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DEVELOPMENT OF STIMULI-RESPONSIVE POLYMERIC VEHICLES FOR siRNA DELIVERY

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MASTER'S THESİS

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The thesis study entitled “Development of Stimuli-responsive Polymeric Vehicles for siRNA Delivery” prepared by Hanim Beyza DOĞAN, has been accepted as a MASTER'S THESIS by the following jury on 28/07/2025 unanimously vote in the Department of Molecular Biology and Genetics of the Institute of Science at Necmettin Erbakan University.

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Beyza DOĞAN

Tarih: 28.07.2025

ÖZET

YÜKSEK LİSANS TEZİ

siRNA Taşınımı için Uyarı-duyarlı Polimerlerin Geliştirilmesi

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Necmettin Erbakan Üniversitesi Fen Bilimleri Enstitüsü
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Kontrollü ilaç taşıma teknolojisi, farklı disiplinden bilim insanlarının katkılarıyla hızla gelişen bir alandır ve geleneksel tedavi yöntemlerine göre daha etkin, daha az toksisite gösteren ve gelişmiş hasta uyumu ve rahatlığı gibi önemli avantajlar sunar. Bu taşıma sistemleri genellikle ilaç taşıyıcı olarak sentetik polimerleri kullanır. Bu taşıyıcılar, daha önce erişilemeyen tedavilere olanak sağlar. Bu yöntem ile siRNA belirli hücrelere iletilebilir. Hedef genleri spesifik olarak susturma yeteneği ile RNA uygulamaları, çeşitli hastalıkların tedavisi için büyük potansiyel gösterir. RNA interferansına yönelik birçok zorluk vardır. Bu problemlerin en önemlilerinden biri siRNA gibi terapötik nükleik asitlerin hedeflenen dokulara ve hücrelere verimli ve seçici bir şekilde iletmektir. Bu projede, nükleik asitlerin hedef üzerinde salımını sağlayacak uyarı-duyarlı polimerik taşıyılar üretilmiştir. Polimer negatif yüklü siRNA'ya bağlanabilmesi için katyonik yüke sahip piridinyum birimleri ile türevlendirilmiştir.

Poli(4-vinil piridin) (P4VP), pKa'sı 5.6 olan zayıf bir polibazdır ve bu pH'ın altında protonlanmış durumda iken hidrofilik, pH 5.6'nın üzerinde ise protonlanmamış durumda hidrofobiktir. P4VP, gen dağıtımında, bölgeye özgü ilaç dağıtımında, biyosensörlerde, antibakteriyel ajanlarda ve hassas kolloid ve yüzey sistemlerinde yaygın olarak kullanılmaktadır. Bu projede bu polimer, siRNA'yı yakalayabilen piridinyum katyonları oluşturmak üzere 4-nitrobenzil bromür ile türevlendirilmiştir. Türevlendirme sonrası zeta potansiyelinin -6.98 mV'dan +22.8 mV'a yükseldiği tespit edilmiş ve ^{1}H NMR ve FTIR yöntemleri ile türevlendirme karakterize edilmiştir. NMR spektrumunda nitrobenzil biriminin piridin yapılarının %10'una bağlandığı görülmüştür. FTIR spektrumunda 1346.27 cm^{-1}'de gözlemlenen yeni pikin yapıya katılan nitro biriminden kaynaklandığı değerlendirilmiştir. nitroredüktaz (NTR) uygulaması sonrasında nitrobenzil piklerinin tamamen kaybolduğu NMR'da tespit edilmiş olup zeta potansiyelinin pozitif değerinde de azalma görülmüştür. Negatif yüklü siRNA polimere elektrostatik etkileşimler yoluyla bağlanmıştırdır. Nitrobenzil birimlerinin hipoksik ortamda NTR enzimlerinin varlığında indirgenerek kendiliğinden yapıdan kopması sonucu katyonik özelliğin azalması, onkogen cMYC'i hedefleyen siRNA'yı bu koşullarda bırakması amaçlanmıştır. Hipoksik insan akciğer hücre hattında yapılan deneylerde cMYC ve bağlantılı yolaklarda yer alan genlerin anlatımında anlamlı bir azalma tespit edilmiştir. Hipoksik mikroçevreye sahip kanserli hücrelerin, normal hücrelere göre daha fazla endojen indirgeyici enzimlere sahip olduğu bilinmektedir. Dolayısıyla bu polimerik sistemin kanserli hücrede daha aktif olması ve kanserli hücredeki spesifik geni susturması sağlanabilmiştir. Tez çalışmasının kanser-mikroçevresine duyarlı yeni taşıyıcıların geliştirilmesine ilham olacağı ve seçici terapi araştırmalarına destek olacağı düşünülmektedir.

Anahtar Kelimeler: siRNA taşınımı, Gen susturma, Uyarı-duyarlı polimer, Nitroredüktaz, Kanser

ABSTRACT

MS THESIS

DEVELOPMENT OF STIMULI-RESPONSIVE POLYMERIC VEHICLES FOR siRNA DELIVERY

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THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE OF NECMETTIN ERBAKAN UNIVERSITY
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Controlled drug delivery technology is a rapidly evolving field driven by contributions of scientists from different disciplines, offering significant advantages over traditional methods, such as enhanced efficacy, reduced toxicity, and improved patient compliance and convenience. These delivery systems commonly utilize synthetic polymers as drug carriers, enabling treatments that were previously inaccessible. With this delivery system, siRNA can be delivered to specific cells. With its ability to specifically silence target genes, RNA interference shows great potential for treating a variety of diseases. There are many challenges to RNA interference. One major problem is to efficient and selective deliver genetic material such as small interfering RNA (siRNA) to targeted tissues and cells. In this project, a stimuli-responsive polymeric carrier systems for efficient target-selective siRNA delivery is developed. Polymer is made up of cationic pyridinium moieties to bind to negatively charged siRNA.

Poly(4-vinyl pyridine) (P4VP) is a weak polybase with a pKa of 5.6 and is hydrophilic in the protonated state below pH 5.6 and hydrophobic in the unprotonated state above pH 5.6. P4VP is widely used in gene delivery, site-specific drug delivery, biosensors, antibacterial agents, and sensitive colloid and surface systems. In this project, this polymer was derivatized with 4-nitrobenzyl bromide to form pyridinium cations with the intension to capture siRNA. After derivatization, the zeta potential was found to increase from -6.98 mV to +22.8 mV and the derivatization was characterized by ^{1}H NMR and FTIR methods. Nitro benzyl peaks appear on the NMR spectrum indicating 10% functionalization. It was evaluated that the new peak observed at 1346.27 cm^{-1} in the FTIR spectrum originated from the nitro unit added to the structure. Nitroreductase (NTR) treatment resulted in nitrobenzyl removal as verified by NMR and also positive charge is reduced as detected through zeta potential. The negatively charged siRNA was bound to the polymer via electrostatic interactions. Positive charge of the polymer is aimed to be reduced upon reduction of nitrobenzyl units in hypoxic environment in the presence of NTR enzymes. Spontaneous detachment of the aniline from the structure will result in the release of siRNA targeting oncogene cMYC under these conditions. In hypoxic human lung cells, a significant decrease in the expression of genes involved in cMYC and related pathways was detected. Cancer cells with hypoxic microenvironment have more endogenous reductive enzymes than normal cells. Therefore, polymer could be made activatable in cancer cells and silence the specific gene in cancer cells. The thesis study would inspire the development of new carriers sensitive to cancer-microenvironment and support the research on selective therapies.

Keywords: siRNA delivery, Gene silencing, Stimuli-responsive polymer, Nitroreductase, Cancer

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H. Beyza DOĞAN
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SYMBOLS AND ABBREVIATIONS

Symbols:

mL: mililiter

μL: microliter

nm: nanometer

μM: Micromolar

mM: Milimolar

°C: degree Celsius

Abbreviations:

ACTB: Actin Beta Gene

ASO: Antisense Oligonucleotides

ATP: Adenosine Triphosphate

BCL-2: B-Cell Lymphoma Gene-2

CDK: Cyclin-Dependent Kinases

c-Myc: Cellular Myelocytomatosis Oncogene

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DAPI: 4',6-Diamidino-2-Phenylindole

CCND-1 :Cyclin D1

CR: Controlled Release

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DOX: Doxorubicin

ECL: Enhanced Chemiluminescence

EDTA: Ethylenediaminetetraacetic Acid

FTIR: Fourier-Transform Infrared

FBS: Fetal Bovine Serum

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GLUT1: Glucose Transporter Gene 1

GSH: Glutathione

HG-DMEM: High Glucose Dulbecco's Modified Eagle Medium

miRNA: Micro Ribonucleic Acid

mRNA: Messenger Ribonucleic Acid

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

MTX: Methotrexate

NADH: Nicotinamide Adenine Dinucleotide

NAPDH: Nicotinamide Adenine Dinucleotide Phosphate

NSCLC: Non-Small Cell Lung Cancer

NF: Nuclease-Free

NMR: Nuclear Magnetic Resonance

NTR: Nitroreductase

ORF: Open Reading Frame

P4VP: Poly(4-vinyl pyridine)

PBS: Phosphate-Buffered Saline

qPCR: Quantitative Polymerase Chain Reaction

RNA: Ribonucleic Acid

RNAi: Ribonucleic Acid Interference

RT-PCR: Real-Time Polymerase Chain Reaction

SD: Standard Deviation

siRNA: Small Interfering RNA

TBST: Tris-Buffered Saline with Tween 20

ΔΔCt: Delta Delta Threshold Cycle

1. INTRODUCTION

Controlled drug delivery technology is a rapidly evolving field driven by contributions from chemists and chemical engineers, offering significant advantages over traditional dosage forms, such as enhanced efficacy, reduced toxicity, and improved patient compliance and convenience. These delivery systems commonly utilize synthetic polymers as drug carriers, enabling treatments that were previously inaccessible. Understanding the motivation for achieving controlled release is crucial, as the field has rapidly expanded and diversified, potentially posing challenges for non-specialists. All controlled release systems aim to enhance drug therapy effectiveness, whether by increasing therapeutic activity while minimizing side effects, decreasing the frequency of drug application, or reducing the necessity of using a certain drug application method such as repeated injections (Uhrich et al. 1999; Bird, Stewai, and Lightfoot 2002; Langer 1998).

The mechanism of controlled drug delivery technology is based on sustained and control release mechanisms. These mechanisms enable targeting of specific diseased area and releasing of drug in aperion of time when it is needed. This technology has adventages over immediate or non-targeted release because side effect of drug in other organs can be decreased and effect of drug can be enhanced.

Various mechanisms have been developed to achieve controlled release of drugs using polymers, driven by the different requirements imposed by various drugs. For instance, drugs intended for extended release in the acidic environment of the stomach necessitate a different system than those for pulsatile delivery in the bloodstream. It's crucial to consider what happens to the polymer following the release of the drug; ideally, polymers excreted out of the body or biodegraded into non-toxic fragments are preferred. Polymers which are nondegradable and stable are prefered in some applications, such as when the delivery system is recycled after drug release or for oral use where the polymer needs to pass through gastrointestinal tract (Flynn, Yalkowsky, and Roseman 1974). Controlled release systems with different mechanisms require polymers with specific physicochemical properties, leading to the development of new polymers adressing these requirements (Langer 1995). There are several mechanisms that enable controlled drug delivery through cell.

2. LITERATURE REVIEW

2.1. Drug Release Mechanisms

2.1.1. Drug release mechanism through diffusion

Diffusion is the spontaneous movement of molecules from one region to another, driven by the goal of equalizing chemical potential or thermodynamic activity (Crank 1979). Despite being a result of random molecular motion, diffusion can be simplified conceptually as involving three main components: the molecules of interest, the diffusional barrier and the medium. The key driving force for diffusion is the concentration gradient, which determines the movement of diffusants within the barrier (Jaeger 1950).

For controlled release systems, diffusion is often studied in two primary contexts: diffusion through a semi-permeable membrane and release from a matrix preloaded with the therapeutic reagent. Thus, diffusion based drug release mechanisms are categorized as matrix and reservour systems that are shown in Figure 2.1 (Schechter 1961; Flynn, Yalkowsky, and Roseman 1974).

2.1.1.1. Matrix systems

Matrix systems consist of a blend of active and inactive ingredients that are uniformly mixed in the dosage form. These systems are the most commonly used oral controlled release (CR) technology, with their popularity attributed to several factors. First, unlike reservoir and osmotic systems, matrix-based products can be manufactured using conventional diffusion-controlled drug delivery systems and standard equipment. Second, matrix systems typically have a shorter development time and lower costs, with no need for additional capital investment. Furthermore, matrix systems are versatile, capable of handling both low and high drug loads, and can accommodate active ingredients with a broad range of physical and chemical properties (Shen, Jasti, and Li 2003).

However, matrix systems have certain limitations. They lack flexibility in adjusting to frequently changing dosage levels, which is often required during clinical trials. When new dosage is needed, a new formulation and additional resources are usually necessary. Additionally, for products that require specialized release profiles (e.g., dual release or delayed and extended release), more complex matrix technologies, such as layered tablets (e.g., Allegra D), may be needed (Qiu, Chidambaram, and Flood 1998). Despite these limitations, matrix systems continue to be popular due to their proven

success across various product types. Matrix systems are typically categorized into two types based on rate-controlling materials: hydrophobic and hydrophilic systems.

