyze the nucleophilic attack by glutathione (GSH) on nonpolar compounds that contain an electrophilic carbon, nitrogen, or sulfur atom.[4, 5] In mammals, GST is divided into three major families: cytosolic, mitochondrial, and membrane-bound microsomal. Mammalian cytosolic GSTs are all dimeric of subunits of 199-244 amino acids in length. Based on the similarities of amino acid sequence, cytosolic family is further designated into seven classes, alpha, mu,

omega, pi, sigma, theta, and zeta.[6, 7] There are 16 cytosolic GSTs subunits existing in human at least. Among seven classes, the alpha and mu classes can form the heterodimers,[5] in which a significant larger number of isoenzymes can be generated from these (Table 3-2).[8] The cytosolic GSTs isoenzymes are majorly found in the cytoplasm. The human transferase, which is closely related to GSTA1-1, has been purified from liver microsomes, and it shows that certain class alpha enzymes have a preference associated with membranes.[9] The GST activity has often been determined via the aid of the chromogenic substrate, 1-chloro-2,4-dinitrobenzene (CDNB), which is suitable for many GSTs. The GST catalyzed formation with CDNB (1-chloro-2,4-dinitrobenzene) forms a dinitrophenyl thioether which can be detected at the absorbance of 340 nm via UV absorptiometer.[10]

Table 3-2. Cytosolic GSTs

Class	***Gene***	***Chromosome location***
Alpha	GSTA1	6p12
	GSTA2	6p12.2
	GSTA3	6p12
	GSTA4	6p12
	GSTA5	6p12.1
Mu	GSTM1	1p13.3
	GSTM2	1p13
	GSTM3	1p13.3
	GSTM4	1p13.3
	GSTM5	1p13.3
Omega	GSTO1	10q25.1
	GSTO2	10q25.1
Pi	GSTP1	11q13-qter
Theta	GSTP1	22q11.2
	GSTT2	22q11.2
Zeta	GSTZ1	14q24.3

The GST activity is highly related to cancer cells. Overexpression of GST in mammalian tumor cells is implicated with resistance to anticancer drugs and thought to be a good target of cancer therapies.[11] In the design of GST inhibitors for increasing the efficacies of chemotherapeutics, one of the best-characterized inhibitor is ethacrynic acid (EA), which acts as a non-competitive inhibitor of GSH for GST binding as well as depleting the GSH cofactor by forming EA-GSH conjugates.[12] The binding to both GST and GSH serves to inhibit enzyme activity. Another GST inhibitor is the GSH conjugate L-γ-glutamyl-(S-9-fluorenylmethyl)-L-cysteinyl-glycine, which is specific to GSTA1-1.[13] This inhibitor has a fluorenylmethyl group and favors to bind to GSTA1-1. L-γ-glutamyl-(S-9-fluorenylmethyl)-L-cysteinyl-glycine was demonstrated to effectively inhibit GSTA1-1, GSTP1-1, and GSTM2-2 with great inhibition efficacies against the alpha isoform.[13]

Another example of prodrug has been designed as inactive agents before approaching the tumor cells and converted to active drug upon exposure to tumor tissues exhibiting high level expression of activating enzymes.[14] The advantage of this approach is minimizing toxicity towards normal tissues whereas increasing delivery of active agents to the tumor tissues. Overexpression of GST in various tumors makes GST as a promising mediate for designing of prodrug achieving treatment purposes. One approach is the designed prodrug containing sulfonamide linkages that activated by GSTs, which acts for a mediate cleavage to sulfonamide linkages.[8] TLK286 [γ-glutamyl-α-amino-β-(2-ethyl-*N, N, N', N*'-tetrakis (2-chloroethyl) phosphorodiamidate)-sulfonyl-propionyl-(R)-(–) phenylglycine], which is a famous candidate of novel prodrug activated in cancer cells by GSTP1.[15] Another series of sulfonylhydrazine prodrugs, KS119

(1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)2-[[1-(4-nitrophenyl) ethoxy] carbonyl] hydrazine) and PNBC (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)2-[[1-(4-nitrophenyl) ethoxy] carbonyl] hydrazine), have been synthesized for utilization of the reductive environment of tumors.[16] Under reduction activation, these prodrugs release the cytotoxic alkylating substance, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl) hydrazine. In the recent study, M. W. van Gisbergen et.al. successfully developed prodrug that are derivatives of anticancer drug, doxorubicin, incorporating sulfonamide moiety. In response to the cleavage by GSH and GSTs, the prodrug released the cytostatic moiety predominantly in GSTs overexpressing cells (Figure 3-1).[17]

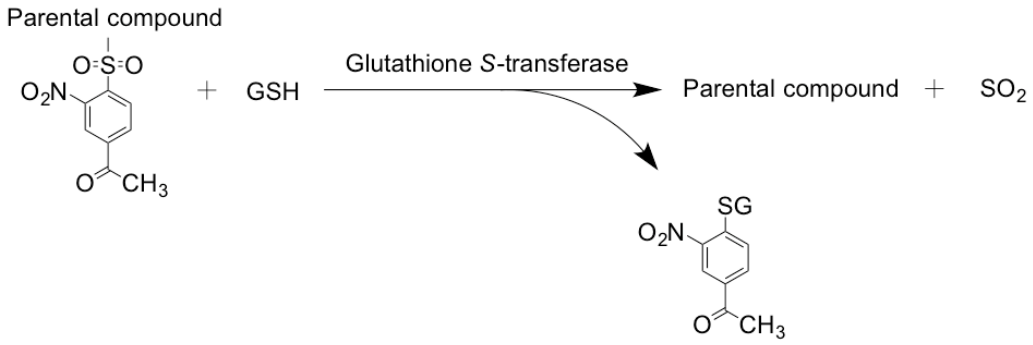

Figure 3-1. Design of produrg (parental compound) with sulfonamide moiety in response to GSH and GST.

In this chapter, in order to demonstrate the utility of 2-nitrobenzenesulfonamide group incorporated into polymer-siRNA conjugates, which releases siRNA in the cell to undergo RNAi pathway, the obtained PEG-siRNA conjugates with different linkages were examined for the extracellular and intracellular stability of each by treatment with GSH and GST, where 2-20 μM GSH was used for mimicked extracellular condition, and 1 mM GSH with/without GSTs for the mimicked intracellular environments. After

determination of stability under extracellular conditions, the cleavability of PEG-siRNA conjugates was also investigated in intracellular conditions. In addition, the stability of PEG-siRNA conjugates with different linkages against enzymatic degradation, including treatment with fetal bovine serum (FBS) and Ribonuclease A (RNase A) were performed. Finally, the stability of PEG-siRNA conjugates with different linkages, which were against light exposure (400-700 nm) and during blood circulation (*in vivo*), were also investigated in this chapter.

3.2 Materials and equipment

3.2.1 Materials

Disodium hydrogenphosphate (Na_2HPO_4) and sodium dihydrogen phosphate dehydrate ($NaH_2PO_4 \bullet 2H_2O$) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Glutathione (reduced form) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Glutathione *S*-transferase from human placenta was purchased from Sigma Aldrich (St. Louis, MO, USA). RNase A was purchased from MACHEREY-NAGEL GmbH & Co. KG (Duren, Germany).

3.2.2 Equipment

Water phase high performance liquid chromatography (HPLC) was performed based on LC-Net II AS2055 system (JASCO, Tokyo, Japan), equipped with Superdex^{TM} 75 10/300GL (GE Healthcare, Illinios, USA), a UV detector at

260 nm, and 10 mM HEPES (pH 7.4) with 150 mM of sodium chloride as an eluent. UV absorbance was determined by UV absorptiometer, V650 spectrophotometer SAH-769 (JASCO, Tokyo, Japan). Fluorescence correlation spectroscopy (FCS) analyses were performed using a confocal laser scanning microscope, LSM710 (Carl Zeiss, Jena, Germany) equipped with a Confocor3 module and a Zeiss C-Apochromat 40x water objective. Ar laser (514 nm) was used for excitation of TAMRA-labeled siRNA and the emission was detected at ~570 nm. Light exposure was performed with light system (400-700 nm, 3 mW/cm^2) at the indicated time. Blood circulation was performed with the mice (BALB/c, male) and determined the fluorescent intensity via fluorometer after intravenous injection.

3.3 Experimental procedures

3.3.1 Cleavage assays in the mimicked cellular environment

The linkage cleavage assays were performed for PEG-sul-siRNA, PEG-disulfide-siRNA, and PEG-car-siRNA conjugates, in the conditions of mimicked intracellular or extracellular environment. In this section, the cleavage assay in the mimicked extracellular environment was performed; 5 μL of PEG-siRNA conjugates (10 μM siRNA concentration, 10 mM HEPES, pH 7.3) was mixed with 495 μL of phosphate buffer (0.1 M, pH 7.4) containing 20 μM GSH, followed by incubation at 37 °C for the indicated periods. For the cleavage

assay in the mimicked intracellular environment, 495 μL of phosphate buffer (0.1 M, pH 7.4) containing 1 mM GSH with/without 0.019 mg/mL of GST was utilized alternatively. The release ratio of siRNA from PEG-siRNA conjugates was analyzed by size exclusion chromatography [column: Superdex 75 (GE Healthcare), detection: UV detection at 260 nm, eluent: 10 mM HEPES (pH 7.4) with 150 mM of sodium chloride]. The release ratio was calculated from the peak intensity ratios of PEG-siRNA and released siRNA.

3.3.2 Enzyme degradation via treatment of FBS

The evaluation of stability of PEG-siRNA conjugates, including PEG-sul-siRNA and PEG-disulfide-siRNA, was performed by treatment with fetal bovine serum (FBS) at the indicated concentration. The conjugates of PEG-sul-5'-TAMRA siGL3, and PEG-disulfide-5'-TAMRA siGL3, were mixed with FBS (1% of final concentration) and incubated for several intervals at 37°C. The solutions were analyzed by fluorescence correlation spectroscopy (FCS) system and calculated by Stokes-Einstein equation, using Rhodamine 6G as the control (Eq. 3.1).

$$D = kT/6\pi r\eta \quad \text{----------Eq. 3.1}$$

Where D is diffusion coefficient [$\mu m^2/\mu s$]

k is Boltzmann constant

T is absolute temperature

r is radius

η is viscosity of liquid

Rhodamine 6G as the control: D is 414 [$\mu m^2/\mu s$] at 25 °C in water

r is 0.6 nm (diameter is 1.2 nm)

3.3.3 Enzyme degradation by RNase A treatment

The further evaluation of stability of PEG-siRNA conjugates was performed using Ribonuclease A (RNase A) with the indicated concentration. The conjugates of PEG-sul-siGL3, PEG-disulfide-siGL3, and free siGL3 (2 μM of final concentration) were added with polycation polymer, PEG *block* poly ornithine (PEG-*b*-POrn, molecular weight: 45,000 Da, DP ~ 37 of poly ornithine, as shown in Figure 3-2) as 5 of N/P ratio, which was determined via amine group (N) contributed by PEG-*b*-POrn and phosphate group (P) contributed by PEG-siRNA, and then added RNase A (20U), further incubated for the indicated time periods at 37 °C. The UV absorbance at wavelength of 260 nm was measured by UV absorptiometer.

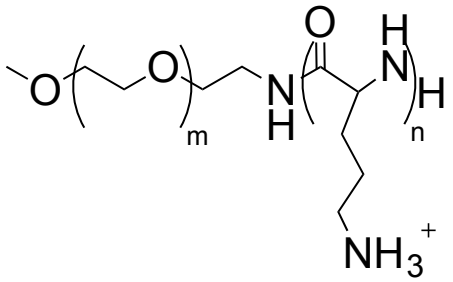

Figure 3-2. Chemical structure of PEG *block* poly ornithine

3.3.4 Stability of PEG-siRNA conjugates upon light exposure

The obtained PEG-sul-siRNA and PEG-disulfide-siRNA conjugates were

exposed to light, in order to compare the resistance to light of the chemical linker. 5 μL of PEG-siRNA conjugates (10 μM siRNA concentration, 10 mM HEPES, pH 7.3) was mixed with 495 μL of phosphate buffer (0.1 M, pH 7.4), followed by light exposure (400-700 nm, 3 mW/cm^2) for the indicated periods. The release ratio of siRNA from PEG-siRNA conjugates was analyzed by size exclusion chromatography [column: Superdex 75 (GE Healthcare), detection: UV detection at 260 nm, eluent: 10 mM HEPES (pH 7.4) with 150 mM of sodium chloride]. The release ratio was calculated from the peak intensities of PEG-siRNA and released siRNA and reposted as mean ± SD based on three independent experiments.

3.3.5 Stability of PEG-siRNA conjugates during blood circulation (*in vivo*)

The PEG-siRNA conjugates containing Cy5-labeled siRNA, i.e., PEG(40k)-sul-5'-siRNA (5'-Cy5-modified siRNA), PEG-car-5'-siRNA (5'-Cy5-modified siRNA), and PEG-disulfide-5'-siRNA (5'-Cy5-modified siRNA) were synthesized as the same procedures in the previous chapter 2. 6 mg of PEG-sul-DBCO, PEG-car-DBCO, or PEG-disulfide-DBCO (0.00015 mmol) was dissolved in 500 μL of HEPES (10 mM; pH 7.3). The 65 μL of siRNA (5'-Cy5-modified) (3 mg/mL) was added to the solution of PEG-sul-DBCO, PEG-car-DBCO, or PEG-disulfide-DBCO, and followed by adding 335 μL of HEPES (10 mM; pH 7.3). The solutions were frozen at -20 °C for overnight and then thawed at 4 °C for 1 hour. The solutions were subsequently characterized and purified via HPLC, where LC-Net II AS2055 system (JASCO, Tokyo, Japan)

was equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260nm, and 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride as eluents.

