Gizem YILDIZ

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POLYMERIC CONJUGATES CONTAINING POEGMA AND CYSTAMINE-MODIFIED PLASMID DNAs FOR POTENTIAL GENE DELIVERY APPLICATIONS

M.Sc. THESIS
SUBMITTED TO THE DEPARTMENT OF BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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SCIENTIFIC ETHICS COMPLIANCE

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M.Sc. thesis titled “Polymeric Conjugates by POEGMA and Cystamine-Modified Plasmid DNAs For Potential Gene Delivery Applications” has been prepared in accordance with the Thesis Writing Guidelines of the Abdullah Gül University, Graduate School of Engineering & Science.

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ACCEPTANCE AND APPROVAL

M.Sc. thesis titled “Polymeric Conjugates by POEGMA and Cystamine-Modified Plasmid DNAs For Potential Gene Delivery Applications” and prepared by Gizem YILDIZ has been accepted by the jury in the Bioengineering Graduate Program at Abdullah Gül University, Graduate School of Engineering & Science.

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APPROVAL:

The acceptance of this M.Sc. thesis has been approved by the decision of the Abdullah Gül University, Graduate School of Engineering & Science, Executive Board dated ….. /….. / …….. and numbered.……..……. .

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ABSTRACT

POLYMERIC CONJUGATES CONTAINING POEGMA AND CYSTAMINE-MODIFIED PLASMID DNAS FOR POTENTIAL GENE DELIVERY APPLICATIONS

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MSc. In Bioengineering
Advisor: Assoc. Prof. İsmail Alper ISOGLU
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August 2024

Polymer-based gene delivery systems have revealed significant advancements in the treatment of various diseases in recent years. Considering the potential of polymeric vectors, it is observed that the improvements in the field of gene therapy enable effective gene transfection and induced therapeutic protein production. In this thesis study, a strategy based on a new conjugation procedure is designed to increase the gene transfer and cellular uptake rate of plasmid DNAs. According to the findings, POEGMA-based carrier and cystamine-modified plasmid DNAs demonstrated successful conjugation through disulfide bond formation. MDA-MB-231 in vitro cellular uptake results of conjugates showed 94-98% cell internalization, indicating excellent results compared to the well-known polymers in the literature. As a result, the new delivery system we developed in this study determined the success of cystamine-modified plasmid DNAs binding to POEGMA polymer chains via a covalent linkage for the first time in the literature and provided a start for future studies.
Keywords: POEGMA, pDNAs, Cystamine, Glutathione

ÖZET

POTANSİYEL GEN DAĞITIMI UYGULAMALARI İÇİN POEGMA VE SİSTAMİNLE MODİFİYE PLAZMİT DNA'LAR İÇEREN POLİMERİK KONJUGATLAR

Gizem YILDIZ
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Ağustos 2024

Polimer bazlı gen taşıyıcı sistemler, son yıllarda çeşitli hastalıkların tedavisinde önemli ilerlemeler ortaya koymuştur. Polimerik vektörlerin potansiyeli göz önüne alındığında, gen terapisi alanındaki gelişmelerin etkili gen transfeksiyonunu mümkün kıldığı ve terapötik protein üretimini yüksek seviyede tetiklediği görülmektedir. Bu tez çalışmasında, plazmit DNA'ların gen transferini ve hücresel alım oranını artırmak için yeni bir konjugasyon prosedürünü temel alan bir strateji tasarlanmıştır. Bulgulara göre POEGMA bazlı taşıyıcı ve sistaminle modifiye edilmiş plazmit DNA'lar, disülfit bağı oluşumu yoluyla başarılı konjugasyon gösterdi. Konjugatların MDA-MB-231 in vitro hücresel alım sonuçları, %94-98 hücre içselleştirmesi gösterdi; bu, literatürde iyi bilinen polimerlerle karşılaştırıldığında mükemmel sonuçlara işaret ediyor. Özetle, bu çalışmada geliştirdiğimiz yeni gen aktarım sistemi, literatürde ilk kez sistaminle modifiye edilmiş plazmid DNA'ların POEGMA polimer zincirlerine kovalent bağlantı yoluyla bağlanma başarısını belirlemiş ve gelecek çalışmalar için bir başlangıç sağlamıştır.