2.1.1.2. Reservoir systems

A reservoir system consists of a core which contains active ingredients and a rate-controlling membrane that can regulate drug release. The release profile is influenced by both the formulation and the solubility of the drug. Unlike single-unit tablets, reservoir systems often use multiple coated units (beads, pellets, minitablets), which help minimize defects and improve release consistency (Shen, Jasti, and Li 2003). A significant advantage is the ability to customize drug release by combining particulates with different release rates, as seen in products like Metadate CD and Ritalin LA. Reservoir systems also offer flexibility in adjusting dosage without needing new formulations, making them ideal for clinical trials where doses change frequently. Key factors for performance include the polymer type, drug load, and solubility, with common materials being ethylcellulose and acrylic copolymers (Mehta, Valazza, and Abele 1986). The manufacturing process may require special equipment and adjustments during scaling, and the dissolution rate can change over time, which is addressed by adding a curing step in the coating process (Mehta 2008).

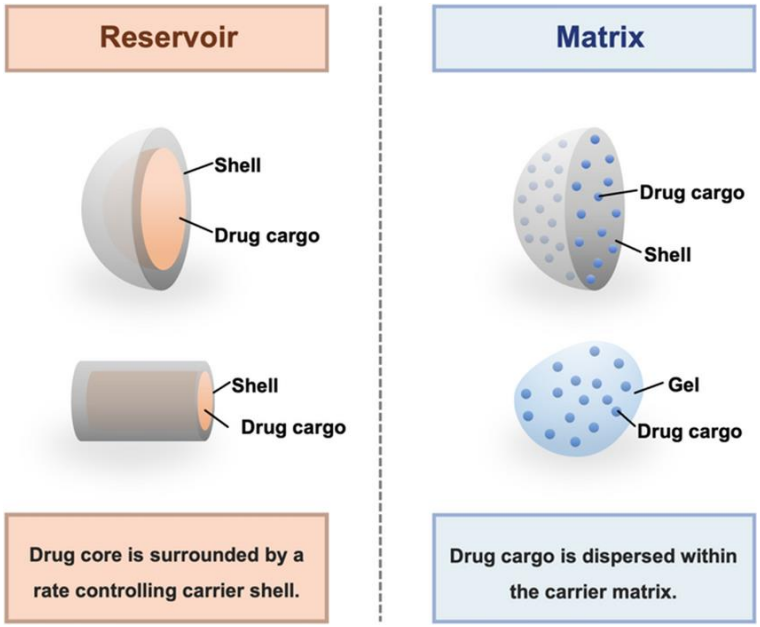


Figure 2.1. Schematic representation of comparision of reservoir systems (core + membrane) and matrix systems (drug dispersed uniformly in polymer matrix). (Shen, Sun, and Sun 2023)

2.1.2 Drug release mechanism through dissolution

The dissolution process involves the detachment of drug molecules from their solid structure into the liquid interface, followed by diffusion into the bulk liquid. This process can be tailored to develop controlled release (CR) systems with specific and desired release patterns. Matrix-based or membrane-based CR systems are used to slow, delay, and control drug release. In matrix systems, the drug is uniformly dispersed in polymers or waxes, while membrane systems have a coating that controls the release shown in Figure 2.2. These systems can be combined into a coated matrix (Ranade 1991; Rhodes and Porter 1998).

In systems made from water-soluble components, dissolution is the rate-limiting step for drug release. Dissolution-controlled release matrix systems offer sustained drug release, providing long-term therapeutic effects. Unlike diffusion-controlled systems, their release profiles do not follow zero-order kinetics but are typically delayed, pulsatile, or sustained. These systems can be applied to various drug delivery routes, beyond oral solid forms (Lachman, Lieberman, and Kanig 1976).

The primary benefit of dissolution-controlled release systems is their capacity to deliver sustained and customized drug release (Cao et al. 2004). However, they can be complex to design and control. The dissolution rate may change over time, requiring steps like curing after coating to stabilize the release. Despite these challenges, these systems offer valuable therapeutic benefits when carefully formulated (Lee and Yeo 2015).

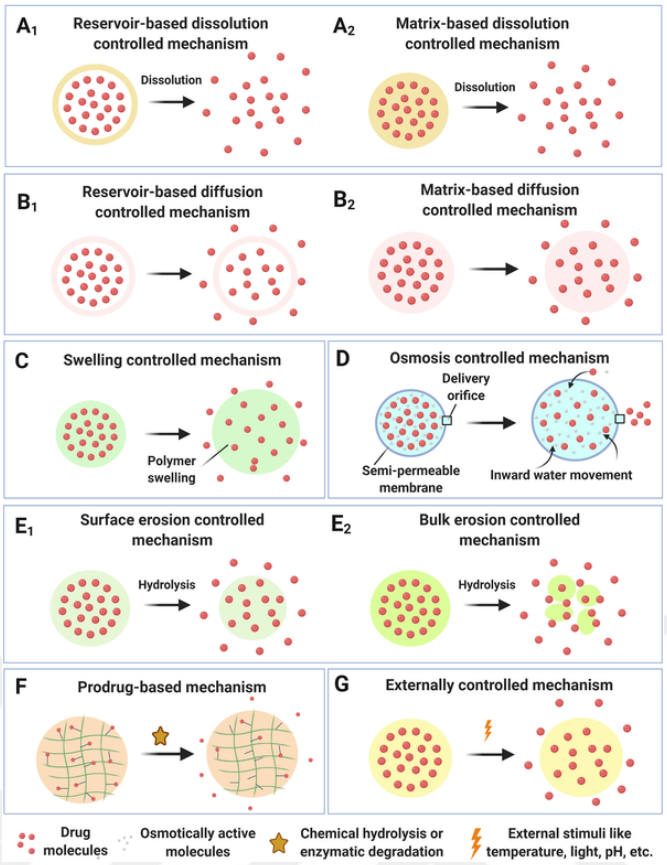


Figure 2.2. Comprehensive schematic showing dissolution controlled (A1, A2), diffusion-controlled (B1, B2), osmotically-controlled (D), stimuli-responsive (G), and chemically-controlled (F) drug release systems (Jamaledin et al. 2020)

2.1.3. Drug release mechanism through osmotic change

Osmosis is the spontaneous flux of the solvent from a region with lower solute concentration to a region having much higher concentration of the solute through a semipermeable membrane. The pressure that prevents solvent movement is termed osmotic pressure. This pressure is a colligative property, depending on the solute concentration. In osmotic delivery systems, osmotic pressure drives the release of drugs, resulting in a constant influx of water, and thus a zero-order release rate, which is ideal for controlled release formulations shown in Figure 2.2.

The main advantage of osmotic systems is their ability to provide precise and consistent drug release over extended periods, regardless of external factors. This ensures a steady plasma concentration, reducing the peaks and troughs associated with conventional drug release forms, and can improve patient compliance by maintaining effective drug levels. Osmotic systems are particularly beneficial for drugs requiring long-term treatment, such as those for diabetes, hypertension, and attention-deficit

disorders. These systems also help reduce side effects by maintaining a consistent plasma level, avoiding the fluctuations that are common in immediate-release forms.

However, osmotic systems are more expensive and complex than concentional systems to manufacture. Despite these challenges, they are increasingly used in once-daily formulations, especially for chronic conditions. Osmotic delivery systems are also showing promise in implantable forms, allowing for controlled drug release over extended periods. As biotechnology continues to develop potent new drugs, osmotic systems will likely play an increasingly significant role in delivering these compounds with precise control (Theeuwes and Higuchi 1974).

2.1.4 Stimuli-responsive drug release

Stimuli-responsive drug delivery systems (SRDDS) are an emerging class of nanocarriers that respond to specific internal or external triggers, enabling spatially and temporally controlled release of therapeutic agents shown in Figure 2.2. These platforms offer promising solutions to longstanding challenges in cancer therapy, such as off-target toxicity, poor bioavailability, and drug resistance (Li and Mooney 2016).

In tumor microenvironments, various physiological differences such as acidic pH, elevated levels of glutathione (GSH), overexpressed enzymes, or hypoxia can be exploited as internal/environmental stimuli. For instance, pH-sensitive nanoparticles are designed to remain stable in neutral blood pH but disassemble in acidic tumor or endosomal environments, releasing their cargo selectively at the tumor site (Wang and Kohane 2017). Likewise, redox-responsive systems utilize disulfide bonds that are cleaved in the presence of high intracellular GSH levels, enabling selective drug release inside tumor cells (Hao et al. 2021).

External factors like temperature, light, magnetic fields, or ultrasound can also be used to initiate drug release in a controlled and non-invasive way. For example, light-responsive carriers can be activated by near-infrared (NIR) irradiation, leading to structural changes or bond cleavage in the carrier that releases the drug on demand (Yavuz et al. 2009).

The integration of stimuli-responsiveness into siRNA or anticancer drug delivery systems enhances their selectivity, reduces systemic side effects, and improves therapeutic efficacy. In the context of this thesis, the design of pyridinium-based polymers for stimuli-triggered release of c-Myc siRNA in lung cancer cells aligns with this strategy, aiming to improve targeted gene silencing with minimal impact on healthy tissues.

2.1.5. Drug release mechanisms through linker cleavage

In the thesis, therapeutic siRNA is aimed to be released through the action of enzymes which ultimately leads to bond cleavage (Wells et al. 2019). Similar strategies in the literature which use bond dissociation for drug release is briefly summarized below:

2.1.5.1. Ester bond hydrolysis

Carboxylic ester-based linkers are commonly used in targeted drug delivery due to their wide applicability and ease of attachment to therapeutic molecules (Perni and Prokopovich 2017). These linkers form a bond between the nanocarrier and drug through functional groups like carboxylic acids and alcohols. The ester bond allows drug release via hydrolysis under physiological conditions, catalyzed by acids, bases, metal ions such as copper (II), and hydrolytic enzymes like esterases. However, the physiological instability ester bonds limits controlled drug release. Drug release can be achieved by two ways; endocytic pathway mediated, intracellular environment mediated (Ding and Li 2017).

Ester hydrolysis mechanisms have been widely exploited in drug delivery systems, including those release of drugs such as Taxol, Methitreate, platinum agents. For instance, Taxol conjugates with nanocarriers release the drug through ester hydrolysis, enhancing efficacy in suppressing tumor growth (Masood 2016). Similarly, Methotrexate and platinum-based agents are conjugated using ester linkages for targeted drug delivery, improving therapeutic efficacy (Thomas et al. 2005; Bhirde et al. 2009). Immunosuppressive agents like tacrolimus and cyclosporine have also been conjugated using ester linkages for controlled release and improved therapeutic efficiency (Yura et al. 1999; Azzi et al. 2010).

2.1.5.2. Amide bond hydrolysis

Amide linkers are frequently employed for drug conjugation to nanocarriers due to their stability against chemical hydrolysis as opposed to ester bonds (Kanamala et al. 2016). This stability results in a longer circulation, as amide bonds require harsh conditions like higher temperatures or strong acid/base catalysts for hydrolysis (Ragozin et al. 2016). Amide cleavage is primarily achieved through enzymatic mechanisms involving hydrolytic proteases such as serine or cysteine proteases, metaloproteases such as zinc-dependent peptidases (Ben-Nun et al. 2017). These enzymes, present in the cell,

in extracellular environments and cell membranes, play a crucial role in drug release, particularly at specific action sites.

Anticancer drugs like methotrexate (MTX), doxorubicin (DOX), and α-tocopheryl succinate (α-TOS) have been attached to nanocarriers via amide linkages to achieve controlled drug release. For instance, MTX conjugates with peptide-based amide linkages are sensitive to overexpressed peptidases on cancer cell surfaces, enabling targeted drug release (Chau, Tan, and Langer 2004). Similarly, DOX-Fe3O4 nanoparticles release DOX through amide bond cleavage in weakly acidic environments, exhibiting controlled drug release and antitumor activity (Chen et al. 2010). α-TOS, conjugated to FA-conjugated G5 PAMAM dendrimers via succinyl amide bonds, demonstrates water solubility and efficacy in inducing apoptosis in cancer cells (Zhu et al. 2014).

2.1.5.3. Hydrozone, imine and oxime bond hydrolysis

Hydrazone-based linkers, having acyl hydrazone, carbonyl hydrazone, and benzenesulfonyl hydrazone moieties, are commonly used in the conjugation of various anticancer therapeutic molecules (Wong and Choi 2015). These linkers allow conjugation with drug molecules containing ketone or aldehyde moieties, facilitating efficient drug delivery systems (Du et al. 2011). Hydrazone bonds enable pH-sensitive drug release, becoming more prone to breakdown in acidic environments (Wong and Choi 2015). Nanoparticle systems employing hydrazone bonds demonstrate efficient anticancer drug delivery by increasing intracellular drug concentration and achieving drug release in acidic subcellular compartments (Du et al. 2011).

Besides hydrazones, imine (Schiff base) and oxime bonds also act as acid-labile linkages, making them valuable for smart delivery vehicles. Imine bonds form between primary amines and aldehydes/ketones, while oxime bonds arise from reactions with hydroxylamines. Both remain stable at neutral physiological pH but cleave selectively under mildly acidic conditions typical of tumor environments or endosomal/lysosomal compartments (Zhuo et al. 2020).