The evaluation of blood circulation (*in vivo*) was performed by the synthesized PEG-siRNA containing Cy5-labeled dye, with the mice (BALB/c, male), followed by tail-vein injection. The PEG-sul-5'-siRNA (5'-Cy5-modified siRNA), PEG-car-5'-siRNA (5'-Cy5-modified siRNA), and PEG-disulfide-5'-siRNA (5'-Cy5-modified siRNA) (20 μg / 200 μL per single injection) were complexed with polycation polymer, PEG *block* poly ornithine (PEG-*b*-POrn, molecular weight: 45,000 Da, DP ~ 37 of poly ornithine), PEG *block* poly ornithine (PEG-*b*-POrn) as 5 of N/P ratio, and then followed by tail-vein injection. The blood of each mouse was collected at the indicated time, and then the fluorescent intensity was measured by fluorometer.

3.4 Results and discussion

3.4.1 Cleavage assays in the mimicked intracellular and extracellular environments

The results of SEC analyses revealed that the peak of PEG-siRNA conjugates with either 2-nitrobenzenesulfonamide or disulfide linkages appeared around the elution volume of 11 mL, followed by the peak of released siRNA from PEG-disulfide-siRNA conjugate around the elution volume of 14.5 mL (6 hours of incubation time with 20 μM of GSH). This exhibited that disulfide system induced siRNA release, whereas no peak of released siRNA from

PEG-sul-siRNA conjugate was observed under the same conditions (Figure 3-3a). Moreover, the enhancement of siRNA release from PEG-disulfide-siRNA conjugate was observed via prolonging the incubation time to 72 hours, whereas nearly no release of siRNA from PEG-sul-siRNA conjugate was observed at the same incubation conditions. The results indicated that the higher stability of PEG-sul-siRNA conjugates than the conventionally used disulfide linkage under mimicked extracellular environment (Figure 3-3c).

When PEG-siRNA conjugates were subjected to the mimicked intracellular environment with higher concentration of GSH (1 mM) for the indicated incubation time (6 hours), the results of SEC analyses, shown in Figure 3-3b, exhibited the peaks of released siRNA from the PEG-siRNA conjugate, similar to incubation in the mimicked extracellular condition. In PEG-disulfide-siRNA conjugate system, almost no PEG-siRNA was observed under high concentration of GSH (1 mM), indicating the quick release of siRNA in disulfide system. This quick release suggests the instability of conventional disulfide linkage possibly leading to unexpected release of siRNA within short incubation time. An extension of incubation time to 72 hours under high concentration of GSH (1 mM) increased the released siRNA from PEG-sul-siRNA conjugate increased by prolonging incubation time (Figure 3-3d).

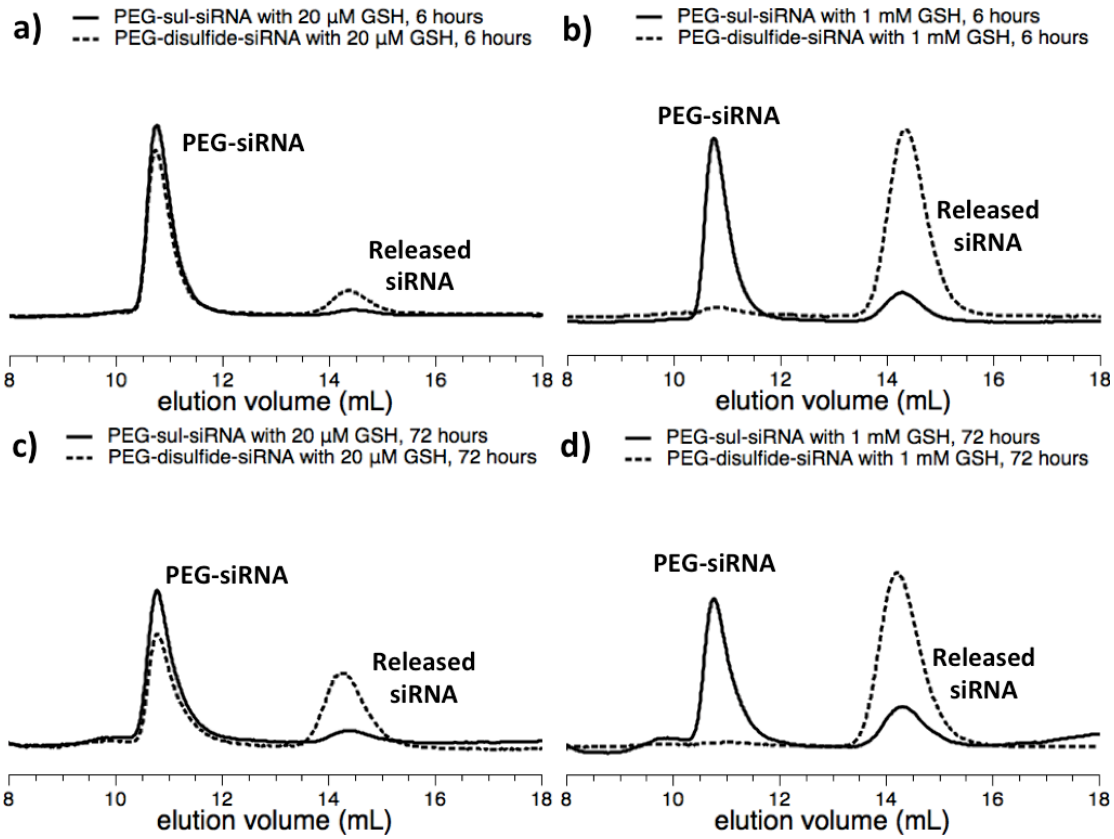

Figure 3-3. SEC chart of solution in cleavage assay of PEG-siRNA conjugates followed by indicated conditions in 0.1 M PB at pH 7.4, a) 20 μM GSH b) 1 mM GSH with 6 hours; c) 20 μM GSH d) 1 mM GSH with 72 hours.

The release ratios of siRNA from PEG-siRNA conjugates were calculated from the intensity of the peaks after the indicated incubation time, including 6 hours, 24 hours, 48 hours, and 72 hours, as shown in Figure 3-4. The PEG-disulfide-siRNA system induced considerable amount of siRNA release (~40%, 72 hours) from PEG-disulfide-siRNA in a mimicked extracellular environment (20 μM GSH, pH 7.4, 37 °C), whereas the dramatically suppressed siRNA release from PEG-sul-siRNA system (~7%, 72 hours), as analyzed by size exclusion chromatography. The result of suppressed siRNA release from PEG-sul-siRNA system suggests that the undesired cleavage of 2-nitrobenzenesulfonamide group and the loss of the conjugated polymer-related

functionality can be reduced, compared to a conventional disulfide linkage outside the cells. Moreover, the siRNA release from PEG-sul-siRNA system was 20% after 72 hours incubation, in the mimicked intracellular environment (1 mM GSH, pH 7.4, 37 °C) (Figure 3-4b). It was primarily due to the concentrated GSH environment. In PEG-disulfide-siRNA system, the release of siRNA was nearly completed within 6 hours (1 mM GSH, pH 7.4, 37 °C) (Figure 3-4a). The rapid release of siRNA from PEG-disulfide-siRNA system suggests that disulfide linkage would be cleaved immediately after entering the cell, as well as would induce unexpected siRNA release before reaching the cytoplasm.

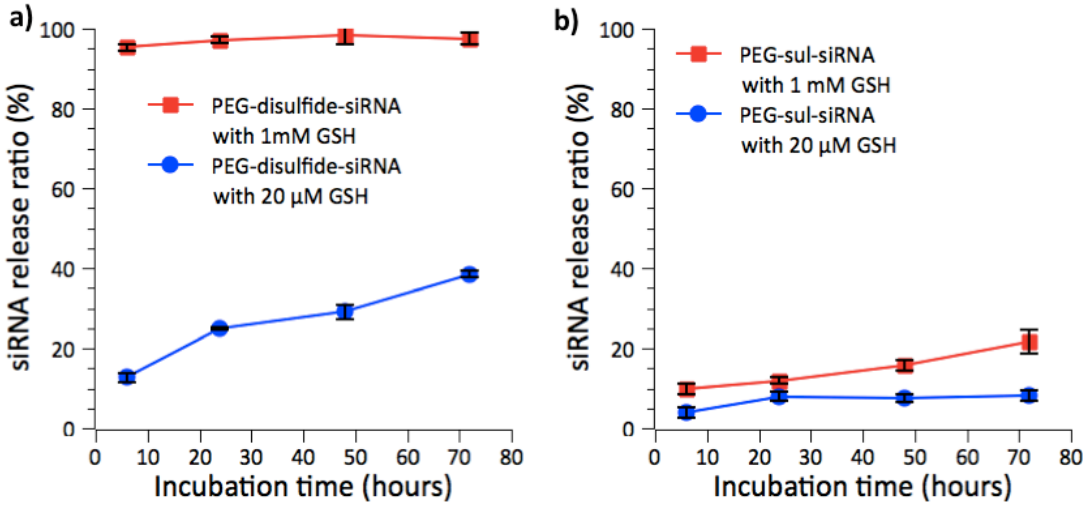

Figure 3-4. Release ratio of siRNA in cleavage assay with 1 mM GSH of mimicked intracellular environment and 20 μM GSH of mimicked extracellular environment a) PEG-disulfide-siRNA b) PEG-sul-siRNA. The siRNA release ratios were reported as mean ± SD of three independent experiments (n = 3).

3.4.2 Cleavage assays in the mimicked intracellular environment with presence of GST

In order to demonstrate the enhanced siRNA release from PEG-sul-siRNA conjugates in response to GSH and GST, which is overexpressed in tumor cells,

the PEG-sul-siRNA conjugate was treated with 1 mM of GSH and 0.019 mg/mL of GST for 72 hours at 37 °C for mimicking the environment of cancer cells. The release of siRNA from PEG-sul-siRNA was also determined via size exclusion chromatography (Superdex 75 anion, GE Healthcare, Illinois, USA) with UV detector at wavelength of 260 nm. The results of SEC analyses revealed that the peak of PEG-sul-siRNA conjugate after incubation with either GSH or co-incubation with GST and GSH appeared at the elution volume of 11 mL, followed by the peak of released siRNA from conjugates at the elution volume of 14.5 mL after 72 hours incubation (Figure 3-5). The siRNA release ratios of siRNA from PEG-sul-siRNA conjugates were calculated from the intensity of the peaks as the same as previous section based on three independent experiments (n = 3) (Figure 3-6). It is worth to note that siRNA release from PEG-sul-siRNA system was significantly facilitated in the presence of GST (~90% of siRNA was released after 72 hours incubation), whereas ~20% of siRNA release was induced in the absence of GST. The result indicates the key role of GST catalysis for GSH and strongly supports the enhanced cleavage of 2-nitrobenzenesulfonamide group toward the effective siRNA release in the environment of cancer cells and suggests the cleavage of 2-nitrobenzenesulfonamide group is highly specific to the presence of GST, such as cytoplasm in cancer cells (Figure 3-7). In comparison with the non-cleavable linkage, the series of PEG-car-siRNA, no siRNA release was observed in the presence of 1 mM GSH (pH 7.4, 37 °C) and additional presence of GST (1 mM GSH, 0.019 mg/mL GST, pH 7.4, 37 °C) (Figure 3-8). The results clearly demonstrated the cleavability of 2-nitrobenzenesulfonamide group included in PEG-sul-siRNA in response to GSH and GST in the mimicked intracellular manner, whereas non-cleavable

ability of carboxylic amide group included in PEG-car-siRNA, was essential for the released siRNA in the further biological evaluations.

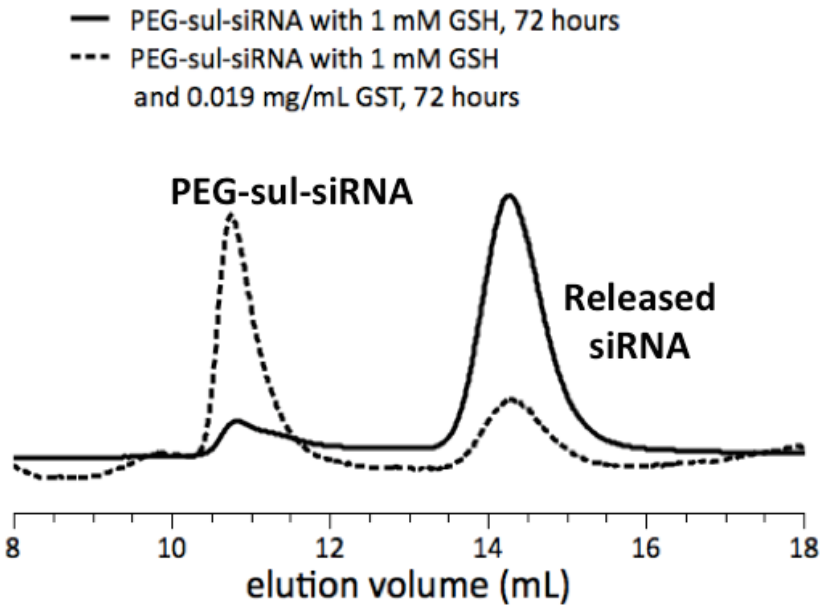

Figure 3-5. SEC chart of solution in cleavage assay of PEG-sul-siRNA conjugates followed by 72 hours incubation with 1 mM GSH and with/without 0.019 mg/mL GST for mimicking intracellular environment.