Anahtar Kelimeler: POEGMA, pDNA'lar, Sistamin, Glutatyon

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LIST OF ABBREVIATIONS

AAV	Adeno-associated viruses
ACVA	4,4'-azobis(4-cyanovaleric acid)
AIBN	Azobisisobutyronitrile
ATRP	Atom Transfer Radical Polymerization
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
CS	Chitosan
CTAs	Chain Transfer Agents
CPLA	Cationic polylactides
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
FDA	US Food and Drug Administration
GSH	Glutathione
GSSG	Glutathione disulfide
LGEA-PU	Diethylamino-ethylamine polyurethane
LRP	Living Radical Polymerization
NPs	Nanoparticles
PC	Polycarbonates
PCL	Poly(ε-caprolactone)
PDMAEMA	Poly(2-(dimethylamino)ethyl methacrylate)
PDS	Pyridyl disulfide
PEI	Polyethyleneimine
PEG	Polyethylene glycol
pDNA	Plasmid DNA
PMAC	Poly(5-methyl-5-allyloxycarbonyl-trimethylene carbonate)
PMAC-g-PEI	Polycarbonate modified with PEI

PMAC-O	Epoxide-functionalized-poly(5-methyl-5-allyloxycarbonyl-trimethylene carbonate)
PLA	Polylactide
POEGMA	Poly(oligo(ethylene glycol) methyl ether methacrylate)
PU	Polyurethanes
PVL	Poly(valerolactone)
RAFT	Reversible Addition-Fragmentation Chain Transfer
RES	Reticulo-endothelial system
RNA	Ribonucleic acid
SCID	Severe combined immunodeficiency
siRNA	Small interfering ribonucleic acid
ssDNA	Single-stranded DNA
TALENs	Transcription activator-like effector nucleases
TCEP	Tris(2-carboxyethyl) phosphine hydrochloride
ZFNs	Zinc finger nucleases

Chapter 1

Introduction

1.1 Historical Perspective of Gene Therapy

Gene therapy is a treatment method that relies on the introduction of engineered genetic material into a patient's cell to enhance life quality by repairing a defect or slowing the progression of a disease using therapeutic protein expression [1], [2]. According to the US Food and Drug Administration (FDA), gene therapy products are administered as viruses, nucleic acids, or genetically modified microorganisms that mediate their therapeutic effects by transcription and/or translation of transferred genetic material and/or integrating into the host genome [3].

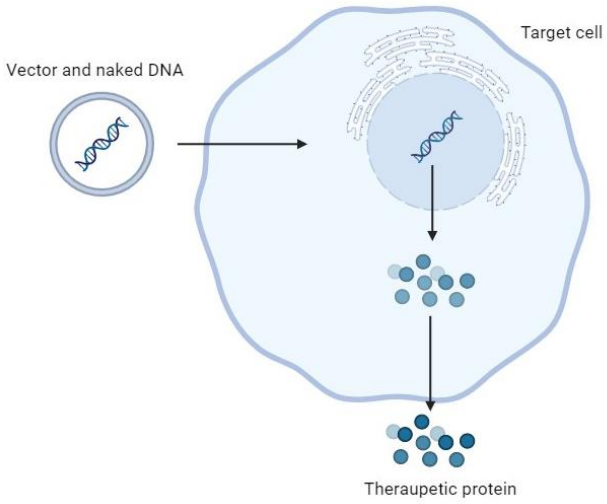

Figure 1.1. Principle of Gene Therapy

Gene therapy is an outcome of a complex historical journey that is considered a revolutionary approach in modern medicine and research and development for the practical implementation of this idea has taken a long time. The fundamentals of gene therapy are built on the discovery of DNA by James Watson and Francis Crick in 1953. This discovery provided the basic concepts of genetics to scientists and established a