For example, PEG-DOX conjugates via Schiff base (imine) bond were shown to self-assemble into nanoparticles that disassemble and release doxorubicin in acidic tumor microenvironments, improving targeted delivery and reducing systemic toxicity (Zhang et al. 2016). Likewise, oxime-linked polysaccharide–doxorubicin conjugates exhibited efficient drug release under acidic intracellular conditions, resulting in enhanced antitumor effects compared to non-acid-sensitive counterparts (Xu et al. 2015).

2.1.5.4. Disulfide bond cleavage

Disulfide linkers are widely utilized in targeted drug conjugation, undergoing cleavage via disulfide exchange reaction with endogenous thiols such as glutathione tripeptide (GSH) or reduction reactions activated by redox enzymes as shown in Figure 2.3 (Rodrigues et al. 2003; Yan et al. 2016). Most cleavage occurs intracellularly, particularly in the cytoplasm, enabling targeted intracellular drug release (Kuśmierek et al. 2009).

Disulfide-linked anticancer therapeutics, such as paclitaxel and cisplatin, demonstrate enhanced efficacy and reduced cytotoxicity through controlled drug release mediated by GSH (Wong and Choi 2015). Nanosized micelle systems utilizing disulfide exchange show enhanced intracellular drug uptake and cytotoxicity, offering promising outcomes in cancer therapy (Yin et al. 2015).

2.1.5.5. Cleavage by hypoxia activation

Hypoxia, characterized by low oxygen levels, is common in solid tumors due to poor blood perfusion, making it a target for selective drug activation (Rooseboom, Commandeur, and Vermeulen 2004). Prodrugs activated by oxidoreductases in hypoxic environments, such as apaziquone and mitomycin C, release active drugs specifically within tumors (Wong and Choi 2015). Hypoxia-activated linkers, categorized into groups like quinone-trimethyl lock systems and nitroaromatic heterocyclics, undergo cleavage by oxidoreductases, releasing drugs within hypoxic tumor microenvironments (Wong and Choi 2015; Du et al. 2011; Rodrigues et al. 2003; Yan et al. 2016; Kuśmierek et al. 2009; Yin et al. 2015; Rooseboom, Commandeur, and Vermeulen 2004; Ong et al. 2008). Reducing agents such as cytochrome P450 oxidoreductase can break hydroquinone linkers, initiating drug release via intramolecular cyclization pathways (Van Haaften et al. 2001). The efficiency of drug release is influenced by linker design and tumor physiological factors.

Hypoxia, characterized by inadequate oxygen supply in the body, represents a significant aspect of solid tumors (Brown and Wilson 2004; Yang et al. 2017). Malignant tumors exhibit heightened oxygen and nutrient consumption during unregulated growth and proliferation, culminating in a profoundly hypoxic microenvironment with localized oxygen concentrations typically ranging from 0-4% (Yang et al. 2017). The development of hypoxic tumors, often accompanied by abnormal microvessels, restricts the perfusion of chemotherapeutic drugs, thereby diminishing their effectiveness and contributing to

tumor recurrence. Consequently, the assessment of hypoxia levels in tumor cells and in vivo holds considerable clinical importance. Early investigations have identified that hypoxia accelerates bioreductive reactions, resulting in increased levels of enzymes such as nitroreductase (NTR), azoreductase, or DT-diaphorase (Xu et al. 2013; Fan et al. 2020).

Nitroreductases (NTRs) are enzymes found in bacteria, fungi, and mammals that catalyze the reduction of nitro functional groups ($-NO_2$) to amino ($-NH_2$) or hydroxylamine (-NH(OH)) groups (Brown and Wilson 2004). This enzyme activity is of particular importance in cancer therapy and drug metabolism because of its ability to selectively activate nitro-containing prodrugs in hypoxic tumor areas. NTR, a flavin-dependent enzyme, has attracted considerable interest due to its effectiveness in reducing nitroaromatic compounds when reduced NADH serves as the electron donor (Zhai et al. 2017). This reduction process happens in physiological settings and is aided by the catalytic activity of NTRs shown in Figure 2.4.

In cancer therapy, the hypoxic environment within solid tumors is appropriate for NTR-mediated selective activation of nitro-containing prodrugs. Furthermore, the hypoxic microenvironment within solid tumors is directly correlated with elevated levels of endogenous NTR. Thus, the assessment of hypoxia status in tumor cells and tissues can be accomplished through the evaluation of NTR activity (Fan et al. 2020). In this thesis, siRNA release is expected to be achieved through nitroreductase enzyme activity in hypoxic tumor cells.

2.2. Gene Silencing Mechanisms with Regulatory RNAs

2.2.1. Theuropatic siRNA

Delivery of siRNA has been the main obstacle for the application of therapeutic siRNAs in humans (Whitehead, Langer, and Anderson 2009; Juliano et al. 2009; Castanotto and Rossi 2009; Miller and Siegwart 2018; Lu et al. 2017). A major obstacle in siRNA delivery lies in its pharmacological characteristics. Compared to small molecule drugs, siRNAs are considerably larger (~13 kDa) and possess a strong negative charge due to the presence of 38 to 50 phosphate groups, hindering their ability to pass through cell membranes. In addition, unmodified siRNAs are prone to degradation in the bloodstream and may trigger immune reactions by interacting with Toll-like receptors (Watts, Deleavey, and Damha 2008). For intravenously delivered siRNA to be effective, it must traverse the vascular endothelium and diffuse through the extracellular matrix. It also needs to evade renal clearance and uptake by unintended cells. Once inside the target

cell, the siRNA must escape from endosomes, dissociate from carriers, and engage with the RNA interference machinery. During the entire process, siRNAs has to be sufficiently resistant to degradation by nuclease enzymes to enable its function successfully (Whitehead, Langer, and Anderson 2009). Therefore, siRNAs have significant delivery challenges that do not exist for small molecule drugs (Gaynor, Campbell, and Cosstick 2010). Significant scientific progress has been made over the years, and currently there are more than 20 ongoing clinical phase studies with siRNA-based therapeutics (Whitehead, Langer, and Anderson 2009; Burnett, Rossi, and Tiemann 2011; de Fougerolles et al. 2007; Kanasty et al. 2013).

Gene silencing refers to the process of inhibiting protein expression by genes, typically occurring at the messenger ribonucleic acid (mRNA) level, effectively halting protein expression post-transcriptionally. This method shows great potential in creating a novel category of molecular therapeutic medications that disrupt genes associated with causing or promoting diseases, especially those that encode "non-druggable" targets inaccessible to traditional treatments. This inhibition can be achieved through the mechanism of ribonucleic acid interference (RNAi) (Van Haaften et al. 2001; Liang and Lam 2012). For the therapeutic use of small interfering ribonucleic acid (siRNA), successful delivery to cells and subsequent release into the intracellular space are essential prerequisites. A delivery system is therefore necessary to safeguard siRNA from enzymatic degradation, enhance its delivery to the target organ, facilitate cellular uptake, and ultimately release it into the cell cytoplasm to enable incorporation into the RNA machinery (Ragelle, Vandermeulen, and Préat 2013). Cationic polymers have emerged as valuable delivery systems for siRNA in vivo, offering improvements in both the pharmacokinetics and pharmacodynamics of siRNA (Buyens et al. 2010). These polymers act as efficient agents for siRNA cell transfer, or transfection, by binding and condensing nucleic acids into stabilized nanoparticles (Whitehead, Langer, and Anderson 2009). Before their use in vivo, the transfection efficiency of such polymers must be evaluated in vitro. Poly 4-vinyl pyridine is one such polymer that can be functionalized for effective delivery of siRNA (Sinani et al. 2023). In the thesis, first example of environment dependent change of pyridinium polymer to pyridine and subsequent release of siRNA targeting c-myc was investigated.

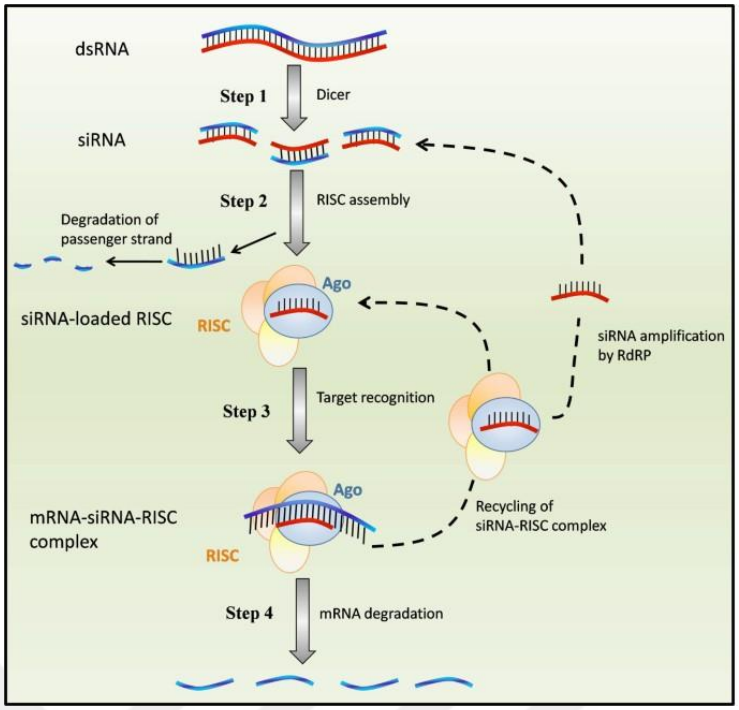


Figure 2.3. Brief depiction of mammalian RNA interference (Cuccato et al. 2011).

2.2.2. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate expression of genes after the transcription. These molecules are typically about 20–24 nucleotides long and function by binding to complementary sequences in messenger RNAs (mRNAs), resulting in either translation repression or mRNA degradation (Bartel 2018). The miRNA biogenesis pathway includes processing by Drosha and Dicer enzymes, ultimately forming a mature miRNA duplex. One strand of this duplex becomes part of the RNA-induced silencing complex (RISC), guiding the complex to target mRNAs, particularly within the 3' untranslated region (3' UTR) (Ha and Kim 2014).

This sequence-specific interaction allows miRNAs to downregulate numerous genes simultaneously, making them critical players in development, cell cycle regulation, and disease progression, especially in cancer (Krol, Loedige, and Filipowicz 2010).

2.2.3. Antisense oligonucleotides (ASOs)

Antisense oligonucleotides (ASOs) are short, synthetic, single-stranded nucleic acids designed to specifically bind to RNA transcripts through Watson-Crick base pairing. Upon hybridization to target mRNAs, ASOs can silence gene expression by triggering RNase H-mediated degradation or by physically obstructing ribosome assembly or splicing events (Bennett and Swayze 2010).

A notable class of ASOs, called gapmers, combines chemically modified regions flanking a central DNA sequence. This configuration enhances both target binding and enzymatic cleavage efficacy. ASO-based therapies are already in clinical use for several genetic disorders and are being investigated for broader therapeutic applications, including cancer and neurodegenerative diseases (Crooke 2017).

2.2.4. CRISPR

The CRISPR interference (CRISPRi) technique utilizes a nuclease-deactivated Cas9 (dCas9) enzyme guided by a single-guide RNA (sgRNA) to bind to specific DNA regions and inhibit transcription without introducing double-strand breaks. When fused to repressive domains like KRAB (Krüppel-associated box), dCas9 can recruit chromatin remodeling complexes to suppress transcription initiation more effectively (Qi et al. 2013; Gilbert et al. 2013).

This programmable system enables precise, reversible gene repression and has become a valuable tool in functional genomics and disease modeling. Unlike RNAi, which acts on transcripts, CRISPRi blocks gene activity at the DNA level, thus offering complementary and sometimes more robust gene silencing (Dominguez, Lim, and Qi 2016).

2.2.5. DNA methylation-based therapy

DNA methylation is an epigenetic change characterized by the attachment of a methyl group to the 5th carbon of cytosine, mainly within CpG sites. This modification can silence gene expression by altering chromatin structure and inhibiting transcription factor binding (Moore, Le, and Fan 2013). In cancer, hypermethylation of tumor suppressor gene promoters is a well-documented mechanism of gene silencing, contributing to uncontrolled cell proliferation and metastasis (Jones and Baylin 2007).

Therapeutic strategies based on reversing aberrant DNA methylation use DNA methyltransferase (DNMT) inhibitors such as azacitidine and decitabine. These agents incorporate into DNA during replication and inhibit DNMT activity, leading to passive demethylation and reactivation of silenced genes (Juergens et al. 2011). Such approaches are already approved for treating myelodysplastic syndromes and are under investigation for broader oncological applications.

2.2.6. Aptamers

Aptamers are short, single-stranded RNA or DNA sequences that adopt distinct three-dimensional shapes, allowing them to bind with high specificity and affinity to particular molecular targets, such as proteins. They are often described as “chemical antibodies” due to their ability to selectively recognize targets (Sun et al. 2014). Aptamers can be engineered to interfere with protein function or serve as delivery agents for gene silencing therapeutics like siRNAs or antisense oligonucleotides.

The selection of aptamers is achieved through a process known as SELEX (Systematic Evolution of Ligands by EXponential enrichment), which enables the identification of high-affinity ligands from large random oligonucleotide libraries (Stoltenburg, Reinemann, and Strehlitz 2007). Due to their low tendency to provoke immune responses, along with their synthetic accessibility and chemical flexibility, aptamers are promising tools for precision gene therapy and targeted drug delivery.