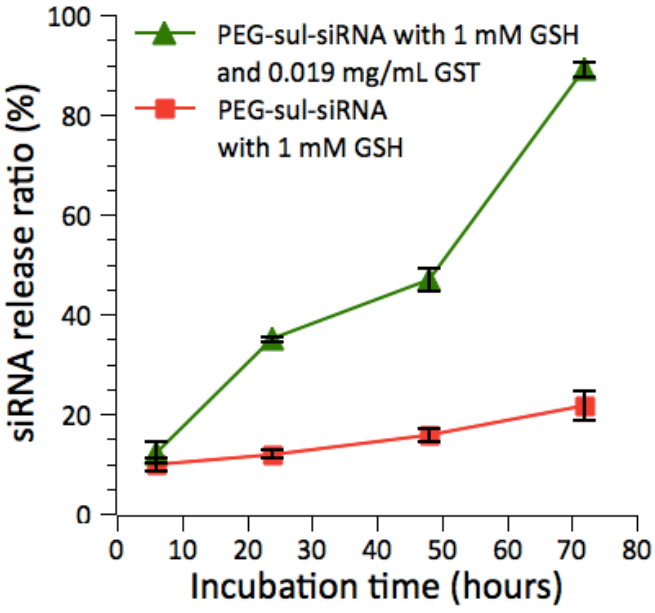

Figure 3-6. Release ratio of siRNA via cleavage assay with 1 mM GSH of mimicked intracellular environment and 20 μM GSH of mimicked extracellular environment a) PEG-disulfide-siRNA b) PEG-sul-siRNA. The siRNA release ratios were reported as mean ± SD of three independent experiments (n = 3).

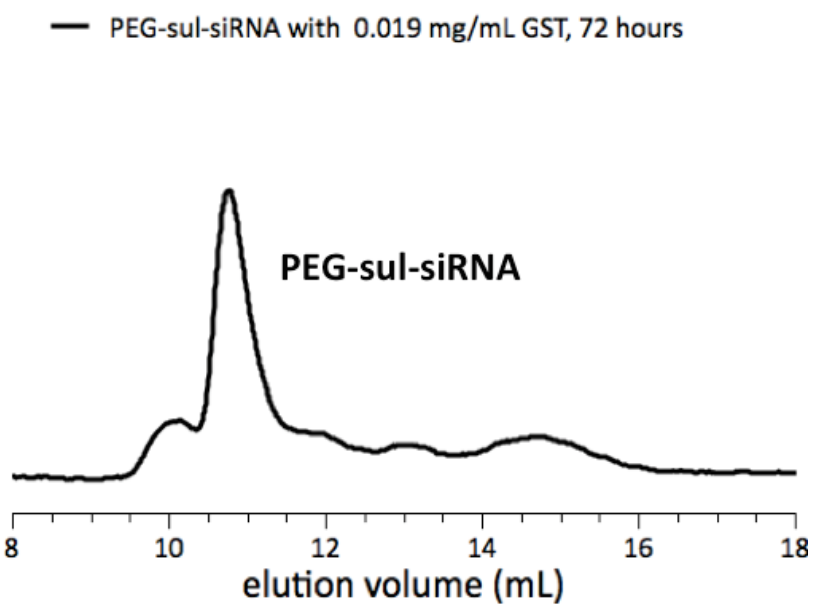

Figure 3-7. SEC chart of solution in cleavage assay of PEG-sul-siRNA conjugates followed by 72 hours incubation with 0.019 mg/mL GST.

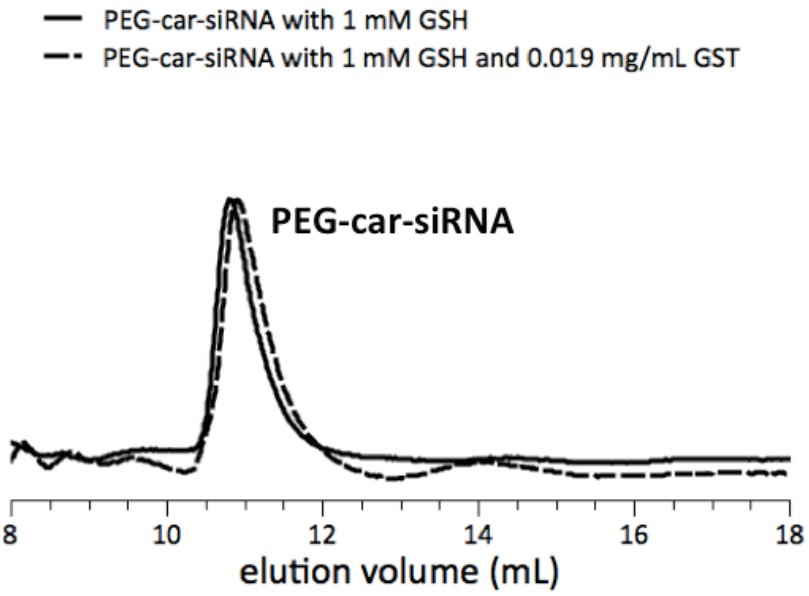

Figure 3-8. SEC charts (UV detection at 260 nm) of PEG-car-siRNA after 72 hours incubation with 1 mM GSH and with/without 0.019 mg/mL GST.

3.4.3 Cleavage assays in the highly reductive condition (10 mM GSH)

For highly reductive condition, 10 mM GSH condition was also performed and it facilitated the siRNA release from PEG-sul-siRNA (pH 7.4, 37 °C), compared to 1 mM of GSH condition, as expected in Figure 3-9, indicating the concentrated GSH can induce the cleavage of 2-nitrobenzensulfonamide group. The 50% of siRNA was released after 6 hours incubation, and the siRNA release was almost completed within 72 hours, which was the similar siRNA release ratio (~ 90%) in the condition of incubation with 1 mM GSH and 0.019 mg/mL GST after 72 hours incubation. However, siRNA release was not detected in PEG-car-siRNA system (non-cleavable carboxylic amide linkage) in the presence of 10 mM GSH (pH 7.4, 37 °C) and the additional presence of GST (10 mM GSH, 0.019 mg/mL GST, pH 7.4, 37 °C) (Figure 3-10). The result further confirms that 2-nitrobenzenesulfonamide group can be cleaved by GSH.

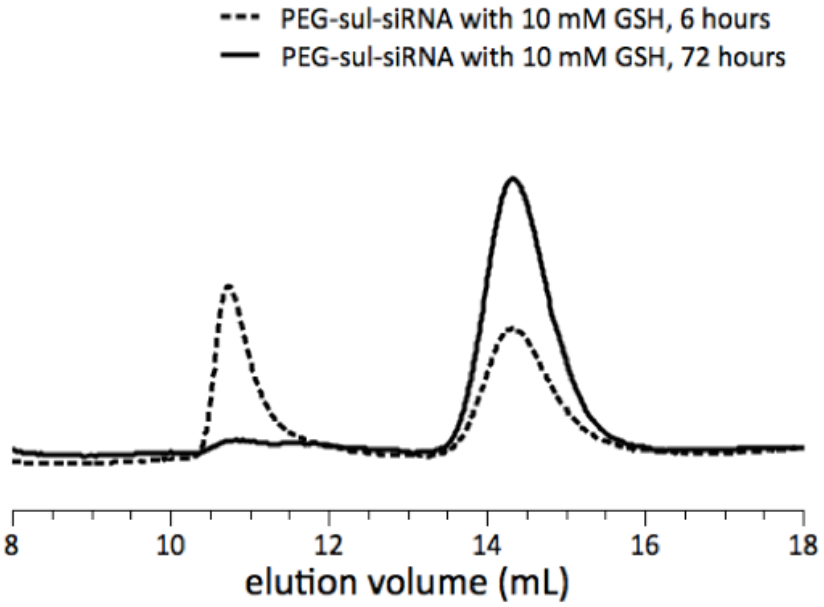

Figure 3-9. SEC chart (UV detection at 260 nm) of PEG-sul-siRNA after 6 hours and 72 hours incubation with 10 mM GSH.

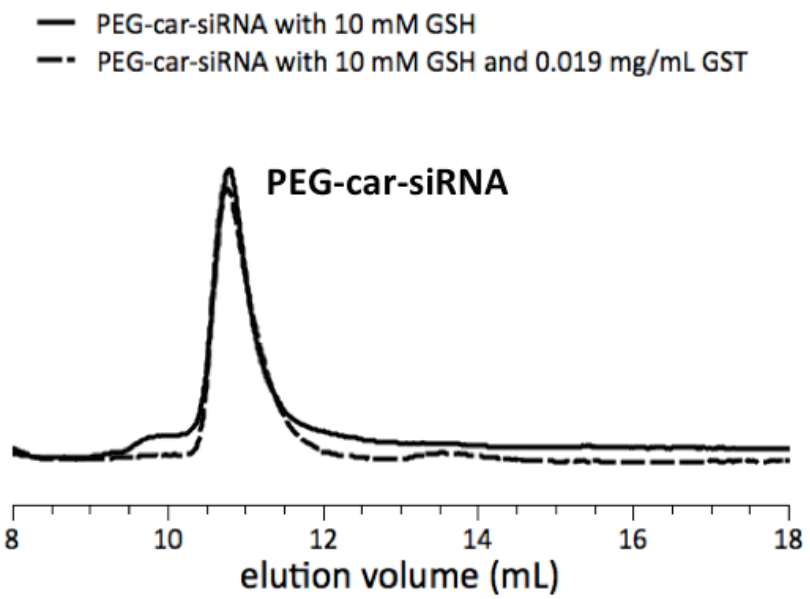

Figure 3-10. SEC chart (UV detection at 260 nm) of PEG-car-siRNA after 72 hours incubation with 10 mM GSH and with/without 0.019 mg/mL GST.

3.4.4 Enzyme degradation via treatment of FBS

From the equation, diffusion time [μs] were obtained by FCS, which is related to the size (diameter) of conjugates via calculation, revealed the PEG-sul-TAMRA siGL3 and PEG-disulfide-TAMRA siGL3 have the similar diameter ~12 nm before incubation with FBS (Table 3-3). Also, in comparison with the unconjugated siRNA, the PEG-sul-5'-TAMRA siGL3 and PEG-disulfide-5'-TAMRA siGL3 conjugates exhibited ~3 folds larger size after forming PEG-siRNA conjugates, which could be acted as protecting shells against enzymatic degradation.[18] After incubating with FBS (1% of final concentration), the size of PEG-sul-siRNA and PEG-disulfide-siRNA conjugates decreased, while increasing incubation time, as shown in Figure 3-11. The PEG-disulfide-siRNA series exhibited a decrease of diameter (85%) after 3 hours

incubation, whereas a 78% decrease of diameter was observed for the PEG-sul-siRNA series. It was probably because the instability of disulfide linkage induced the unexpected cleavage as well as siRNA degradation, compared to PEG-sul-siRNA. The results suggest the 2-nitrobezenesulfonamide group was suitable for construction of polymer-conjugated siRNA system toward better resisting ability against enzymatic degradation, as well as maintained the conjugated form for achieving protecting ability.

Table 3-3. Diffusion time (μs) and diameter (nm) of PEG-siRNA conjugates with at 37 °C via determination of fluorescence correlation spectroscopy (FCS)

	Diffusion time (μs)	**Diameter (nm)**	**STD (nm)**
Rhodamide 6G (R6G)	**22.39**	**1.2**	**---**
Unconjugated siRNA	**83.04**	**4.45**	**±0.58**
PEG-sul-siRNA	**229.02**	**12.27**	**±1.42**
PEG-disulfide-siRNA	**248.81**	**13.33**	**±0.89**

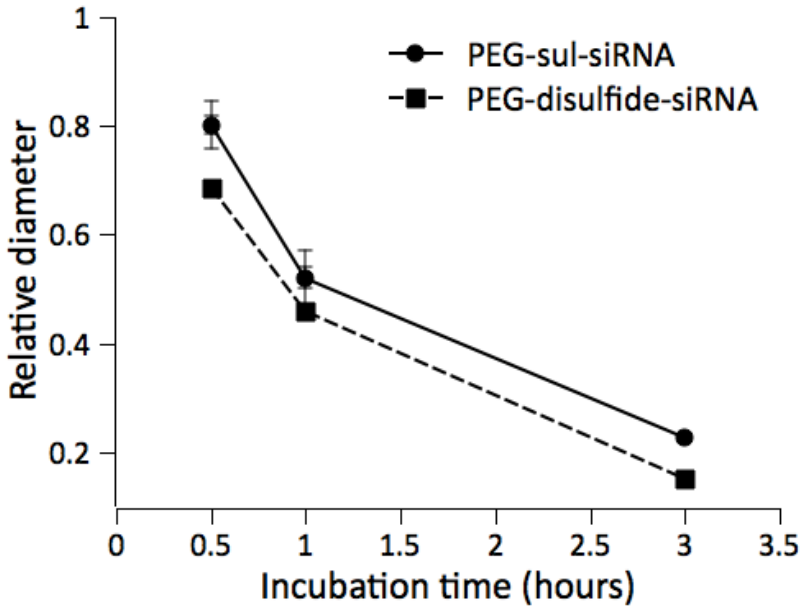

Figure 3-11. Relative diameter of PEG-siRNA conjugates with incubation of FBS (1% of final concentration) at 37 °C via determination of Fluorescence correlation spectroscopy (FCS). The values were reported as mean ± SD of three independent experiments (n = 3).

3.4.5 Enzyme degradation by RNase A treatment

From the results, all of the three series, including PEG-sul-siRNA, PEG-disulfide-siRNA, and unconjugated siRNA, exhibited increases of UV absorbance at 260 nm which was dependent on incubation time (Figure 3-12), due to the treatment of RNase A toward the siRNA into small fragments. The unconjugated siRNA exhibited a ~34 % increase of UV absorbance at 260 nm after 3 hours incubation. It was probably due to siRNA without conjugation to PEG segment, which induced faster increase of UV absorbance, indicated the role of PEG providing the protecting ability against degradation of RNase A treatment, compared to PEG-sul-siRNA and PEG-disulfide-siRNA. Of note, comparing with two different linkages, 2-nitrobenzenesulfonamide and disulfide groups, PEG-disulfide-siRNA exhibited a higher increase of UV absorbance at 260 nm

(~15%), whereas PEG-sul-siRNA system drastically suppressed the increase of UV absorbance at 260 nm (~6%) after 3 hours incubation. It was probably due to the instability of disulfide linkage induced the undesired cleavage, which led to siRNA degradation, compared to PEG-sul-siRNA. RNase A is a class of nuclease that catalyzes the degradation of RNA molecule into small segments.[19, 20] One of the major obstacles for inefficient siRNA delivery is the degradation and inactivation of siRNA by nuclease enzymes. The results suggest that 2-nitrobenzenesulfonamide group, contributed the potent stability, as well as maintaining the conjugated structure against the nuclease degradation toward protecting ability.

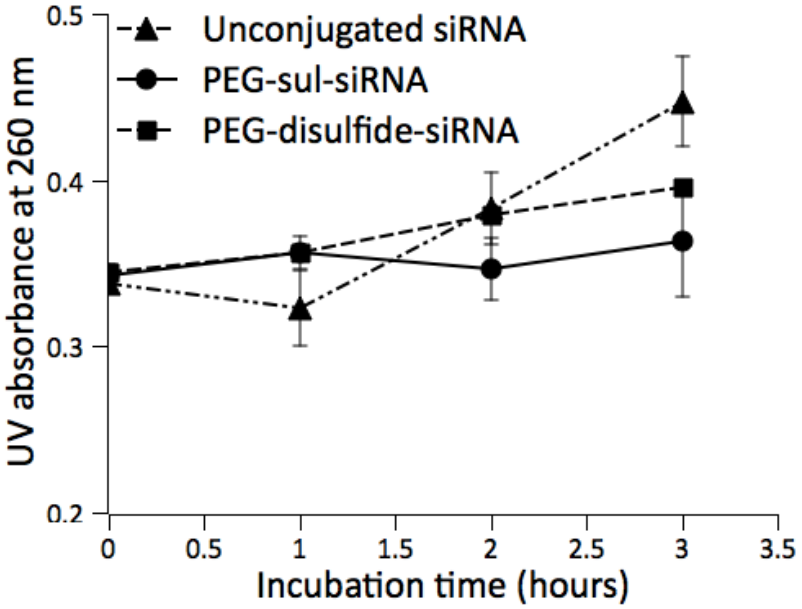

Figure 3-12. Results of stability of PEG-siRNA conjugates and unconjugated siRNA via treatment of RNase A at the indicated time. The values were reported as mean ± SD of three independent experiments (n = 3).

3.4.6 Stability of PEG-siRNA conjugates upon light exposure

It is worthy of note that 2-nitrobenzenesulfonamide group exhibited higher

stability upon exposure to light, compared to disulfide group (Figures 3-13 and 3-14). PEG-disulfide-siRNA system induced ~15% of siRNA release after six hours exposure to light (400-700 nm, 3 mW/cm^2), whereas PEG-sul-siRNA system significantly suppressed siRNA release (~3%), probably due to the radical production upon light excitation leading to photolysis of disulfide linkage. In general, disulfide group can be degraded in response to light exposure during their production, packing, or any other administration processes, thereby carefully handling (or storage) of disulfide-derived materials in a dark is often required.[21] The results suggest the 2-nitorobenzenesulfonamide-related materials system provided the enhanced stability against light exposure and the potency of easy handling, compared to disulfide-related materials.

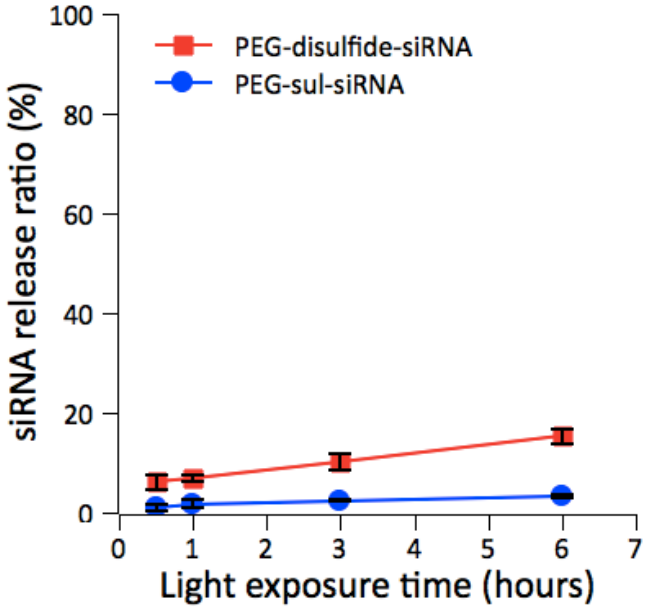

Figure 3-13. Release ratio of siRNA from PEG-sul-siRNA and PEG-disulfide-siRNA upon light exposure (400-700 nm, 3 mW/cm^2) at indicated periods. The siRNA release ratios were reported as mean ± SD of three independent experiments (n = 3).

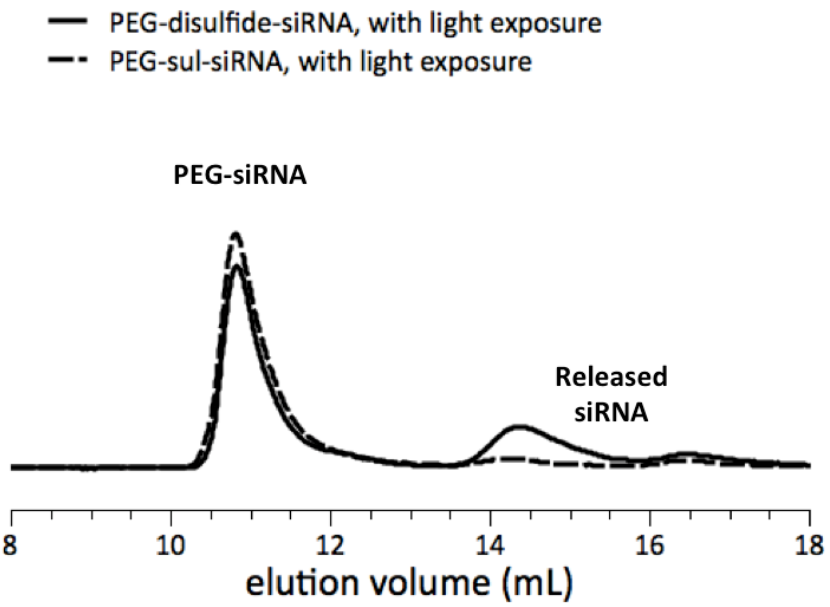

Figure 3-14. SEC chart (UV detection at 260 nm) of PEG-sul-siRNA and PEG-disulfide-siRNA after 6 hours of light exposure (400-700 nm, 3 mW/cm^2).

3.4.7 Stability of PEG-siRNA conjugates during blood circulation (*in vivo*)

The synthesized PEG-siRNA conjugates containing Cy5-labeled dye were applied for the animal model, in order to determine the stability during circulation in bloodstream. In general, the polymer-siRNA conjugates containing disulfide linkage, that is one of the commonest linkers, results in half-life period of several hours in weakly reductive blood condition.[22] Thereby, the further improvement of stability of linkage for incorporating into polymer-siRNA conjugate should be considered.

From the results, all of the three series, including PEG-sul-siRNA, PEG-car-siRNA, and PEG-disulfide-siRNA, exhibited time dependent decrease of fluorescent intensity while circulation in bloodstream, due to the degradation

of PEG-siRNA conjugates under the reductive environment in bloodstream. However, in comparison with 2-nitrobenzenesulfonamide and disulfide groups, the PEG-sul-siRNA exhibited the enhanced stability during blood circulation after 2 hours of injection, whereas PEG-disulfide-siRNA series induced the faster degradation during circulation in bloodstream. Moreover, the PEG-car-siRNA (non-cleavable system), revealed the similar blood circulation property with PEG-sul-siRNA series against degradation in the blood (Figure 3-15). The enhanced blood circulation property is required for polymer-siRNA conjugates toward successful delivery. The results suggest that 2-nitrobenzenesulfonamide group could be a good candidate for construction of polymer-conjugated siRNA system for enhanced ability against degradation during blood circulation.

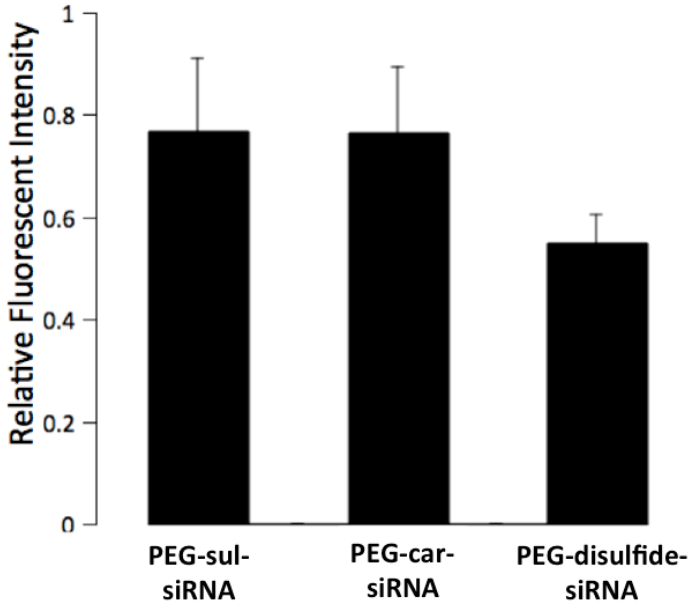

Figure 3-15. Blood circulation evaluation of PEG-siRNA conjugates following tail-vein injection (20 μg / 200μL per single injection). Relative fluorescent intensity was determined and calculated as mean ± SD of three independent experiments (n = 3) after 2 hours injection.

3.5 Conclusion

In this chapter, the cleavage assays of PEG-siRNA conjugates, i.e., PEG-sul-siRNA, PEG-car-siRNA, and PEG-disulfide-siRNA, were performed in the mimicked extracellular environment (20 μM GSH, pH 7.4, 37 °C) and intracellular environment (1 mM GSH, pH 7.4, 37 °C). The results clearly demonstrated the powerful stability of PEG-sul-siRNA, compared to PEG-disulfide-siRNA. In addition, PEG-sul-siRNA also exhibited the higher stability against enzymatic degradation, e.g. FBS treatment and RNase A treatment, than PEG-disulfide-siRNA system. It was pivotal for handling in the biological milieu. During storage or handling the PEG-siRNA conjugates, PEG-sul-siRNA series also revealed the stronger stability against light exposure than PEG-disulfide-siRNA series, indicates the easy handling of 2-nitorobenzenesulfonamide-related materials. Furthermore, PEG-sul-siRNA systems were demonstrated to have the potent stability during circulation in the reductive bloodstream environment, compared to PEG-disulfide-siRNA series. Of note, the cleavability of 2-nitrobenzenesulfonamide group in response to combination of GSH and GST was determined via performing cleavage assay in the mimicked intracellular environment with additional presence of GST (1 mM GSH, 0.019 mg/mL GST, pH 7.4, 37 °C), which represents the environment of cancer cells. 2-nitrobenzenesulfonamide group serves as a chemical linker for construction of polymer-siRNA system, which overcomes the weak point, instability, of conventional disulfide linkage, before entering into cytosol.

Next, the biological evaluations of PEG-siRNA conjugates, including gene silencing efficacies, cytotoxicity, and cellular uptake, will be described in the following chapter.

3.6 References

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Chapter 4

Evaluation of biological activities of PEG-siRNA conjugates (*in vitro*)

4.1 Introduction

In the previous chapter 3, the various evaluations of PEG-siRNA conjugates were performed. 2-nitrobenzenesulfonamide group, which was incorporated into the PEG-siRNA system (PEG-sul-siRNA series), exhibited the potent stability under extracellular environment, against enzyme degradation, and against light exposure, compared to the conventional disulfide linkage. In addition, the enhanced siRNA release from PEG-sul-siRNA series was achieved via treatment of combination of GSH and GST in the mimicked intracellular condition, which represented the environment in cancer cells. The evaluations of biological activities of PEG-siRNA will be investigated by *in vitro* experiments in the present chapter.