2.2.7. Long noncoding RNAa

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that do not code for proteins but play critical roles in regulating gene expression at various levels, including chromatin modification, transcription, and post-transcriptional processing (Statello et al. 2021). Unlike small RNAs like miRNAs, lncRNAs exhibit more diverse modes of action due to their ability to form complex secondary and tertiary structures, interact with proteins, DNA, and other RNAs, and localize in both the nucleus and cytoplasm.

A major mechanism by which lncRNAs mediate gene silencing is through recruitment of chromatin-modifying complexes. For example, the well-characterized lncRNA XIST facilitates X-chromosome inactivation by recruiting Polycomb Repressive Complex 2 (PRC2) to the inactive X chromosome, leading to histone methylation and transcriptional silencing (Chu et al. 2015). Other lncRNAs, such as HOTAIR, guide epigenetic regulators to specific genomic loci, altering chromatin state and repressing gene expression in trans (Rinn et al. 2007).

Additionally, lncRNAs can act as molecular decoys for transcription factors or microRNAs, sponges that sequester miRNAs, or scaffolds that bring together multiple regulatory proteins. The versatility of lncRNAs enables them to be essential regulators in processes such as development, maintenance of stem cell pluripotency, and cancer formation (Statello et al. 2021; Wang and Chang 2011).

2.3. Tumor and Tumor Environment

Cancer is a complex disease defined by uncontrolled cellular growth, which leads to a development of abnormal tissue masses known as tumors. These tumors arise from a variety of anatomical places, interfering with physiological processes such as cell division and renewal. In healthy people, cells develop and divide in a controlled manner to maintain tissue homeostasis and function. However, disturbances caused by genetic mutations or environmental stressors can disrupt this equilibrium, resulting in unregulated cellular growth and tumor formation. Following the acquisition of genetic changes, these aberrant cells gain the ability to infiltrate adjacent tissues and spread to distant anatomical locations via hematogenous or lymphatic channels, a phenomenon known as metastasis. Cancers have many forms, ranging from benign to malignant tumors. Benign tumors are localized growths that lack the potential for invasiveness or distant spread, and are typically treatable with surgical excision and low recurrence rates (Hanahan and Weinberg 2011). Malignant tumors, which epitomize cancerous lesions, have invasive properties, infiltrating surrounding tissues and metastasizing to distant sites, posing serious health risks and potentially fatal outcomes. While solid tumors, which arise from epithelial tissues, are a common type of cancer, hematological malignancies, such as leukemia, arise from blood-forming cells and spread throughout the body without forming discrete masses. The complex nature of cancer etiology emphasizes the importance of understanding the underlying mechanisms that drive abnormal cellular proliferation and dissemination. Extensive research into the genetic, molecular, and environmental drivers of cancer etiology has resulted in important advances in diagnostic, therapeutic, and preventive strategies for combating this complicated disease (Health 2019).

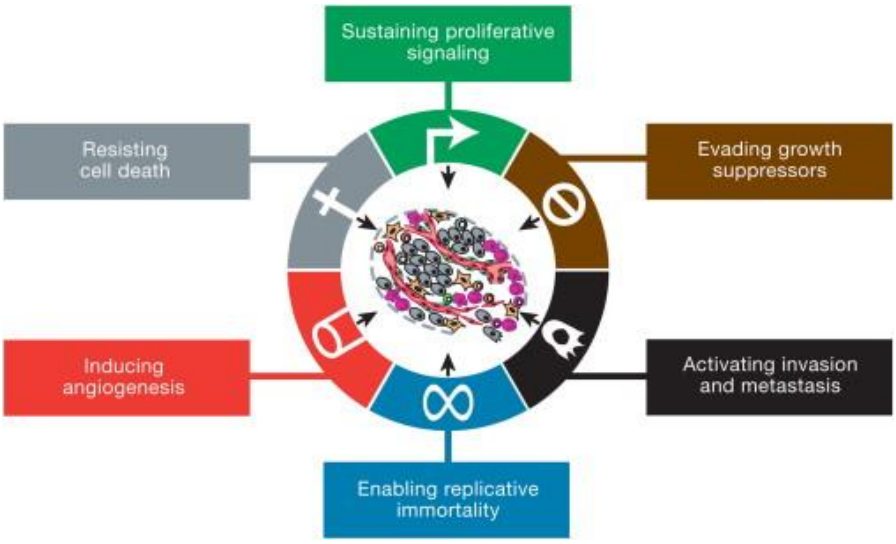


Figure 2.4: Six hallmarks of cancer cell (Hanahan and Weinberg 2011).

2.3.1. c-Myc gene as a cancer therapeutic target

The c-myc gene encodes a nuclear phosphoprotein that functions as a transcription factor, playing a central role in controlling a wide range of cellular activities, including proliferation, growth, metabolism, differentiation, and apoptosis (Dang 2012). Under physiological conditions, the expression and function of c-Myc are tightly regulated through multiple layers such as transcriptional, translational, and post-translational mechanisms. However, in pathological contexts, especially in malignancies, c-myc is often dysregulated due to genetic alterations like gene amplification, chromosomal translocations, or enhancer hijacking, which leads to its aberrant overexpression (Meyer and Penn 2008). This overexpression contributes to tumor initiation and progression by disturbing cell cycle checkpoints, promoting genomic instability, and cooperating with other oncogenes.

Importantly, non-small cell lung cancer (NSCLC), which accounts for approximately 85% of all lung cancer cases, frequently exhibits c-myc disregulation. Studies have shown that MYC amplification or overexpression correlates with poor prognosis, aggressive tumor behavior, and resistance to certain therapies in NSCLC (Chabanon and Postel-Vinay 2022). Given its critical role in sustaining malignant phenotypes, c-Myc is considered a classic example of “oncogene addiction,” wherein tumor cells rely heavily on its continuous expression for survival and proliferation. Interestingly, targeting c-myc in preclinical lung cancer models has induced tumor regression with minimal toxicity to normal tissues, highlighting its potential as a therapeutic target (Soucek et al. 2008).

In addition to c-Myc itself, several of its downstream effectors play significant roles in supporting the oncogenic program. One such effector is GLUT1 (encoded by SLC2A1), a key glucose transporter that facilitates the elevated glucose uptake necessary for tumor growth. c-Myc has been shown to transcriptionally upregulate GLUT1, thereby enhancing aerobic glycolysis—a phenomenon known as the Warburg effect—which supports the energetic and biosynthetic demands of proliferating cancer cells (Osthus et al. 2000). Overexpression of GLUT1 is a common feature in NSCLC and has been associated with increased invasiveness and poor clinical outcomes.

Another important factor in this oncogenic network is BCL2, a central regulator of the mitochondrial apoptotic pathway. While c-Myc drives proliferation, it can also sensitize cells to apoptosis in the absence of survival signals. Overexpression of BCL2 counteracts this pro-apoptotic pressure by inhibiting cell death pathways, thereby enabling c-Myc-driven cells to survive and expand uncontrollably. Studies have demonstrated a functional cooperation between c-Myc and BCL2, particularly in solid tumors, including lung cancer, where their co-expression contributes to resistance against therapy and tumor progression (Eischen et al. 2001).

To overcome challenges associated with direct targeting of transcription factors like c-Myc, alternative approaches such as antisense oligonucleotides (ASOs) and RNA interference (RNAi) have been explored. These strategies allow sequence-specific inhibition of MYC expression, and recent advancements in delivery systems and chemical modifications have improved their stability and efficacy. In this thesis, we introduce a novel strategy for c-myc silencing using stimuli-responsive, pyridinium-based polymer carriers for siRNA delivery. Moreover, the study also investigates the expression patterns of GLUT1 and BCL2 in lung cancer cell lines to better elucidate their interplay with c-Myc-mediated signaling and to evaluate their potential as co-targets in NSCLC therapy.

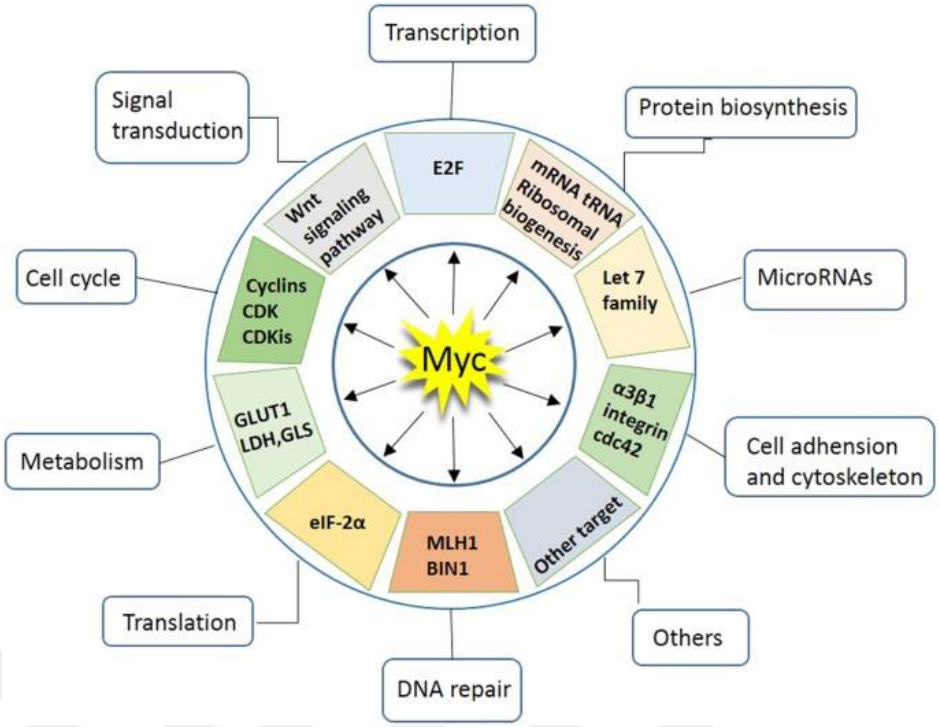


Figure 2.5. Myc regulates a spectrum of cellular functions. Myc regulates a large number of protein-coding or non-coding genes that are involved in distinct cellular functions, including cell cycle (CCND-), metabolism (GLUT-1), signal transduction, transcription, and translation (eIF-2A), among others (Chen, Liu, and Qing 2018).

2.4. Cell Cycle

The cell cycle is a series of carefully regulated stages through which cells grow, duplicate their DNA, and divide into two daughter cells. It consists of four main phases: G1 (first gap), S (DNA synthesis), G2 (second gap), and M (mitosis). Key molecular regulators, including cyclins, cyclin-dependent kinases (CDKs), and various checkpoint pathways, ensure proper transition between these phases and safeguard genome integrity. Disruptions in these control mechanisms can trigger abnormal proliferation of the cells, a common feature of cancerous cells (Bretones Sánchez, Delgado Villar, and León Serrano 2015).

Among the central regulators of cell cycle control is the c-Myc proto-oncogene, which encodes a transcription factor that influences a broad spectrum of genes associated with cellular growth, metabolism, and programmed cell death (Meyer and Penn 2008). When c-Myc is overexpressed, it accelerates cell cycle progression by stimulating the transcription of pro-proliferative genes, such as cyclin D, and E2F family members, particularly enhancing the G1/S phase transition (Dang 2012; Bretones Sánchez, Delgado Villar, and León Serrano 2015). On the other hand, diminished c-Myc expression may cause arrest at the G1 phase or decelerate progression, depending on the cellular context

(Meyer and Penn 2008). Moreover, persistently high levels of c-Myc can promote genomic instability and induce apoptosis if not counteracted by survival signals (Ahmadi et al. 2021).

Gaining insight into how c-Myc influences cell cycle dynamics is vital for understanding its oncogenic potential and developing targeted cancer therapies (Dang 2012).

3. MATERIAL AND METHOD

This study focuses on the synthesis of stimuli responsive polycationic compound 1, which is intended to deliver siRNA targeting the suppression of c-Myc gene expression. Structural characterization of the synthesized molecule was performed using ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectroscopy. Additionally, the human lung caner (A-549) cell line was used for cell culture studies. Cytotoxicity analysis of the molecule was conducted on that cell line, and following siRNA delivery Real-Time Polymerase Chain Reaction (RT-PCR) analysis was performed to understand the changes in RNA expression levels within the cells.

3.1. Design of Molecule

The tumor microenvironment is characterized by several distinctive features that differ from normal tissue physiology, among which hypoxia is one of the most critical hallmarks (Brown and Wilson 2004). Hypoxia not only contributes to cancer progression and metastasis but also serves as a biomarker for tumor-specific targeting strategies (Rankin and Giaccia 2016). In this context, the development of hypoxia-responsive nanocarriers offers a promising route to improve therapeutic selectivity and efficacy, especially for targeting oncogenes like c-Myc, which are often overexpressed in hypoxic solid tumors such as non-small cell lung cancer (NSCLC).

To date, a number of polymer-based platforms have been designed for c-Myc siRNA delivery; however, most of these systems lack stimuli-responsiveness, particularly hypoxia sensitivity. For example, cationic polymers like polyethylenimine (PEI) or lipid-based systems have been used to deliver MYC-targeting nucleic acids, but they suffer from non-specific release and cytotoxicity (Yin et al. 2014). These limitations underline the necessity for more selective and controlled approaches.