The main function of siRNA based on RNA interference (RNAi) process is silencing gene expression via targeting the specific mRNA.[1] In the process of RNAi, the ribonucleoprotein complex RISC is the actual effector, which is

guided by the siRNA to the complementary target mRNA.[2] Only one strand of the two strands of the siRNA duplex is contained in the RISC. Both strands of siRNA can be competent to achieve RNAi process.[3] However, it is known that the 5' terminus of antisense strand of siRNA duplex exhibited a lower internal stability than the 3' terminus in the functional siRNA based on the statistical analysis.[4-6] Thermodynamic properties of siRNA play a critical role in determining strand and terminus selections for RISC formation to achieve RNAi process (Figure 4-1).[5] In this regard, the different conjugated sites of siRNA in the design of PEG-siRNA conjugates should be considered and investigated from the point of view of the biological activities. Further, it was reported that the siRNA recognition was hindered by steric hindrance effect of conjugated polymer on RNAi pathway in the siRNA-conjugated polymer systems.[7] Therefore, the biological activities in the design of PEG-siRNA conjugates with different chemical linkages, i.e., cleavable disulfide group and 2-nitrobenzenesulfonamide group, non-cleavable carboxylic amide group, were also investigated and described in the following paragraph.

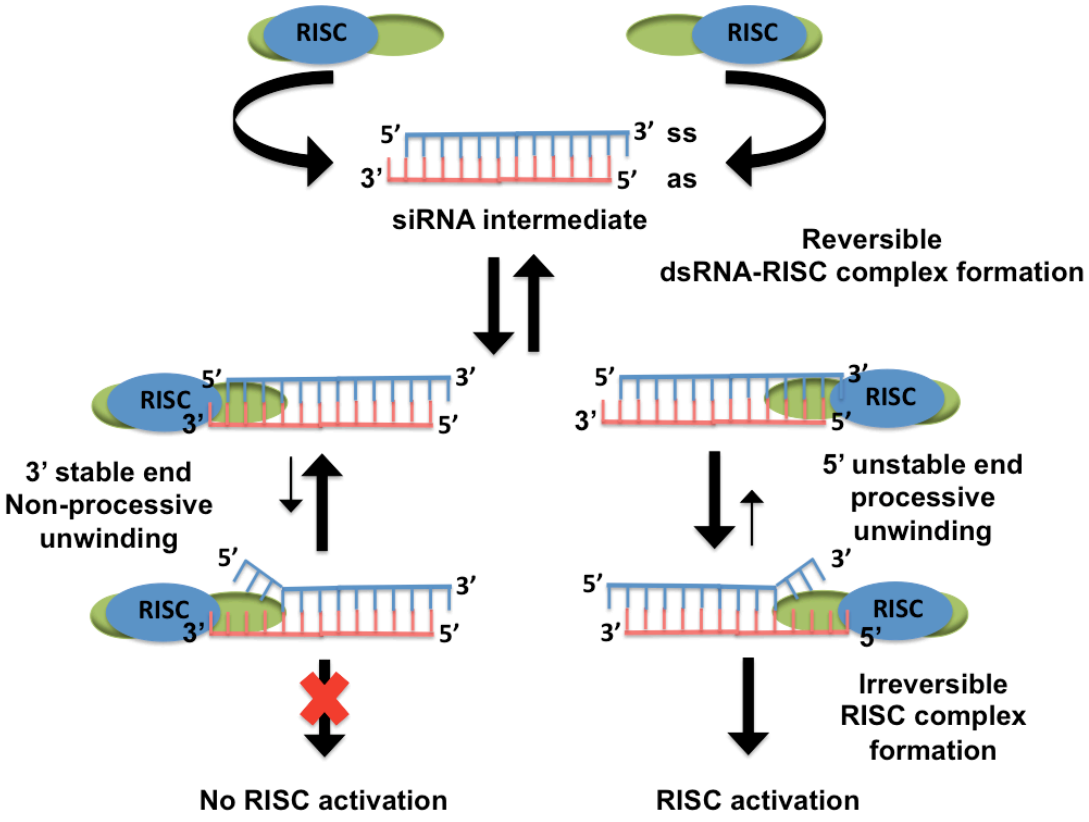


Figure 4-1. Mechanism of efficient RNAi based on thermodynamic characteristics within siRNA.

In this chapter, the obtained PEG-siRNA conjugates with different linkages (i.e., 2-nitrobenzenesulfonamide, carboxylic amide, and disulfide group), as well as different conjugated site of siRNA (i.e., 5' terminus, 3' terminus, and 3' and 5' termini) were applied for human cervical cancer cells stably expressing luciferase (HeLa-Luc) to examine the gene silencing ability via gene silencing assays, using Lipofectamine RNAiMAX. Luciferase-targeting sequence (siLuc) and scrambled siRNA sequence (siScr) were used to confirm sequence-specific gene silencing efficacies. After determination of gene silencing efficacies, the cell viability of the synthesized PEG-siRNA conjugates was also estimated for further confirmation of cytotoxicity of PEG-siRNA conjugates. Ultimately, the endocytosis of the PEG-siRNA was required for the conjugates entering in the

cell and achieving efficient gene silencing efficacies. In this regard, the synthesized PEG-siRNA conjugates with dye-labeled siRNA were prepared and subjected to the cultured cells. The cellular uptake of synthesized PEG-siRNA conjugates was investigated by flow cytometry. All of the biological evaluations were described in the following paragraphs.

4.2 Materials and equipment

4.2.1 Materials

Dulbecco's phosphate buffered saline (DPBS) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's modified eagle's medium (DMEM) and trypsin-EDTA solution were purchased from Sigma Aldrich (St. Louis, MO, USA). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) solution (1 M, pH 7.3) was purchased from AMRESCO Inc. (Solon, OH). The luciferase-expressing human cervical cancer cell line, HeLa-Luc, was purchased from Caliper LifeScience (Hopkinton, MA, USA). Fetal bovine serum (FBS) was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Lipofectamine RNAiMAX was purchased from Invitrogen (Carlsbad, CA). The Luciferase Assay System Kit was purchased from Promega Co. (Madison, WI). The Cell Counting Kit-8 (CCK 8) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Series of siRNAs were synthesized by Hokkaido System Science Co. Ltd. (Hokkaido, Japan). The sequences are as follows:

(1) siGL3 (3'-TAMRA-modified) :

5'-(N_3)-CUU ACG CUG AGU UCG AdTdT-3' (sense strand),

5'-UCG AAG UAG UCA GCG UAA GdTdT(-TAMRA)-3' (antisense strand)

(2) 3' modified terminus:

5'-CUU ACG CUG AGU ACU UCG AdTdT-(N_3)-3' (sense strand),

5'-ACG UGA CAC GUU CGG AGA AdTdT-3' (antisense strand)

(3) 3' and 5' modified terminus:

5'-(N_3)-CUU ACG CUG AGU ACU UCG AdTdT-(N_3)-3' (sense strand),

5'-ACG UGA CAC GUU CGG AGA AdTdT-3' (antisense strand)

4.2.2 Equipment

Water phase high performance liquid chromatography (HPLC) analyses were performed LC-Net II AS2055 system (JASCO, Tokyo, Japan) equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260 nm, and 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride as an eluent. Luciferase assays were performed by GLOMAX 96 Microplate luminometer (Promega, Madison, WI). Cell viability assay was performed by Cell Counting Kit-8, and measured by BIORAD iMark Microplate Reader (BIORAD, California, US). Cellular uptake was investigated by flow cytometry, guava easy cyte 6-12L system (Merck Millipore, Darmstadt, Germany).

4.3 Experimental procedures

4.3.1 Preparation of PEG-siRNA conjugates with TAMRA-labeled siRNA and different conjugated site of siRNA

In this section, PEG-siRNA conjugates with TAMRA-labeled siRNA, including PEG-sul-5'-siRNA (3'-TAMRA-modified) and PEG-car-5'-siRNA (3'-TAMRA-modified), were synthesized as the same procedures in chapter 2. 6 mg of PEG-sul-DBCO conjugate or PEG-car-DBCO conjugate (0.00015 mmol) was dissolved in 500 μL of HEPES (10 mM; pH 7.3). The 65 μL of siRNA (3'-TAMRA-modified) (3 mg/mL) was added to the solution of PEG-sul-DBCO conjugate or PEG-car-DBCO and followed by adding 335 μL of HEPES (10 mM; pH 7.3). The solutions were frozen at -20 °C for overnight and then thawed at 4 °C for 1 hour. The solutions were subsequently characterized and purified via HPLC, where LC-Net II AS2055 system (JASCO, Tokyo, Japan) was equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260 nm, and 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride as eluents (Figure 4-2).

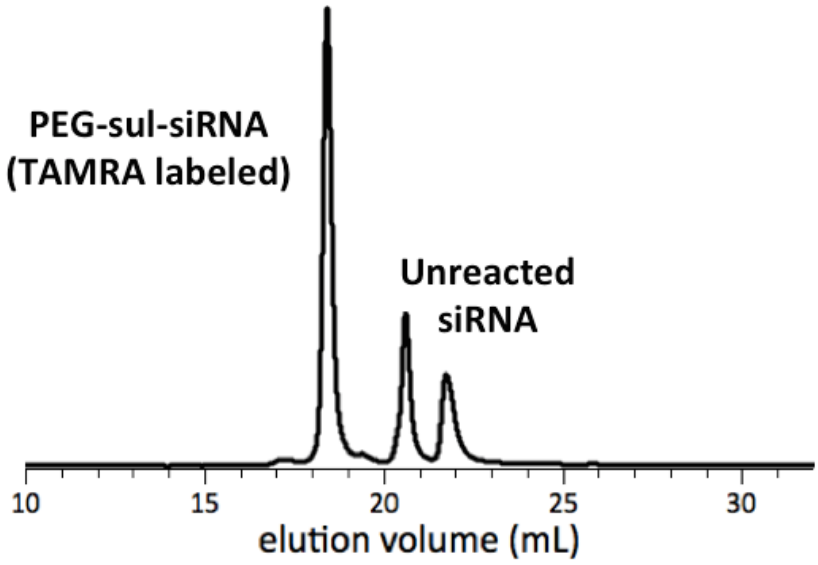


Figure 4-2. HPLC chart of the reaction solution of PEG-sul-DBCO and TAMRA-labeled azide-siRNA.

In addition, PEG-disulfide-5'-siRNA (3'-TAMRA-modified) was also synthesized as the same procedures in chapter 2. 6 mg of PEG-disulfide-DBCO was dissolved in 500 μL of HEPES (10 mM; pH 7.3). The 65 μL of siRNA (3'-TAMRA-modified) (3 mg/mL) was added to the solution of PEG-disulfide-DBCO conjugate and followed by adding 335 μL of HEPES (10 mM; pH 7.3). The solutions were frozen at -20 °C for overnight and then thawed at 4 °C for 1 hour. The solutions were subsequently characterized and purified via HPLC, where LC-Net II AS2055 system (JASCO, Tokyo, Japan) was equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260 nm, and 10 mM HEPES (pH 7.4) without sodium chloride and with 1 M of sodium chloride as eluents (Figure 4-3).

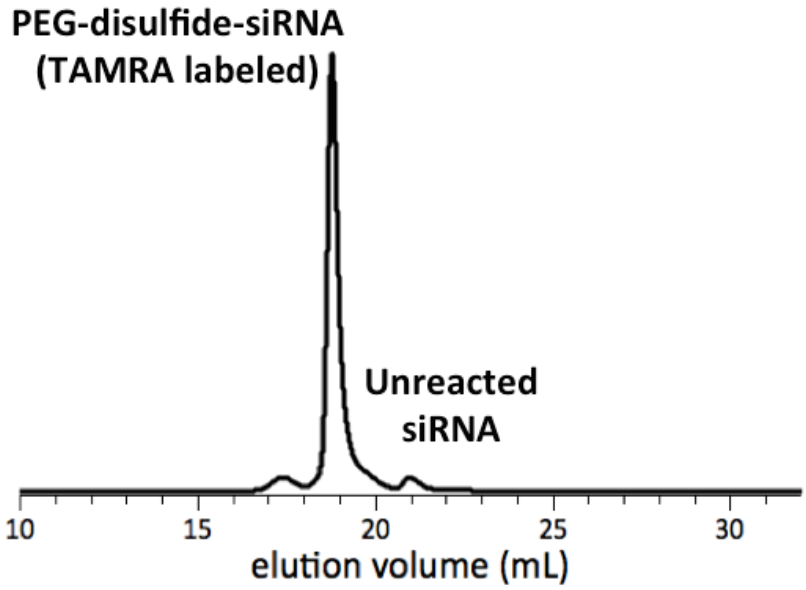


Figure 4-3. HPLC chart of the reaction solution of PEG-disulfide-DBCO and TAMRA-labeled azide-siRNA.