Poly(pyridinium) derivatives, although previously explored for gene delivery due to their high cationic charge density and ability to condense nucleic acids (Zhang et al. 2015), have not yet been adapted into hypoxia-responsive carriers. In this thesis, we propose the first example of a stimuli-responsive delivery system using pyridinium-based polymers engineered to undergo structural transformation in hypoxic conditions, thereby releasing c-Myc-targeting siRNA selectively within tumor cells. This novel design enables environment-specific activation, minimizing off-target effects in normoxic healthy tissues.

The chemical rationale lies in modifying pyridinium moieties with hypoxia-sensitive linkers (e.g., azo or nitroaromatic groups), which are cleaved or reduced in the presence of hypoxia-inducible reductases (Brown and Giaccia 1998). Upon exposure to low oxygen levels, these moieties are reduced, leading to the disassembly of the nanostructure and the intracellular release of the siRNA payload.

This work not only fills a gap in the current stimuli-responsive gene delivery literature, where c-Myc-targeting systems remain largely non-responsive, but also introduces a first-in-literature application of hypoxia-sensitive pyridinium polymers, positioning the platform as a novel, tumor-targeted therapy for aggressive cancers such as lung cancer.

In this thesis, poly(4-vinylpyridine) (P4VP) was functionalized with 4-nitro benzyl units through pyridine quaternization. Thus, this polymer was converted into a polycationic molecule. This positive charge is intended to enable loading of negatively charged siRNA, targeting c-myc oncogene. In a hypoxic environment, expression of nitroreductase enzymes are known to increase. When this siRNA loaded molecule goes into a hypoxic environment like a tumor microenvironment, the nitroreductase enzyme reduces the nitro unit leading to self-emolative removal of the benzyl from the pyridine so the positively charged molecule reduces its positive charge or is neutralized. Then, as the electrostatic interaction between polymer delivery vehicle and siRNA weakens, siRNA release can be achieved (Figure 3.1.). This released siRNA load can bind to mRNA of c-myc target gene that is important for cell proliferation. In return, this release is expected to cause a decrease in the level of c-myc expression and the expression of genes in the downstream pathway.

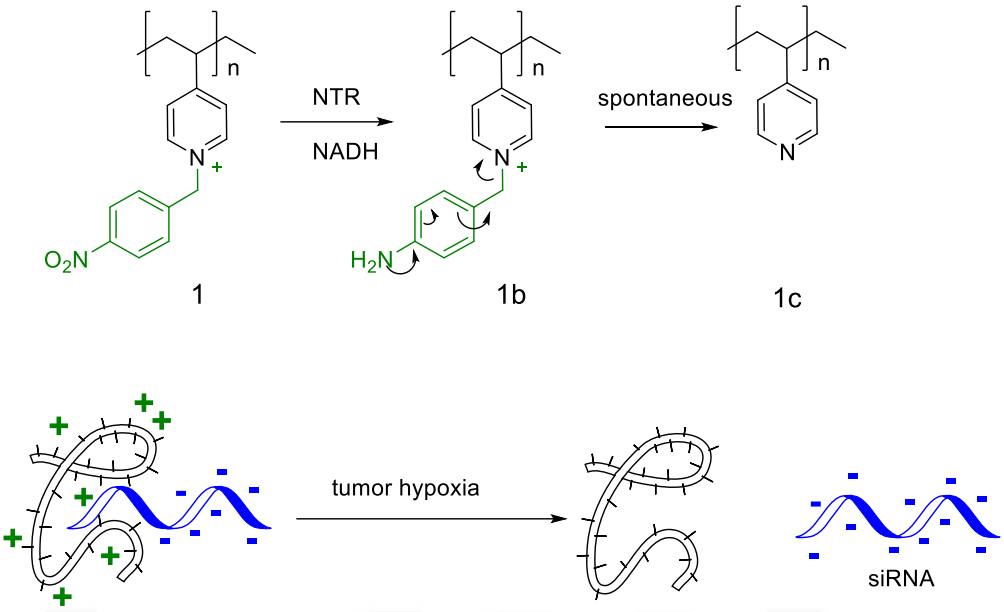
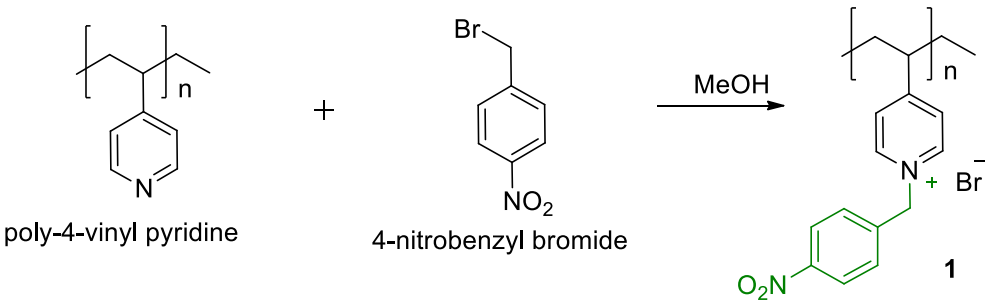


Figure 3.1. Mechanism of siRNA release. Binding of negatively charged siRNA (blue) to pyridinium functionalized polycationic polymer (1) is enabled by electrostatic interaction. Reduction (1b) followed by elimination of nitrobenzyl unit (1c) in the presence of nitro-reductase (NTR) enzyme and cofactor NADH in hypoxic cancer cell microenvironment is expected to release siRNA.

3.2. Synthesis and Characterization

Synthesis is performed by following the steps shown in Scheme 3.1. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra (Bruker, 400 MHz) at Necmettin Erbakan University, Konya) was taken to confirm the purity of compounds. Compound is purified by precipitation.



Scheme 3.1. The synthesis pathway of stimuli-responsive polymeric vehicle (1).

600 mg poly-(4-vinylpyridine) (0.01 mmol) and 100 mg 4-nitrobenzyl bromide (0.46 mmol) were dissolved with 30 mL methanol. The reaction was stirred for 2 days. After 2 days, excess 4 nitrobenzyl bromide was removed by washing the sample with solvent mixture (Hexane: Ethyl acetate, 3:1 volume ration) 2 times and decanting the solvent and then the crude product was washed one more time with acetone. Product is

obtained as white solid precipitate in quantitative yields. NMR and FTIR analysis proved the binding nitrobenzyl units to the polymer.

3.3. Enzyme Cleavage Experiment

1.6 mg nitroreductase (NTR, Sigma-Aldrich, E. Coli, N9284) enzyme was dissolved in 200 μL D_2O to obtain 160U enzyme solution. This mixture was labeled as A. 1.3 mg (1.74 μmol) Nicotinamide Adenine Dinucleotide Phosphate (NAPDH) was dissolved in 183 μL D_2O and the solution was labelled as solution B. After dissolving 5 mg of **compound 1** in 0.5 μL deuterated-DMSO, 100 μL A and 150 μL B were added. Solution was incubated at 37 ºC for 20 hours. After incubation of this solution, **compound 1** and naked polymer (P4VP) were tested by NMR in order to understand the cleavage of nitrobenzyl units from **compound 1**.

3.4. Zeta Potential Experiments

Zeta potential analysis were performed with the unfunctionalized polymer (P4VP) and **compound 1**. For this analysis, compounds were dispersed in the water and measurements were done for 3 times using Particulate Systems NanoPlus HD device at BİTAM Necmettin Erbakan University.

3.5. Cell Culture Experiments

A549 human lung cancer cells were cultured in High-Glucose Dulbecco's Modified Medium (HG-DMEM, with pyruvate) which contains 10% Fetal Bovine Serum (FBS) and 1% Gentamcin. The cell medium contains carbon sources, growth factors, aminoacids, vitamins, hormones and inorganic salts in order to support cell growth. The cells were incubated in a humidified CO_2 incubator which provides the A549 cells the desired temperature, moisture, the proper amount of O_2, and CO_2 which mimic the human body. The optimal incubation condition for cells is 37°C. Sudden temperature changes stress the cells, leading to alterations in transcription and protein synthesis. Additionally, the use of exogenous 5% CO_2 and 20% O_2 in the incubator is crucial for maintaining the pH balance of the culture medium (Masters and Stacey 2007).

First of all, the cells stored in the cryovial was suspended in 1 mL complete culture medium. And then, they were transfered to a 15 mL falcon. The cells at the falcon were centrifugated for 5 minutes at 1400 rpm. After centrifugation the supernatant was removed and 3 mL culture medium was added to the pellet. Sample containing cells was added to a T25 flask. The cells were incubated for 2 days. During this time cells were

able to attach to the surface of the flasks and grow. Cell lines can grow on surfaces in two ways: adherent and suspension. Cells used in this study is adherent.

Cells must be passaged before they reach almost 90% confluency. In cell culture studies, it is crucial to ensure cells have adequate nutrients in the flask. Therefore, passaging and refreshment of medium are necessary. Passaging is performed when cells cover approximately 90% of the flask's surface, allowing for nutrient replenishment and dilution of cell density.

Cells grow in three main phases:

Lag Phase: Cells adapt to new conditions, dependent on the medium and cell density.

Log Phase: Cells enter exponential growth.

Stationary Phase: Proliferation rate becomes equal to the rate of cell death due to waste accumulation and space limitations.

The frequency of medium refreshment depends on the nutrient consumption rate of the cells. Decreased nutrients and increased metabolic waste lower the pH, often turning the phenol red containing medium into yellow. Without adequate nutrients, exponential growth ceases, ultimately leading to cell death (Masters and Stacey 2007).

In order to passage the cell, the medium was gently pipetted from the flask with a Pasteur pipette. 1.5 mL trypsin-EDTA was added to the T25 flask to detach the cells from the surface of flask. The enzyme targets the adhesion proteins, cadherins, which mediate cell-cell and cell-matrix interactions. While Ca^{2+} ions act as activators for these proteins, EDTA in the trypsin binds Ca^{2+}, preventing its action and inactivating cadherins. After addition of trypsin-EDTA, the flask was returned to the incubator for 5 minutes. The cells were checked under the phase contrast microscope whether they were detached from the surface or not. After detachment of the cells, 6 mL fresh growth medium was added to the flask. And then the cells were added to a 15 ml falcon and centrifugated for 5 minutes at 1400 rpm. After centrifugation the supernatant was removed and the pellet was resuspended with 1 mL growth medium. Then, 6 mL fresh medium was added into falcon, and then this cell culture was distributed to 2 different T25 flasks. However, for future use, fraction of the cells were freezed by using freezing solution which is dimethylsulfoxide (DMSO). For that, after detachment and centrifugation step, cells were resuspended in 1 mL DMSO in falcon and tranferred to the cryovial and stored at -80°C.

When it was necessary, medium is chaged with the fresh growth medium. To do that, all the medium was gently pipetted by a Pasteur pipette and then 3 mL Phosphate

Saline Buffer (PBS, pH 7.4) was added to wash the flask. After the dead cells detached, 3 mL fresh growth medium was added to the T25 flask.

Throughout the text, “medium” or “complete medium” is used for HG-DMEM supplemented with 10% FBS and 1% gentamicin, unless otherwise stated.

3.5.1. Cytotoxicity experiment

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay is the most commonly used technique to detect cell viability in a 96 well-plate which is suitable for high-throughput screening. By doing this technique, cytotoxicity of molecule can be understood. Behind this assay mechanism, there is conversion of the tetrazolium salt MTT into formazan crystal dyes. NAD(P)H-dependent cellular oxidoreductase enzymes which can be found when there is mitochondral activity reduces the tetrazolium salt MTT into formazan purple dye with an absorption wavelength at 570 nm (Tl, Moravec, and Niles 2004). This conversion proves that cells are viable and there is mitochondrial activity. The resulting formazan crystal dyes are solubilized by DMSO or ethanol. After the addition of DMSO or ethanol, the change in the number of viable cells can be detected by measuring the formazan concentration by measuring optical density (OD) with ELISA plate reader at 570 nm. If decrease in viable cell numbers is seen that means cell growth is inhibited by the agent. After this assay IC50 value can be calculated. IC50 value is known as 50% growth inhibitory concentration of the tested molecule.