In this section, PEG-siRNA conjugates with the different conjugated sites of siRNA, including PEG-sul-3'-siRNA and PEG-sul-5'-siRNA-3'-sul-PEG, were synthesized as the similar procedures in chapter 2 (Figure 4-4). 6 mg of PEG-sul-DBCO (0.00015 mmol) was dissolved in 500 μL of HEPES (10 mM; pH 7.3). The 65 μL of siRNA (3' azide modified) (3 mg/mL) was added to the solution of PEG-sul-DBCO and followed by adding 335 μL of HEPES (10 mM; pH 7.3). In addition, 12 mg of PEG-sul-DBCO (0.00030 mmol) was dissolved in 500 μL of HEPES (10 mM; pH 7.3). The 65 μL of siRNA (5' and 3' azide modified) (3 mg/mL) was added to the solution of PEG-sul-DBCO and followed by adding 335 μL of HEPES (10 mM; pH 7.3). The solutions were frozen at -20 °C for overnight and then thawed at 4 °C for 1 hour. The solutions were subsequently characterized and purified via HPLC, where LC-Net II AS2055 system (JASCO, Tokyo, Japan) was equipped with Mono Q^{TM} 5/50GL (GE Healthcare, Illinois, USA), a UV detector at 260 nm, and 10 mM HEPES (pH 7.4)

without sodium chloride and with 1 M of sodium chloride as eluents (Figure 4-5).

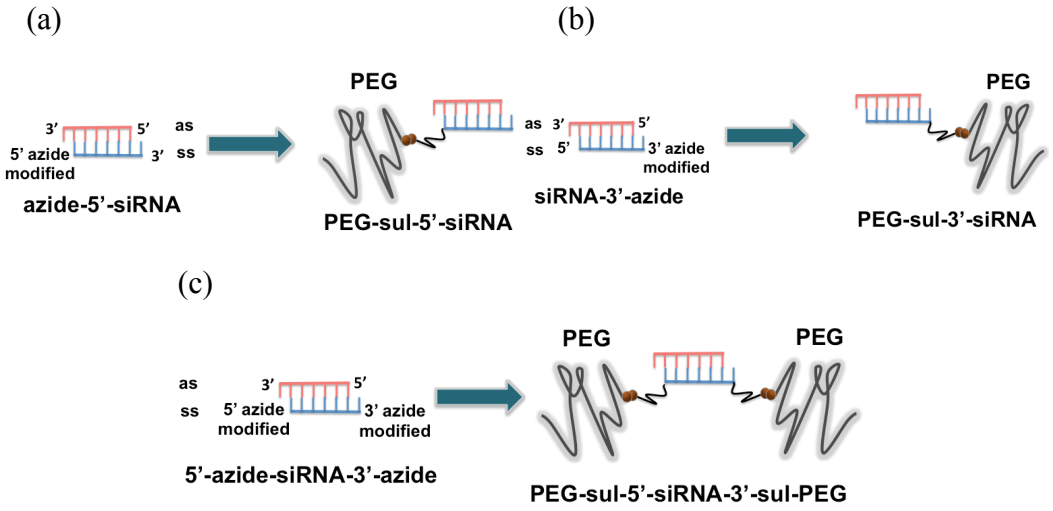


Figure 4-4. Illustration of PEG-sul-siRNA conjugates with different conjugated sites of siRNA (a) 5' conjugated site of siRNA (5' azide modified siRNA) (b) 3' conjugated site of siRNA (3' azide modified siRNA) (c) 5' and 3' conjugated sites of siRNA (5' and 3' azide modified siRNA).

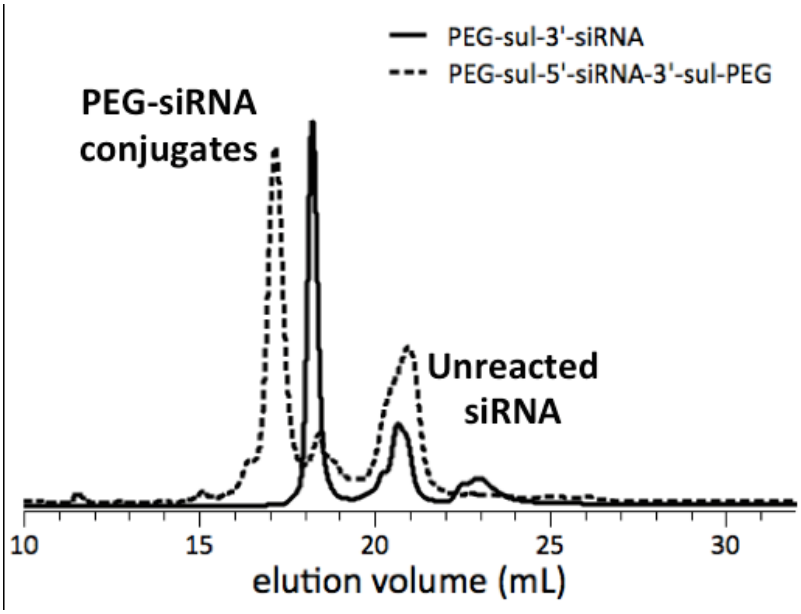


Figure 4-5. HPLC chart of the reaction solution of PEG-sul-DBCO, 3' azide modified siRNA, and 5' and 3' azide modified siRNA.

4.3.2 Luciferase assays

To examine whether the biological activity of PEG-siRNA conjugates

containing different linkages, the PEG-siRNA conjugates were subjected to the gene silencing assay for cultured cells. The human cervical cancer cells which stably expresses luciferase (HeLa-Luc) were cultured and seeded (1000 cells per well) with DMEM (containing 10% FBS), and followed by 24 hours incubation at 37 °C. The PEG-siRNA conjugates were applied to the cells via complexing with Lipofectamine RNAi MAX at the indicated siRNA concentrations (50 nM), followed by further 72 hours incubation. Luminescence intensity for each well was measured by Luciferase Assay System (Promega).

4.3.3 Cell viability

To examine the cytotoxicity of PEG-siRNA conjugates containing different linkages or different conjugated sites of siRNA, and the collected compound (released siRNA collected from PEG-sul-siRNA via treatment of GSH and GST), cellular viability assay was performed as the same conditions with luciferase gene silencing assays by using Cell Counting Kit-8.

4.3.4 Cellular uptake efficacy

Cellular uptake assays were performed and the efficacy for PEG-siRNA systems was estimated by flow cytometric analysis. The PEG-siRNA conjugates containing TAMRA-labeled siRNA were prepared (in the previous section 4.3.1). The human cervical cancer cells which stably expresses luciferase (HeLa-Luc) were seeded on the 12 wells culture plate with 50000 cells and 400 μL of DMEM (containing 10%) with FBS per well, and followed by 24 hours incubation at 37

°C. The medium was replaced with fresh medium, followed by addition of solution of PEG-siRNA conjugates containing TAMRA-labeled siRNA, i.e., PEG-sul-5'-siGL3, PEG-sul-3'-siGL3, PEG-sul-5'-siGL3-3'-sul-PEG, PEG-car-5'-siGL3, and PEG-disulfide-5'-siGL3, which were complexed with Lipofectamine RNAi MAX at 50 nM siRNA concentration, and followed by the indicated incubation time. After incubation, the cells were washed 3 times with PBS to remove extracellular TAMRA fluorescence. After detachment of the cells by trypsin from the culture plate, the cells were re-suspended in PBS for flow cytometry analysis.

4.4 Results and discussion

4.4.1 Luciferase assay for evaluation of silencing efficacies with PEG-siRNA conjugates

The result was shown in Figure 4-6, the PEG-sul-5'-siGL3 exhibited a significantly stronger silencing efficacies (with ~60% of Relative Luminescence Unit (RLU) at 50 nM of siRNA) than PEG-car-5'-siGL3 (with ~80% of Relative Luminescence Unit (RLU) at 50 nM of siRNA) and PEG-disulfide-5'-siGL3 (with ~80% of Relative Luminescence Unit (RLU) at 50 nM of siRNA). The results are presumably due to the cleavability of 2-nitrobenzenesulfonamide linkage in response to expression of GSH and GST inside the cell, compared to non-cleavable carboxylic amide linkage. Interestingly, PEG-disulfide-5'-siGL3 exhibited a lower silencing efficacy, probably due to higher possibilities of undesired cleavage of disulfide linkages under extracellular environment. The

results clearly demonstrated that the cleavability of 2-nitrobenzenesulfonamide linkage in PEG-siRNA conjugate under intracellular conditions for eliciting siRNA with enhanced silencing ability.

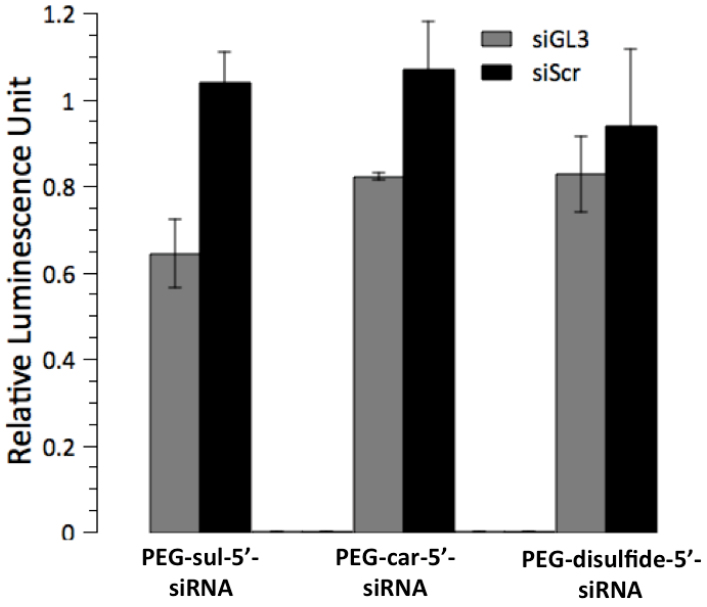


Figure 4-6. Relative luminescence unit (RLU) for cultured HeLa-Luc cells after the treatment with PEG-siRNA conjugates for 72 hours using Lipofectamine RNAiMAX (50 nM of siRNA). The data were reported as mean ± SD of six independent experiments (n = 6).

In Figure 4-7, PEG-sul-5'-siGL3 exhibited the significant silencing efficacies with ~60% of Relative Luminescence Unit (RLU) at 50 nM of siRNA, whereas the PEG-sul-3'-siGL3 exhibited ~80% of Relative Luminescence Unit (RLU) at 50 nM of siRNA. It was presumably due to the less unstable thermodynamic properties of 5' azide modified terminus of siRNA. The siRNA acted with a function of silencing ability, which was triggered from RISC formation process, was described in the previous paragraph. The selective RISC assembly is principally based on the evaluation of thermodynamic stability. In the previous studies, it was found that during the RNAi machinery, the terminus selection was due to the thermodynamic stability of the 5' end of a siRNA strand,

especially the strand with a relatively less stable 5' terminus was preferentially selected.[4, 5]

Moreover, the PEG-siRNA conjugates containing both 5' and 3' modified termini of siRNA whether with cleavable or non-cleavable linkage exhibited no silencing efficacies. It was presumably due to the increase of the steric hindrance effect of conjugated polymer while conjugating to two terminus of siRNA, which drastically affected the siRNA recruitment into RNAi pathway for achieving ineffective gene silencing ability.

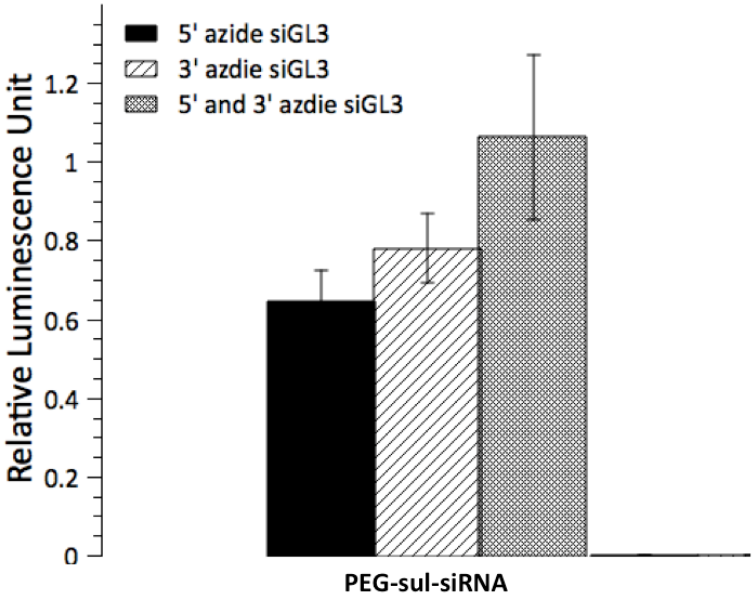


Figure 4-7. Relative luminescence unit (RLU) for cultured HeLa-Luc cells after the treatment of PEG-sul-siRNA conjugates containing different conjugated sites of siRNA for 72 hours using Lipofectamine RNAiMAX (50 nM of siRNA). The data were reported as mean ± SD of six independent experiments (n = 6).

4.4.2 Silencing efficacies with the collected compound (PEG-sul-5'-siGL3 conjugate after cleavage assay) evaluated by Luciferase assays

Further, the compound at 14.5 mL in SEC chart (chapter 3) was collected after treatment with GSH and GST for PEG-sul-siRNA and the concentration was adjusted based on UV absorbance at 260 nm (major absorbance of nucleotide). To examine the biological activity of the collected compound, the collected compound was applied for gene silencing assay for cultured cells. The experimental procedures of luciferase assay were the same as previous section. The result exhibited the gene silencing activity of the collected compound, where comparable gene silencing efficacy to unconjugated siRNA was observed for the collected compound (Figure 4-8), indicating that the peak at 14.5 mL in SEC chart should be released siRNA from PEG-sul-siRNA and the released siRNA exerts its function after cleavage of 2-nitrobenzenesulfonamide linkage.