The cytotoxic effects of the agent was analyzed by MTT assay after 24 h treatment in A549 cell line. A549 cells were cultured at HG-DMEM medium which is supplemented with 10% FBS and 1% gentamicin at 37°C and 5% CO_2 in the incubator. When almost enough number of cell is found in the T25 flask, the medium in the flask was removed and A549 cells were washed with 1 mL PBS. After that in order to decath cells from the surface of flask, 3 mL trypsin-EDTA was added and flask is incubated for 5 minutes in the 37°C, 5% CO_2 incubator. After 5 minutes, the cells were checked under the phase contrast microscope whether they were detach from the surface or not. When detachment was confirmed, 6 mL of fresh culture medium was added to flask and the cells were transferred to 15 mL falcon. The sample was centrifugated for 5 minutes at 1400 rpm. After centrifugation the supernatant was discarded and the pellet was resuspended with 1 mL growth medium. 20 µL of cells and 20 µL trypan blue dye were mixed and cells were counted using the Thoma chamber. 5000 cells/well were cultured

in 96-well plate with fresh growth medium. Thi plate was placed in the incubator for 24 hours. After incubation, medium was removed and HG-DMEM medium including 10% FBS, 1% gentamycin and 100 μM $CoCl_2.6H_2O$ were added into each well. Plate was incubated for 24 hours again. After incubation, A549 cells were treated with different concentrations (0-16 μM) of the **compound 1** and incubated for 24 hours in a 96 well-plate which contains a final volume 200 μL/well with medium includin 100 μM $CoCl_2.6H_2O$. All groups were treated with DMSO, which is the solvent of compound 1. Therefore, 4% DMSO was applied to the control groups to eliminate the potential effects of the solvent itself. Experiment was done with four replicates. For control goup, same amount of DMSO was added to culture medium without any compound 1. Then, plate was incubated for 24 hours. 10 μL MTT solution (5 mg/mL) was added to each well after 24 hours incubation and mixed gently. After 4 hours, the medium was removed and 100 μL DMSO was added to each well to solubilize the precipitate. The cells viability was estimated by measuring absorbance at 570 nm using a Quant ELISA plate reader (Bio-tek Instruments, USA).

The same MTT analysis was also done under normoxia. During this experiment, $CoCl_2.6H_2O$ was not added to culture medium.

The cell viability was also measured by using this formula;

Relative cell viability % = (ODtreatment-ODblank) /(ODcontrol-ODblank) ×100

Where OD refers to absorbance of indicated sample at 570 nm.

3.5.2. siRNA expression analysis

Gene expression was analyzed to observe the change in c-myc mRNA level after c-myc siRNA (sc-29226 Santa Cruz) application which is transferred into cell by hypoxia-activatable **compound 1**. For this analysis, the transfer agent and siRNA were applied to cells. After this step gene expression analysis was performed in three steps. First step of analysis was RNA isolation from the cells, and then cDNA is produced from these isolated RNAs. The last step involves quantitiative Real Time Polymerase Chain Reaction (RT-qPCR) by using produced cDNAs. In order to observe the change in gene expreesion, RT-qPCR is most commonly used technique. During this technique, the target amplification is obtained at a value which is called cycle threshold (Ct). Ct represents the time at which fluorescence intensity is greater than background fluorescence. So, if the quantity of interested mRNA in starting material is high then the fluorescence signal can be read at lower PCR cycles, which causes lower Ct value. In this study, it is expected

that **compound 1** will transfer the siRNA to the cell in order to silence the c-myc expression by releasing the siRNA in hypoxic cancer condition. So, c- myc Ct value is expected to be higher which means fold change of c-myc expression in siRNA applied cells are expected to be lower than untreated cell control.

When cell confuency and cell number is enough, cells were counted to culture in 6 well plate for siRNA experiment. First of all, the medium was gently pipetted from the T25 flask with a Pasteur pipette. 3 mL of PBS was added to the flask to wash the cells. Then, 3 mL of trypsin-EDTA was added to the T25 flask to detach the cells from the surface of flask. After addition of trypsin-EDTA, the flask was returned to the incubator for 5 minutes. The cells were checked under the phase contrast microscope whether they were detach from the surface or not. When detachment is checked, 6 mL of fresh culture medium is added to flask and transferred to 15 mL falcon. The medium cell complex was centrifugated for 5 minutes at 1400 rpm. After centrifugation the supernatant was removed, and the pellet was resuspended with 1 mL growth medium. 20 μL of cells and 20 μL trypan blue dye was mixed and cells were counted with a Thoma chamber. 200.000 cells/well were cultured in 12 well plate with growth medium which have no antibiotic because antbiotic addition can decrease the effect of siRNA with 2 replicate. 2 well for control group, 2 wells for siRNA and **compound 1** addition, 2 wells for siRNA and its tranfection agent, 2 wells for **compound 1**. The plate was incubated for 24 hours in 37ºC incubator. After a day, the culture medium was replaced with medium that has 100 μM $CoCl_2.6H_2O$ without any antiobiotic gentamycin. Then, plate was incubated for a day at 37ºC incubator. After 24 hours incubation, for siRNA+ **compound 1** group solution A and B, for siRNA and tranfection agent solution A and B1 were prepared separately and mixed. The procedure for siRNA application was adopted from supplier recomeded procedure (siRNA targeting c-myc: sc-29226 Santa Cruz; transfection reagent: sc-29528 Santa Cruz; transfection medium: sc-36868 Santa Cruz; Dilution Buffer: sc-29527 Santa Cruz). Composition of these solutions are given below:

Solution A: 8 μL of siRNA and 92 μL of transfection medium

Solution B: 5 μL of **compound 1** stock (6.08μM) and 100 μL of transfection medium.

Solution A1: 8 μL of siRNA and 92 μL of transfection medium

Solution B1: 8 μL of transfection agent and 92 μL of transfection medium.

Those mixtures were incubated for 30 minutes at room temperature. After incubation cells were washed with medium without FBS and antibiotic and with 100 μM $CoCl_2.6H_2O$. For each transfection, 0.8 mL of transfection medium was added into both A+B solution and A1+B1 solution. These were mixed gently and distributed to 2 separate washed cells.

For wells that have **compound 1**, the 0.5 mL medium that antibiotic-free having 100 μM $CoCl_2.6H_2O$ was added. For control group, antibiotic free hypoxic medium was added to the cells without **compound 1**. The cells were incubated for 6 hours.

After 6 hours fresh medium that has 100 μM $CoCl_2.6H_2O$, 20 FBS and %2 gentamycin medium was added into 12-well plate. And then, cells were incubated for 20 hours at 37^oC incubator. After incubation mediums were aspirated and fresh growth medium with 100 μM $CoCl_2.6H_2O$. Cells were further incubated for 40 hours.

After that, RNAs were isolated from the cells by using GeneJET RNA purification kit (Thermo Scientific, GeneJet, K0731) according to the manufacturer's protocol. First of all, buffer A and B were prepared by addition of ethanol to the wash buffer 1 and 2 according to the protocol. And then, the growth medium was removed from MCF-7 cells by rinsing with PBS. PBS was discarded. After that, the cells were detached from the culture plate by adding 2 mL trypsin-EDTA and they were transfered into microcentrifuge tubes and centrifuged for 5 min at 250 ×g (Hettich Universal 320). The cells at the pellet were resuspended in 600 μL Lysis buffer which were previously supplemented with β-mercaptoethanol and were vortexed for 10 seconds. 360 μL of ethanol (96-100 %) was added to the sample and mixed by pipetting. 700 μL of cell lysate was transfered to the GeneJET RNA purification column inserted in a collection tube and then centrifuged for 1 min at ≥12000 ×g (Beckman Coulter Microfuge 16). The flow-through was discarded and the purification column was inserted into the collection tube. 700 μL buffer A and 600 μL buffer B were added to the purification column and centrifuged for 1 min at ≥12000×g at each step. The flow-through was discarded. 250 μL wash buffer 2 was added to the purification column and the sample was centrifuged for 2 min at ≥12000×g. The collection tube was discarded, and the purification column was transfered into a sterile 1.5 mL Rnase-free microcentrifuge tube. Finally, 100 μL nuclease-free water was added and centrifuged for 1 min at ≥12000×g in order to isolate RNA. The amount and purity of isolated RNAs was calculated by using NanoDrop. In order to do that, the isolated RNAs and nuclease-free water were placed into the spectrophotometer (Epoch 2

Microplate Reader). Firstly, 1 µL nuclease-free water (blank solution) was loaded to the well. And then the well was cleaned and 1 µL RNA sample was loaded (Select option: RNA-40). This step was repeated three times and A260/A280 and A260/A230 ratios were measured and also the amount of RNA samples in ng/µL were obtained.

The second step is to synthesize cDNA from isolated RNA samples. cDNAs were synthesized by using PrimeScript 1st strand cDNA synthesis kit (Takara: 6110A-50) reactions according to the protocol provided by the kit. First of all, the following mixtures were prepared in a microtube; 1 µL oligo dT primer (50 µM), 1 µL of each dNTP mixtures (100 µM), 3000 ng isolated RNA template, and finally Rnase-free water. The total volume of the microtube was 10 µL. The mixture was heated at 65 ºC for 5 minutes and then was immediately cooled on ice. The reaction mixture (20 µL) was prepared with 10 µL extracted RNA template/primer mixture, 4 µL 5X PrimeScript Buffer, 20 units Rnase inhibitor (40 U/µL), 100 units PrimeScript RT (200 U/µL), and finally Rnase-free water. The mixture was gently mixed and then the cDNA synthesis reaction was performed by using the following protocol; at 30 ºC for 10 minutes, at 42 ºC for 40 minutes and then the mixture was heated at 70 ºC for 15 minutes and was cooled on ice. The amount of synthesized cDNA was calculated by using NanoDrop.

The final step is to perform RT-qPCR with synthesized cDNA samples and interested forward and reverse primers (C-MYC, GLUT1, BCL-2, ACTB, CCND-1). Primers are obtained from Sentebiolab and their sequences are given in Table 2. Briefly, each PCR tubes contained 2 µL cDNA solutions (100 ng), 5 µL 2X SYBR Green Supermix (Bio-Rad), 12.5 pmol reverse and forward primers (0.5 µL each of primers). The final reaction volume was 10 µL. This reaction mixture was separately prepared for each cDNA samples and each reverse and forward primers. RT-qPCR protocol was performed as initial denaturation at 95 ºC for 30 seconds, denaturation at 95 ºC for 15 seconds, annealing at 60 ºC for 30 seconds, and extension at 72 ºC for 30 seconds, respectively. PCR reaction was performed as 39 cycles. RT-qPCR analysis was evaluated using Bio-Rad CFX Connect$_{TM}$ Real-Time System. Analysis of melting curves was performed by progressive heating from 65 to 95 ºC (0.1 ºC increment/sec). GAPDH gene was used as a reference gene in the experiment. All experimental data were repeated three times with data shown as mean±SD. The RT-qPCR analysis was performed by using the $2^{(-\Delta\Delta Ct)}$ method. The comparisons between groups have been statistically evaluated using 'unpaired student t-test' with Graphpad software. Any comparison with a p value smaller than or equal to 0.05 is considered as statistically significant.

Table 3.1. Primer sequences used in Real Time-qPCR analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
ACT-B	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
C-MYC	CCTGGTGCTCCATGAGGAGAC	CAGACTCTGACCTTTTGCCAGG
BCL-2	ATCGCCCTGTGGATGACTGAGT	GCCAGGAGAAATCAAACAGAGGC
GLUT1	GCTACAACACTGGAGTCATCAA	ACTGAGAGGGACCAGAGC
CCND-1	TCTACACCGACAACTCCATCGC	TCTGGCATTTTGGAGAGGAAGTG

4. RESULTS AND DISCUSSION

4.1. Characterization of Compound 1

In order to verify the formation of target compound 1, ^{1}H NMR spectra of compound 1 and polymer (poly-4-vinyl pyridine) were taken and the results were compared in Figure 4.1. Upon functionalization with 4-nitrobenzyl bromide new peaks resonating at 8.91 ppm, 7.64 ppm and 5.92 ppm appears on ^{1}H NMR spectrum (peaks c, d and e respectively). These protons are assigned as nitrobenzyl peaks and the integral of each peaks found out to be nine fold smaller than the peaks of the polymer. This result suggest that about 10% of the pyridines are functionalized with 4-nitrobenzyl group.

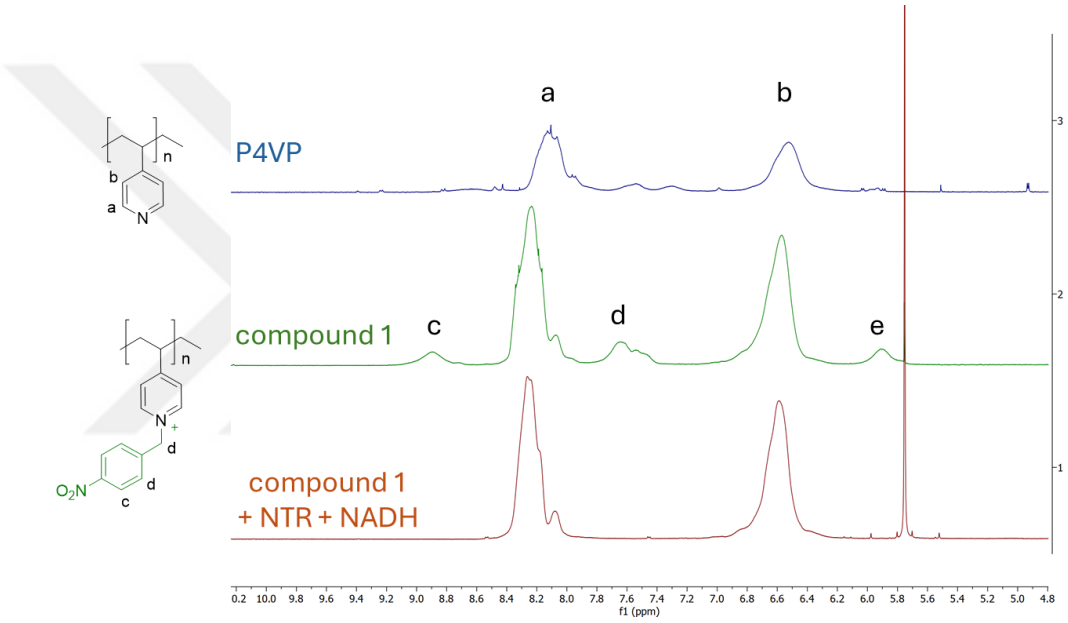


Figure 4.1. ^{1}H NMR spectra of unfunctionalized polymer (P4VP, blue, top), **compound 1** (green, middle) and enzyme treated compound 1 (brown, bottom). NMR was recorded in D_2O:*d*-DMSO mixture (2:1 volume ratio). 107 U/mL NTR enzyme and 1.27 mM NADH were used in the experiment.

FTIR spectra of compound 1 and polymer (poly-4-vinyl pyridine) were taken and the results were compared in Figure 4.2. Fourier-transform infrared (FTIR) spectroscopy detects characteristic absorption bands that correspond to specific functional groups within molecules. This method analyzes how various biomolecules—including lipopolysaccharides, lipids, carbohydrates, nucleic acids, and proteins—interact with infrared radiation. Because the absorption patterns of these groups are well-established, substances can be identified by comparing their spectral profiles to reference data [101].

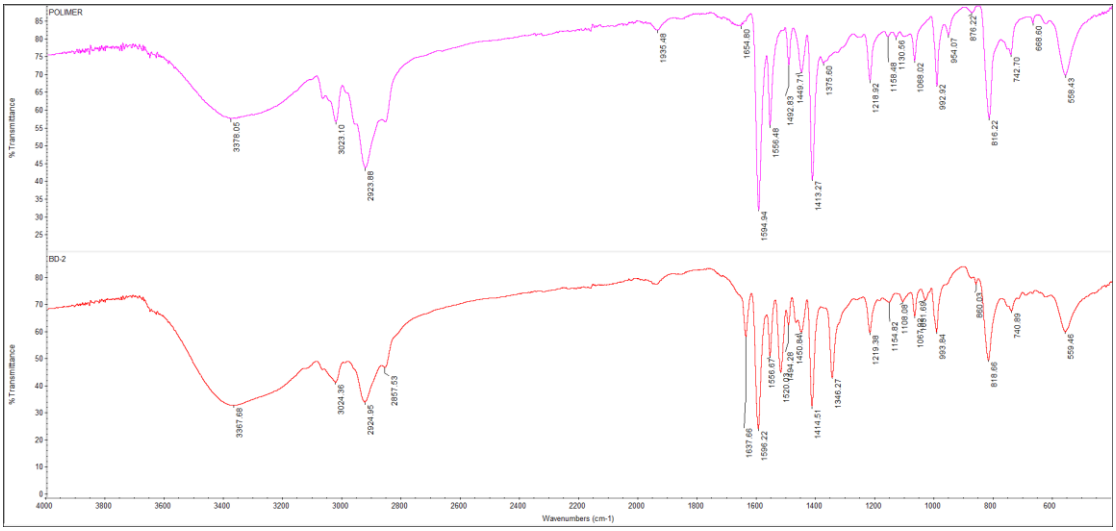


Figure 4.2. FTIR result of unfunctionalized polymer (P4VP, pink) and **compound 1** (red). (n=3).

FTIR spectral analysis revealed significant changes consistent with the successful functionalization of P4VP with a nitrobenzyl group. The emergence of a new peak at 1637.66 cm^{-1} and increased intensity at 1346.27 cm^{-1} confirmed the presence of aromatic and nitro functionalities, respectively. In addition to that, emergence of new peaks between 1050 and 1150 cm^{-1} confirmed presence of quaternary N^{+}. Meanwhile, the reduction and loss of peaks at 668 cm^{-1} and 954 cm^{-1} indicate structural modifications within the original polymer backbone. These findings collectively support the covalent attachment of the nitrobenzyl group to the P4VP structure.

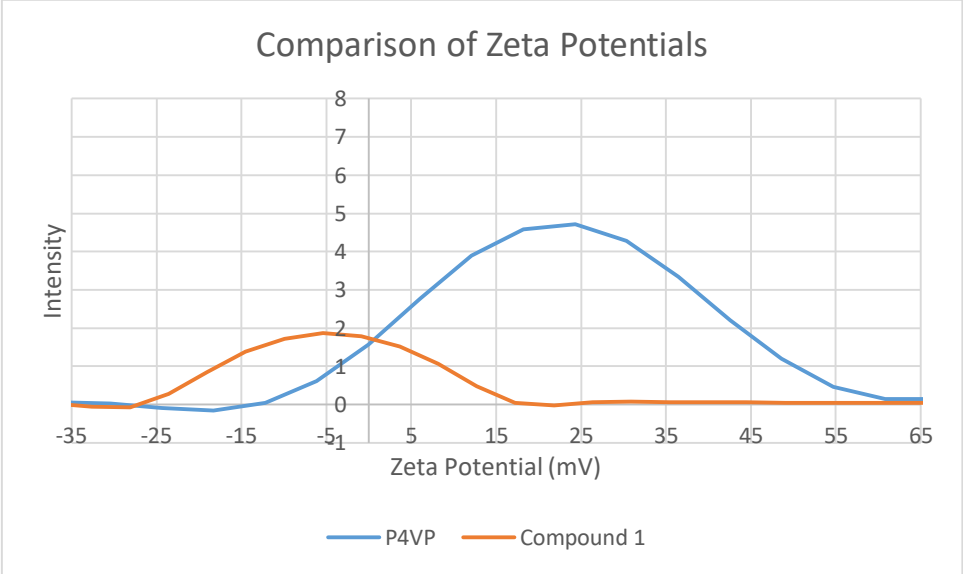


Figure 4.3. Zeta potential result of unfunctionalized polymer (P4VP, blue) and **compound 1** (red). (n=3).

For surface potential analysis compound 1 and the unfunctionalized polymer (poly-4-vinyl pyridine) were dispersed in water. All measurement were done as 3 replicates. As shown in Figure 4.3, addition of the nitro benzyl units to generate pyridinium polycationic polymers increased the surface potential of the P4VP from -6.98 to +22.8 mV. This result clearly verify that pyridine functionalization with nitro benzyl units on PVP4 is successfull.

4.2. Enzyme Cleavage Experiment

Compound 1 has pyridines quaternized with 4-nitro benzyl units which makes the molecule polycationic so that it can bind to and deliver negatively charged siRNA. NADPH and nitroreductase (NTR) that are found in high level in cancer cells are used to reduce the nitro benzyl unit of **compound 1** which then leads to spontaneous elimination of resulting 4-amino benzyl unit, generating neutral pyridines. Thus, siRNA is expected to be release from the delivery vehicle.

To verify enzyme mediated cleavage, ^{1}H NMR analysis was performed (Figure 4.1). Results suggest the disappearance of the 4-nitrobenzyl units following NTR (107 U/mL) and NADH (1.27 mM) treatment. Surface potential of the enzyme-treated compound 1 is found to be 18.14 which is approximately 4 unit less than compound 1 (Figure 4.3.). This result indicates the reduction in positive charge consistent with hypothesis. The reason why the value is still higher than the unmodified polymer may result from incomplete dissociation of benzyl units from pyridiniums or positive charge of the co-factor NADH might also interfere with the result.

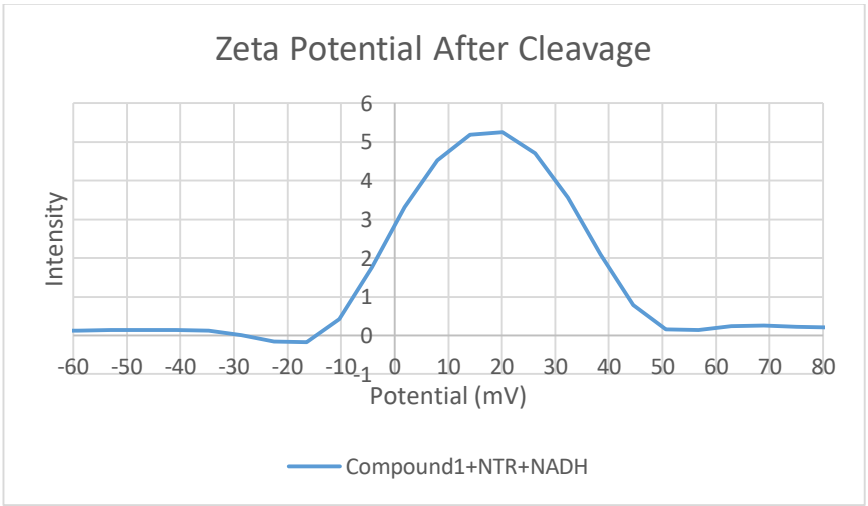


Figure 4.3. Surface potential of compound 1 (5 mg) treated with 107 U/mL NTR enzyme and 1.27 mM NADH cofactor for 24 h.

4.3. Cytotoxicity Experiment

The cytotoxicity of **compound 1** and naked polymer which is P4VP were investigated A549 lung cancer cells in both normoxic and hypoxic conditions. Hypoxic microenvironment is enabled by treateing the cells with 100 μM cobalt chloride 24 h prior to application of polymers. MTT test was used for viability analysis and the viabilities were calculated after 24 h polymer or compound 1 application. As shown in Figures 4.4 and 4.5, compound 1 does not have significant cytotoxic effect up to 8 μM under both hypoxic and normoxic conditions, which is higher than the application dose.

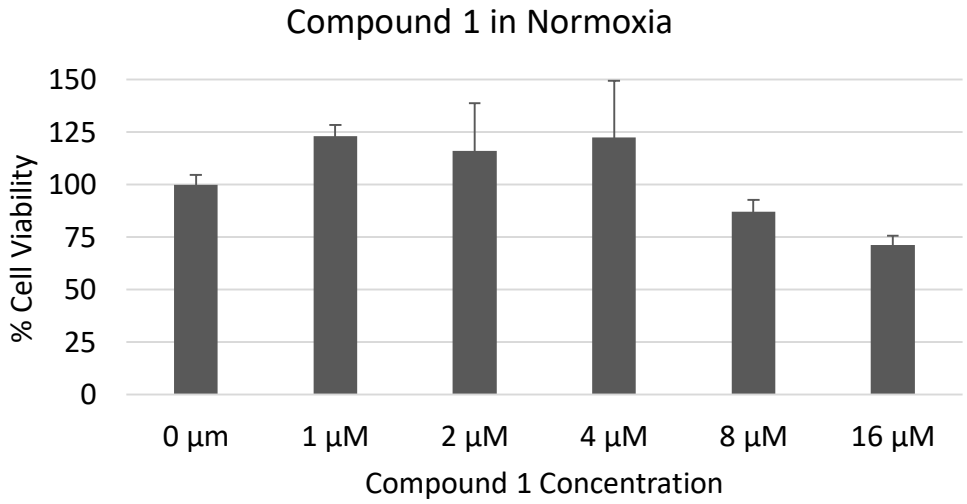


Figure 4.4. Effect of compound 1 (0-16 μM) on the viability of A549 cells under normoxic conditions. (n = 3)

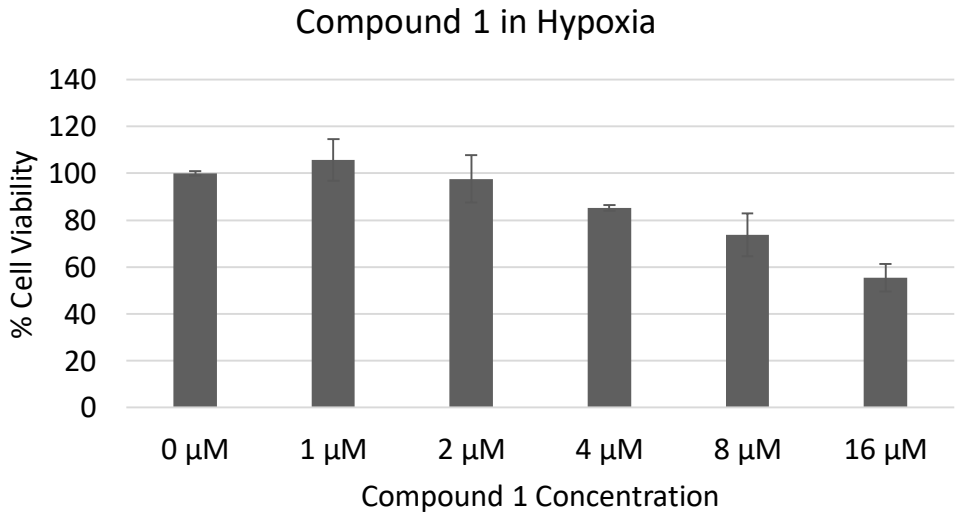


Figure 4.5. Effect of compound 1 (0-16 μM) on the viability of A549 cells under hypoxic conditions. (n = 3)

As shown in Figures 4.6 and 4.7, P4VP polymer does not have significant cytotoxic effect up to 8 µM under both hypoxic and normoxic conditions, which is higher than the application dose. Since free polymer P4VP is expected to be generated after elimination of nitrobenzyl unit under hypoxia, resulting polymer needs to be non-toxic.