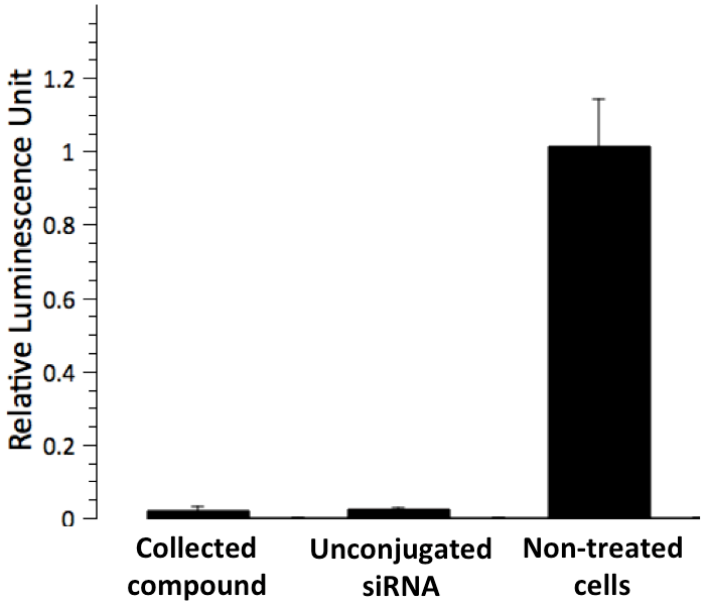


Figure 4-8. Relative luminescence unit (RLU) for cultured HeLa-Luc cells after the treatment with the collected compound (released siRNA from PEG-sul-siRNA) and unconjugated siRNA for 72 hours using Lipofectamine RNAiMAX (50 nM of siRNA). The data were reported as mean ± SD of six independent experiments (n = 6).

4.4.3 Cell viability

The results revealed that PEG-siRNA conjugates, i.e., PEG-sul-5'-siGL3, PEG-car-5'-siGL3, PEG-disulfide-5'-siGL3, PEG-sul-3'-siGL3, and PEG-sul-5'-siGL3-3'-sul-PEG, induced no significant cellular death at the concentration of siRNA subjected (50 nM) (Figures 4-9 and 4-10). The results demonstrated that siRNA after conjugating to PEG to form PEG-siRNA conjugates enhanced no obvious cytotoxicity to the treated cells with remaining gene silencing efficacies under the indicated siRNA concentration. In addition, the collected compound (released siRNA collected from PEG-sul-siRNA via treatment of GSH and GST), also induced no significant cytotoxicity after treatment of cleavage assay (Figure 4-11).

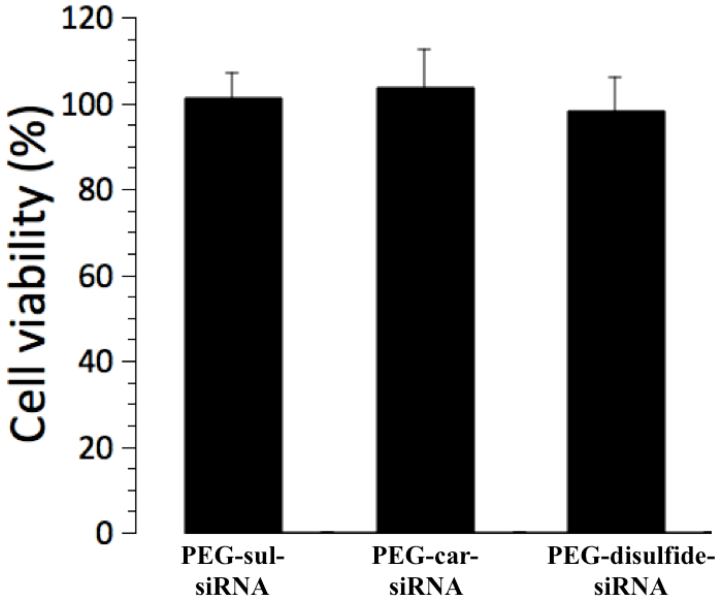


Figure 4-9. Cell viability for cultured HeLa-Luc cells after the treatment with PEG-siRNA conjugates containing different linkages for 72 hours using Lipofectamine RNAiMAX (50 nM of siRNA). The data were reported as mean ± SD of six independent experiments (n = 6).

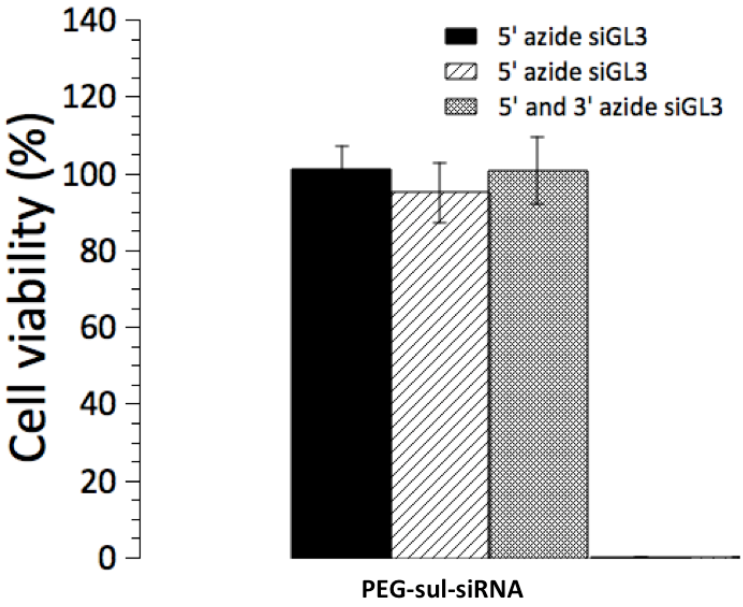


Figure 4-10. Cell viability for cultured HeLa-Luc cells after the treatment with PEG-sul-siRNA conjugates containing different conjugated sites of siRNA for 72 hours using Lipofectamine RNAiMAX (50 nM of siRNA). The data were reported as mean ± SD of six independent experiments (n = 6).

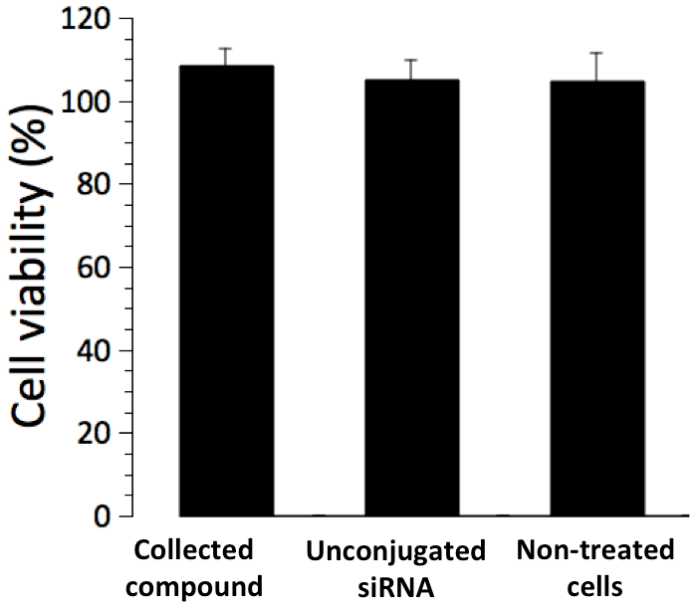
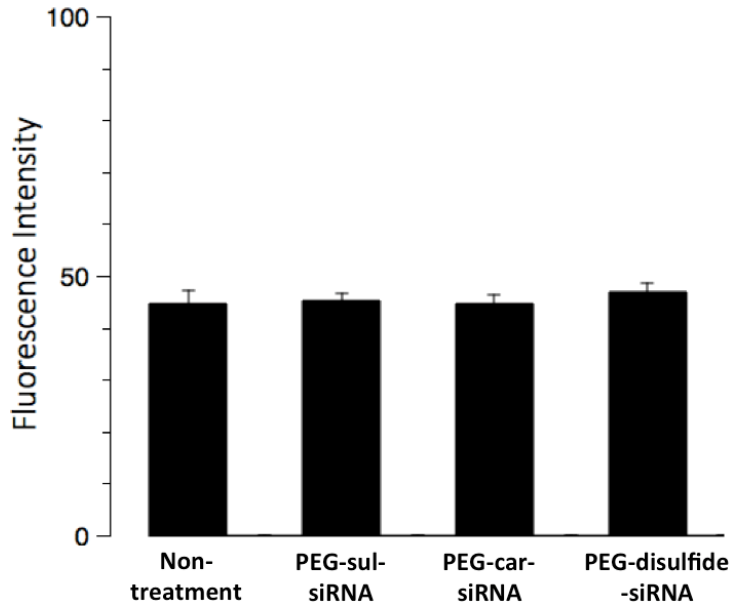


Figure 4-11. Cell viability for cultured HeLa-Luc cells after the treatment with the collected compound (released siRNA from PEG-sul-siRNA) and unconjugated siRNA for 72 hours using Lipofectamine RNAiMAX (50 nM of siRNA). The data were reported as mean ± SD of six independent experiments (n = 6).

4.4.4 Cellular uptake efficacy of PEG-siRNA conjugates

From the results of 24 hours incubation (Figure 4-12), inefficient cellular uptake was observed for all PEG-siRNA conjugates containing different linkages, and different conjugated site of siRNA, compared to the non-treated cells. The results suggest that the prolonged incubation time was required for the PEG-siRNA conjugates entering in the cell for achieving efficient gene silencing ability.

(a)



(b)

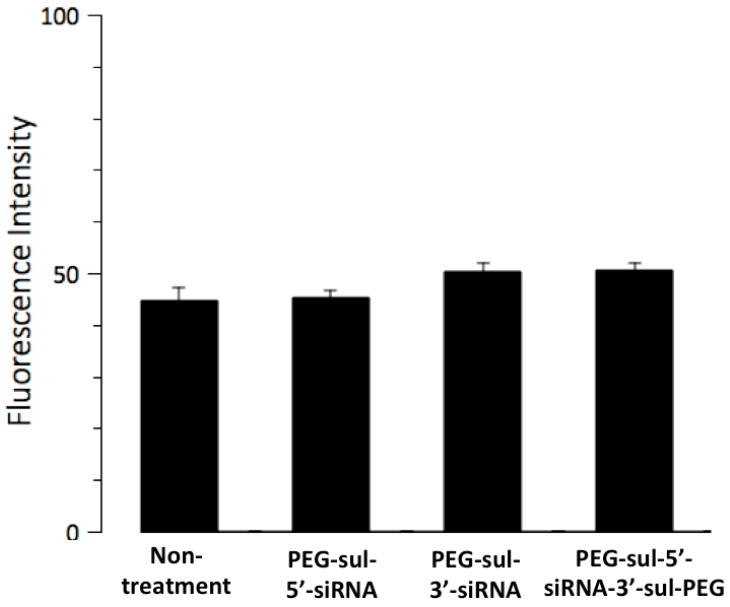
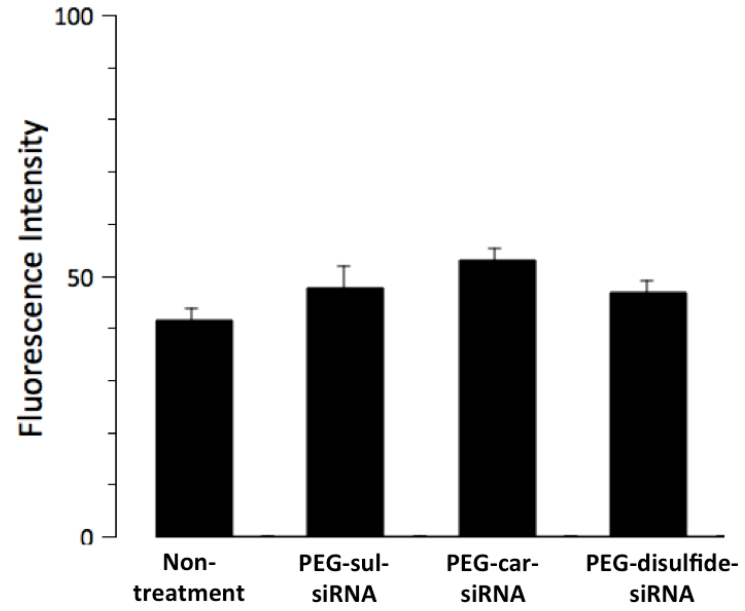


Figure 4-12. Cellular uptake activity of PEG-siRNA conjugates complexing with Lipofectamine RNAiMAX at the concentration of 50 nM of siRNA after 24 hours incubation varying with (a) different linkages (b) different terminus modified siRNA.

While prolonging the incubation time to 72 hours (results shown in Figure 4-13), the apparent cellular uptake was observed for all PEG-siRNA conjugates, comparing with non-treatment cells. However, there was no significant difference of fluorescence intensity shown in PEG-siRNA conjugate containing different linkages (Figure 4-13a) and different conjugated site of siRNA (Figure 4-13b), indicating that the linkers for PEG conjugating to siRNA do not affect cellular uptake efficacy and the cleavability of the linker would be a factor to affect the gene silencing efficacies.