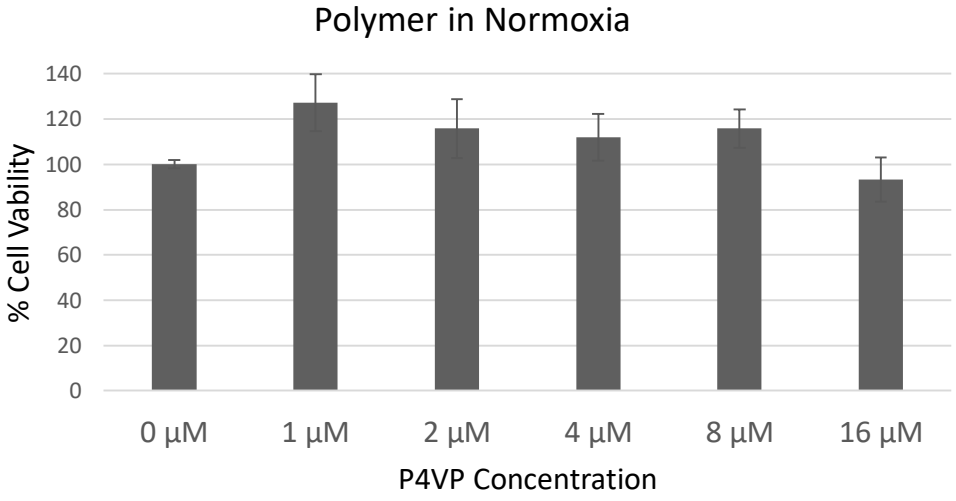


Figure 4.6. Effect of P4VP polymer (0-16 µM) on the viability of A549 cells under normoxic conditions. (n = 3)

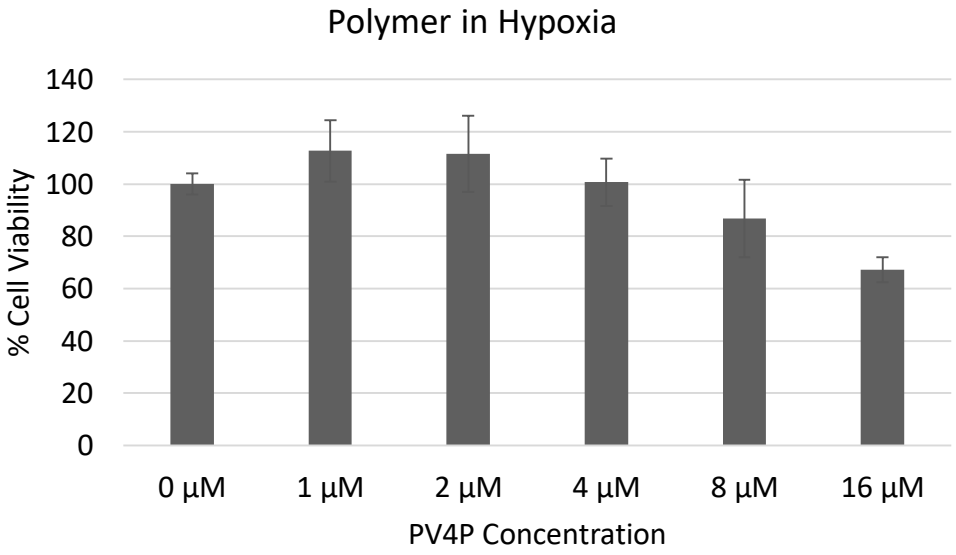


Figure 4.7. Effect of P4VP polymer (0-16 µM) on the viability of A549 cells under hypoxic conditions. (n = 3)

4.4. Gene Expression Analysis

By using A549 cell line, expressions of c-MYC, BCL2, GLUT-1, CCND-1 gene expression with the use of **compound 1** that tranfers c-MYC siRNA into cell was investigated. β-Actin gene was used as housekeeping gene. Under normoxic conditions, no statistically significant decrease in c-MYC gene expression was observed; however, a significant reduction was observed with the use of a siRNA transfection agent (Figure 4.8). This means that under normoxia siRNA release is not observed.

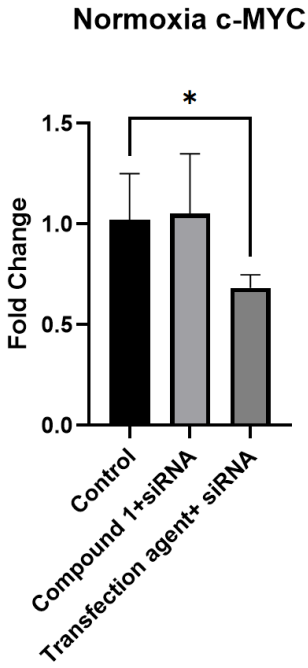


Figure 4.8. Relative expression of c-MYC in A549 cells treated siRNA with compound 1 and transgection agent. (*p value:0.0216)

In hypoxic condition, significant decrease (approximately 4*fold) in c-MYC expression is seen (Figure 4.9). This result indicates that siRNA can be released and shows its inhibitory effect in the hypoxic condition, in the presence of nitroreductase enzyme.

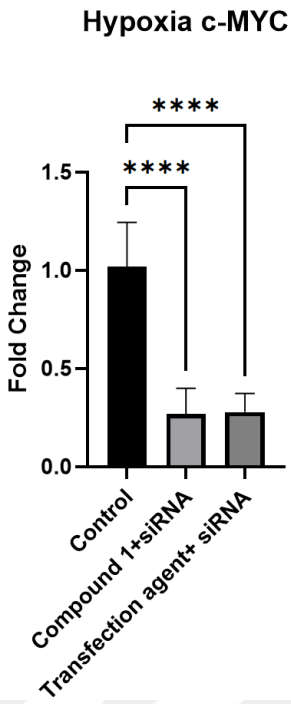


Figure 4.9. Relative expression of c-MYC in A549 cells treated siRNA with compound 1 and transfection agent under, hypoxic condition . (***p value<0.0001)

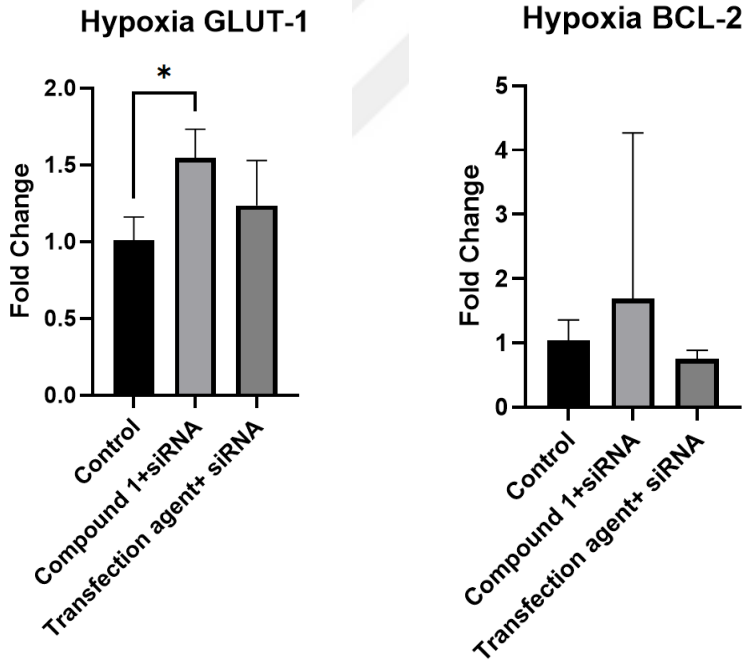


Figure 4.10. Relative expression of GLUT1 in A549 cells treated siRNA with compound 1 and transgection agent in hypoxic condition (**** p value<0.0001), relative expression of BCL-2 in A549 cells treated siRNA with compound 1 and transgection agent in hypoxic condition.

In order to understand the change in gene expression of cMYC downstream pathways, GLUT-1 and BCL-2 gene expressions were analyzed. After tranferring siRNA by using compound 1, 1.5 fold increase in the level of GLUT-1 is observed (Figure 4.10).

BCL-2 gene expression did not change significantly with the usage of c-MYC siRNA, however considering the high error bar, more replicates needs to be done validate the data.

In order to understand the effect of c-MYC silencing in the cell cycle, CCND-1 and EIF-2A gene expressions were analyzed. Since Cyclin D1 plays a critical role in promoting the transition from the G1 to S phase of the cell cycle, c-MYC enhances cell proliferation by stimulating CCND1 transcription in cancer cell. After tranferring siRNA by using compound 1, significant decrease in the level of CCND-1 is observed (Figure 4.11.). This can be caused by the decrease in level of c-MYC.

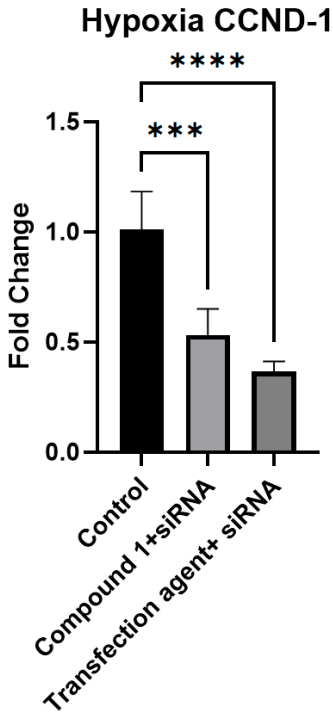


Figure 4.11. Relative expression of CCND-1 in A549 cells treated siRNA with compound 1 and transgection agent in hypoxic condition (*** p value: 0.0002, **** p value < 0.0001)

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusion

In this thesis, a novel hypoxia-responsive gene delivery platform was developed which utilize pyridinium-functionalized polymers engineered to selectively release siRNA targeting the oncogene c-Myc under tumor-like hypoxic conditions. The core of this strategy involves the chemical modification of poly(4-vinylpyridine) (P4VP) with 4-nitrobenzyl groups, creating a polycationic polymer capable of forming stable complexes with negatively charged siRNA molecules. FTIR spectral analysis revealed significant changes consistent with the successful functionalization of P4VP with a nitrobenzyl group. The emergence of a new peak at 1637.66 cm^{-1} and increased intensity at 1346.27 cm^{-1} confirmed the presence of aromatic and nitro functionalities, respectively. Upon functionalization of the polymer with 4-nitrobenzyl bromide new peaks resonating at 8.91 ppm, 7.64 ppm and 5.92 ppm appears on ^{1}H NMR spectrum. These protons are assigned as nitrobenzyl peaks and the integral of each peaks found out to be nine fold smaller than the peaks of the polymer. This result suggest that about 10% of the pyridines are functionalized with 4-nitrobenzyl group. Pyridinium polycationic polymer has a surface +22.8 mV which is higher than the naked polymer. Compound 1 does not have significant cytotoxic effect up to 8 μM under both hypoxic and normoxic conditions, which is higher than the application dose.

The hypoxia sensitivity of the system is based on the enzymatic activity of nitroreductases, which are overexpressed in the hypoxic microenvironments of solid tumors. Upon encountering such conditions, these enzymes reduce and cleave the nitrobenzyl moieties, leading to a loss of positive charge on the polymer. This charge neutralization disrupts the electrostatic interactions between the polymer and siRNA, thereby facilitating the controlled release of the siRNA payload within tumor cells. NTR treatment resulted in release of the nitrobenzyl unit as verified by NMR. Surface potential is reduced upon enzyme application indicating the decrease in the positive charge of the polymer carrier.

Experimental results validated the hypoxia-specific behavior of the system. Under normoxic conditions, siRNA remained bound to the carrier, and no significant decrease in c-Myc mRNA levels was detected, indicating a low risk of off-target effects in healthy tissues. In contrast, under hypoxic conditions, a significant downregulation of c-Myc expression was observed, supporting the efficiency of Compound 1 as a hypoxia-activated siRNA delivery vehicle. Furthermore, the silencing of c-Myc also led to

modulation of downstream oncogenic pathways, reinforcing the therapeutic potential of this approach in targeting aggressive cancers such as lung cancer.

This study presents the first demonstration of a c-Myc-targeting siRNA delivery system based on hypoxia-sensitive pyridinium polymers. By combining environmental responsiveness with molecular specificity, this platform addresses key limitations of conventional delivery systems, such as lack of targeting and off-tissue toxicity. The approach holds significant promise for the development of next-generation gene therapies with high precision and minimal systemic side effects, contributing to the expanding field of stimuli-responsive nanomedicine.

5.2. Recommendations

Based on the encouraging results of this study, several future directions are recommended to fully explore and expand the potential of the developed hypoxia-responsive siRNA delivery platform. First, detailed analyses of the broader transcriptomic and proteomic effects following c-Myc silencing could provide valuable mechanistic insights and identify complementary pathways for combination therapies. The design of the polymer could also be further optimized—for instance, by adjusting the degree of quaternization, altering linker chemistry, or incorporating tumor-targeting ligands to improve delivery specificity and transfection efficiency. Co-delivery strategies that integrate this siRNA platform with chemotherapeutics or immunomodulatory agents may also enhance therapeutic outcomes, especially in treatment-resistant cancers. Furthermore, this platform's modularity allows for its adaptation to deliver siRNAs targeting other oncogenes or disease-related genes, offering potential applications in a wide range of hypoxia-associated diseases beyond cancer. Overall, this work establishes a strong foundation for tumor-targeted, environmentally responsive gene therapy, with substantial room for refinement and clinical advancement.

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