(a)



(b)

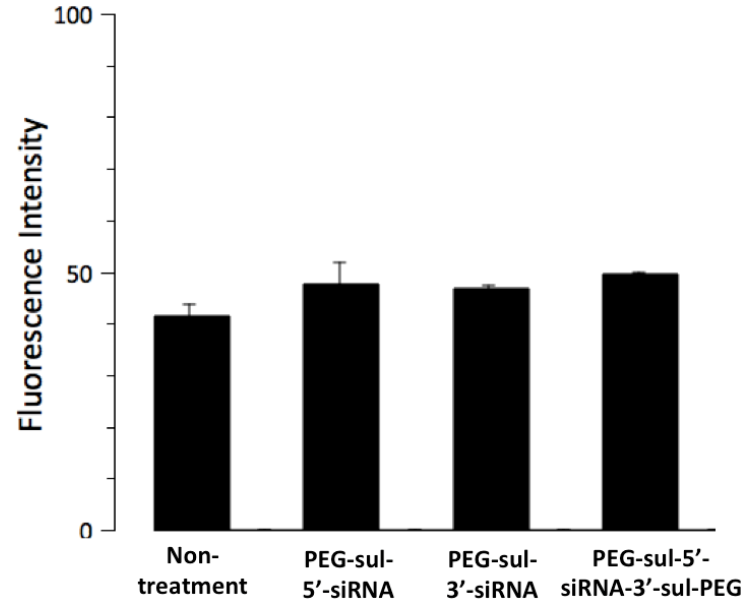


Figure 4-13. Cellular uptake efficacy of PEG-siRNA conjugates complexing with Lipofectamine RNAiMAX at the concentration of 50 nM of siRNA after 72 hours incubation varying with (a) different linkages (b) different terminus modified siRNA.

4.5 Conclusion

In this chapter, the PEG-siRNA conjugates containing TAMRA-labeled siRNA and different conjugated position of siRNA, were successfully synthesized and purified by HPLC systems for biological evaluations. The luciferase assay for evaluation of gene silencing ability of PEG-siRNA conjugate was performed for the cultured cells. The relative luminescence unit (RLU) was measured by Luciferase Assay System. The results exhibited enhanced gene silencing efficacy of PEG-siRNA conjugate containing 2-nitrobenzenesulfonamide linkage, due to the cleavability of 2-nitrobenzenesulfonamide group in response to expression of GSH and GST inside the cell, compared to PEG-siRNA conjugates containing the conventional disulfide linkage and non-cleavable carboxylic amide linkage. In addition, the gene silencing ability also depends on the conjugated sites of siRNA to PEG. The PEG-sul-5'-siGL3 exhibited the significant silencing efficacies, compared to the PEG-sul-3'-siGL3. It was because while PEG conjugated at the site of 5' terminus of sense strand of siRNA, the less unstably thermodynamic properties of 5' terminus of antisense strand of siRNA didn't affected by conjugated PEG and preferentially selected. Moreover, all series of PEG-siRNA conjugates contributed similar cell viability and similar cellular uptake ability, indicated the PEG-siRNA conjugates induced no significant cytotoxicity after conjugation processes and the ability of PEG-siRNA conjugates entering into the cells. Of note, the comparable gene silencing efficacy to unconjugated siRNA was observed for the collected compound, which was released from PEG-sul-siRNA

conjugate after treatment of GSH and GST. The result indicates the function of released siRNA after cleavage of the linker was maintained and emphasizes the pivotal role of cleavable 2-nitrobenzenesulfonamide linkage in response to GSH and GST for polymer-siRNA conjugate system.

4.6 References

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Chapter 5

Summary

5.1 Summary of the present study

In this study, a novel molecular design of siRNA-conjugated polymer system that responds to intracellular redox potential was developed, and its optimized formulation and biological activities were also investigated. The experimental results revealed that the design of siRNA-conjugated polymer system with potent extracellular stability against degradation as well as the cleavability in response to intracellular environment with high specificity, and thereby, achieved the improved biological activities. As described in Chapter 1, siRNA has attracted great interest and been recognized as a potential medicine for cancer therapies. Due to various barriers of siRNA delivery, many research groups pay great efforts to overcome the challenges for efficient delivery of siRNA. Many methodologies have been developed for successful construction of siRNA. siRNA-conjugated polymer system potentially provides the new functionalities for improved biological activities. For example, siRNA conjugated with poly(ethylene glycol) (PEG) provides improved stability against degradation, but recruitment of siRNA into RNAi pathway is hindered by the steric hindrance effect of conjugated polymer, which leads to the compromised

gene silencing activity. Taking advantage of utility of stimuli-responsive system for siRNA conjugated polymer systems, siRNA could be released in the right time and position due to decrease of steric hindrance effect of conjugated polymer, and then contributed the efficient gene silencing efficacies.

In Chapter 2, I described that my design of siRNA-conjugated polymer system was fabricated from poly(ethylene glycol) (PEG) conjugated to siRNA via the copper free click reaction with redox-sensitive 2-nitrobenzenesulfonamide linkage between PEG and siRNA. In my design, PEG-siRNA conjugate containing 2-nitrobenzenesulfonamide group served as a excellent candidate for construction of siRNA derivative, providing potent stability under the extracellular environment as well as cleavability in response to intracellular redox potential. In addition, PEG-siRNA conjugate containing conventional disulfide group and PEG-siRNA conjugate containing non-cleavable carboxylic amide group were also synthesized for control groups. The PEG-siRNA conjugates, i.e., PEG-sul-siRNA, PEG-car-siRNA, and PEG-disulfide-siRNA, were successfully synthesized and purified by ion-exchange HPLC system.

In Chapter 3, several evaluations of synthesized PEG-siRNA conjugates, e.g. stability and cleavability in the mimicked cellular conditions via cleavage assays, stability in biological systems, were investigated. The results of cleavage assays clearly demonstrated the strong stability of PEG-sul-siRNA system, compared to PEG-disulfide-siRNA under the mimicked extracellular environment (20 μM GSH, pH 7.4, 37 °C). In addition, the enhanced release of siRNA from PEG-sul-siRNA was also shown in the results of cleavage assay, which was performed in the mimicked intracellular environment with additional

presence of GST (1 mM GSH, 0.019 mg/mL GST, pH 7.4, 37 °C). The results demonstrated that 2-nitrobenzenesulfonamide group with the extracellular stability and intracellular cleavability, in response to combination of GSH and GST, serves as a great candidate of chemical linkage for polymer-siRNA system. Furthermore, PEG-sul-siRNA exhibited the higher stability against enzymatic degradation, e.g. FBS treatment, RNase A treatment, and circulation in bloodstream, compared to PEG-disulfide-siRNA system.

In Chapter 4, biological evaluations, e.g. gene silencing ability, cell viability, cellular uptake ability, were investigated. The results of luciferase assays exhibited the enhanced gene silencing efficacy of PEG-siRNA conjugate containing 2-nitrobenzenesulfonamide linkage, due to the cleavability of 2-nitrobenzenesulfonamide group in response to expression of GSH and GST inside the cell, compared to PEG-siRNA conjugates containing the conventional disulfide linkage and non-cleavable carboxylic amide linkage. Further, the gene silencing ability of PEG-siRNA conjugates containing different conjugated sites of siRNA were also estimated. The results revealed the effective gene silencing activity of PEG-sul-5'-siRNA, compared to PEG-siRNA conjugates containing 3' conjugated site of siRNA and containing 5' and 3' conjugated sites of siRNA, suggesting the cleavability of 2-nitrobenzenesulfonamide group in the cell, as well as dependency on the conjugated position of siRNA molecule.

5.2 Future prospects

Through this study, a successful construction of siRNA-conjugated polymer system was developed for improved therapeutic activities. In design, a

newly redox-sensitive 2-nitrobenzenesulfonamide linkage was created and engineered into polymer-siRNA conjugate system. Although, the conventional methodology for polymer-siRNA conjugate system was already developed, there are still various challenges have to overcome for realization in clinical use, such as the widely used disulfide group with insufficient stability against degradation under extracellular environment. The enhanced extracellular stability was achieved in my design, compared to the conventional methodology. Moreover, the siRNA release ability inside the cell was also achieved by incorporating redox-sensitive 2-nitrobenzenesulfonamide linkage into PEG-conjugated siRNA, that responds to redox potential. The facilitated siRNA release was induced by additionally presence of GST, providing the potential possibility of specific siRNA release, due to overexpression of GST in tumor cells. The developed 2-nitrobenzenensulfonamide linkage and methodology could serve as a potential candidate for not only construction of siRNA-conjugate, but also other bioconjugation.

Consequently, the new design of siRNA-polymer conjugate containing redox sensitive 2-nitrobenzenensulfonamide linkage with optimized formulation was well developed toward improved biological activities as well as therapeutic activities for efficient construction of siRNA conjugate in this study. The author strongly believes that the developed methodology and basis of molecular design will contribute to the development of siRNA-based therapeutics toward realization of next generation of medicine, and further application in the biomedical field.

6 Achievement

1. Publication

C. H. Huang *et al.*, Utility of 2-nitrobenzenesulfonamide group as a chemical linker for enhanced extracellular stability and cytosolic cleavage in siRNA-conjugated polymer system. *ChemMedChem* **2016,** *12* (1), 19-22

2. Patent

フアン　チー　ハオ、武元宏泰、西山伸宏、野本貴大、友田敬士郎、松井誠，特許出願番号 2016-113664，結合体

3. Conference

C. H. Huang, H. Takemoto, K. Tomoda, T. Nomoto, M. Matsui, and N. Nishiyama, 2-Nitrobenzenesulfonamide groups as a chemical linker with enhanced extracellular stability and redox-sensitive cleavability for siRNA-conjugated polymer system, The 11th SPSJ International Polymer Conference, Fukuoka, Japan, Dec 2016

[Appendix]

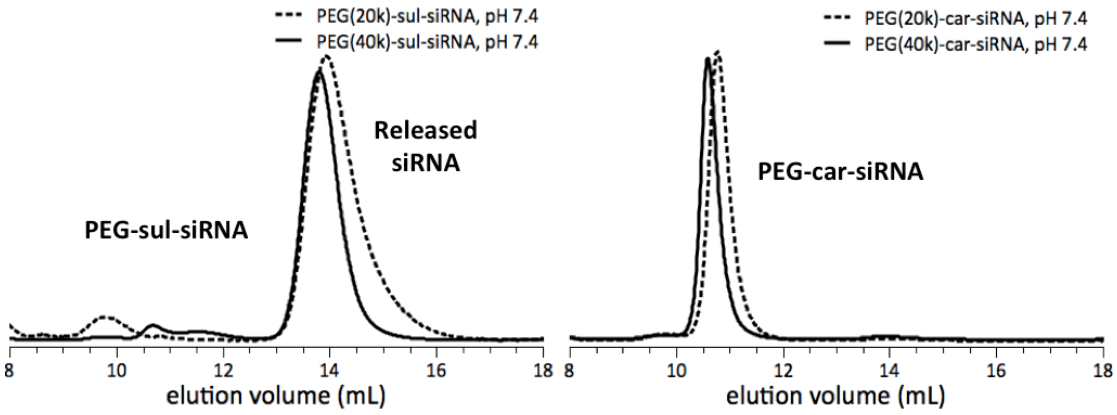

Figure S-1. SEC chart of solution in cleavage assay a) PEG-sul-siRNA b) PEG-car-siRNA conjugates followed by 72 hours incubation with 5 mM GSH and 0.05 mg/mL GST in 0.1 M PB at pH 7.4.

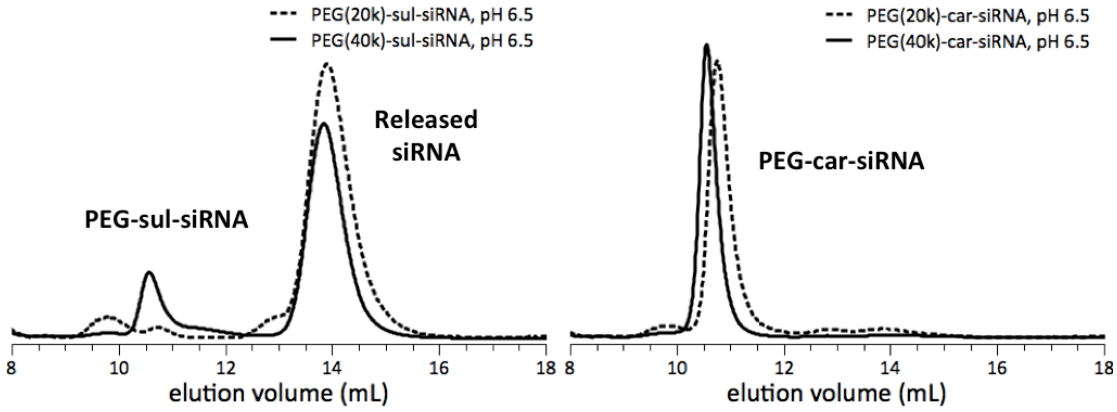

Figure S-2. SEC chart of solution in cleavage assay a) PEG-sul-siRNA b) PEG-car-siRNA conjugates followed by 72 hours incubation with 5 mM GSH and 0.05 mg/mL GST in 0.1 M PB at pH 6.5.