ground for the research on disease treatment by altering or manipulating genes. In 1978, Arber, Nathans, and Smith discovered restriction enzymes and assisted in making progress in recombinant DNA technology enabling gene manipulation and cloning. This technology gave way to developing new techniques for the delivery of therapeutic genes into targeted cells [4], [5]. In 1980, the first clinical trial of gene therapy was accomplished by hematologist Martin Cline [6]. He applied genetically modified cells to two patients having beta-thalassemia which is an inherited blood disorder. The bone marrow cells of two patients were isolated and subjected to treatment using plasmid DNA with an integrated β-globin gene. The patients' bone marrows were subsequently replenished with the altered cells. Even though this experiment was unsuccessful, it is regarded as the first usage of recombinant DNA in clinical treatment since it showed the possibility of gene transfer in humans [6], [7]. In 1990, the treatment of two patients with severe combined immunodeficiency (SCID) caused by failure of B- and T lymphocyte development ended with success, this led to the gene therapy experiments being approved by the Food and Drug Administration (FDA). Scientists used retroviral vectors for the introduction of adenosine deaminase (ADA) cDNA to isolated peripheral T-lymphocytes and the cells were reinjected into patients' bodies in order to restore immune function. The treatment resulted in an improved and more functional immune system. Although this successful treatment was promising, it led to safety concerns since some patients developed leukemia caused by the genome integration of viral vectors [8]. After the success of ADA treatment in SCID, the gene therapy studies were accelerated in various genetic diseases, but all preclinical experiments had to comprise detailed and comprehensive research. Throughout the 2000s and 2010s, vector design, delivery systems, and genome editing technologies such as clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) contributed to the evolution of gene therapy applications. These advances extended the application fields including not only genetic disorders but also cancer, infectious diseases, and complex disorders [9], [10], [11]. Despite the progress, significant challenges including high-profile failures, the death of a patient in a clinical trial in 1999, and the development of serious side effects emerged various controversies such as concerns about safety, efficacy, and ethical implications. These have encouraged regulatory agencies to implement oversight of gene therapy research more strictly [5]. In recent years, gene therapy has received renewed interest and investment with the

promising clinical results and approval of gene therapy products for rare genetic diseases [12]. The field offers an approach for conditions that could not be treated previously, in addition to triggering innovations in biotechnology and medical science. However, optimizing delivery methods, overcoming immune responses, and long-term safety remain as important challenges [1]. In the gene therapy field, the selection and design of vectors for the delivery of genetic material have also become crucial in recent years. Vectors are vehicles that are used to introduce therapeutic genes into target cells, and they have specific characteristics and efficacy on long-term outcomes in different applications [13]. In particular, viral vectors including adenoviruses, adeno-associated viruses (AAV), and lentiviruses are widely used in the field of gene therapy [12], [14]. In addition to viral vectors, significant progress has been made in the development of non-viral vectors and they have the potential to overcome some disadvantages possessed by viral vectors [15]

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1.2 Role of Efficient Gene Delivery Systems in Gene Therapy

The main objective of gene therapy is to cure diseases or repair a dysfunction resulting from genetic defects by inserting a functional gene into the targeted cell that functions as the drug. The genetic material that is delivered to target cells can be in the form of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), plasmid DNA, anti-sense oligonucleotides (ASON), and small interfering RNA (siRNA) [16]. Ensuring that the therapeutic gene introduces the targeted cell without undergoing any kind of biodegradation is fundamental to the success of gene therapy [17]. However, DNA cannot penetrate passively through the cell membrane due to its large size, hydrophilic polyanionic structure, and sensitivity to the nuclease of the biological media [18]. Injection of naked DNA encoding the therapeutic protein is the simplest way of gene transfer; but, due to its limited efficiency, particular substances and techniques are required to enhance gene delivery. Therefore, DNA needs to be carried by a vector or delivery mechanism that introduces the therapeutic gene into the targeted cell, preventing its nuclease degradation and ensuring its transcription inside the cell [17], [19]. The ideal delivery system should satisfy several functions, such as transporting

nucleic acids whatever their size, shielding the genes from destruction, facilitating the transfer of genes without triggering a strong immune response, guaranteeing gene transcription, and leading to sustained and regular expression of its genetic cargo within the cell [16]. Only disease-specific cell types must receive the gene from the vector, especially when the target cells are dispersed across the body or comprise a heterogeneous population. A therapeutic application must be achievable for the optimal delivery systems. The vectors must be prepared and purified easily at high concentrations and have low-cost production [16], [19]. Optimal gene delivery systems increase the effectiveness of gene therapy and increase the likelihood of successful outcomes by delivering therapeutic genes into precisely targeted cells, maximizing the desired effect of the treatment while minimizing side effects.

1.3 Types of Gene Delivery Systems

Gene delivery systems can be characterized into two subgroups which are physical and carrier-based/particulate comprising viral and non-viral vectors (Figure 1.2). The viral vectors used for gene transduction are such as retroviral, adenoviral, and adeno-associated viral vectors, and non-viral vectors used for gene transfection are polysaccharides, peptides, liposomes, and polymeric systems. However, none of these types are considered optimal for both safe and effective gene delivery and adequate gene expression each vector has its own advantages and disadvantages [19]. In the context of the investigation and analysis of gene transfer and expression, non-viral and viral vectors will be placed on primary emphasis in this review.

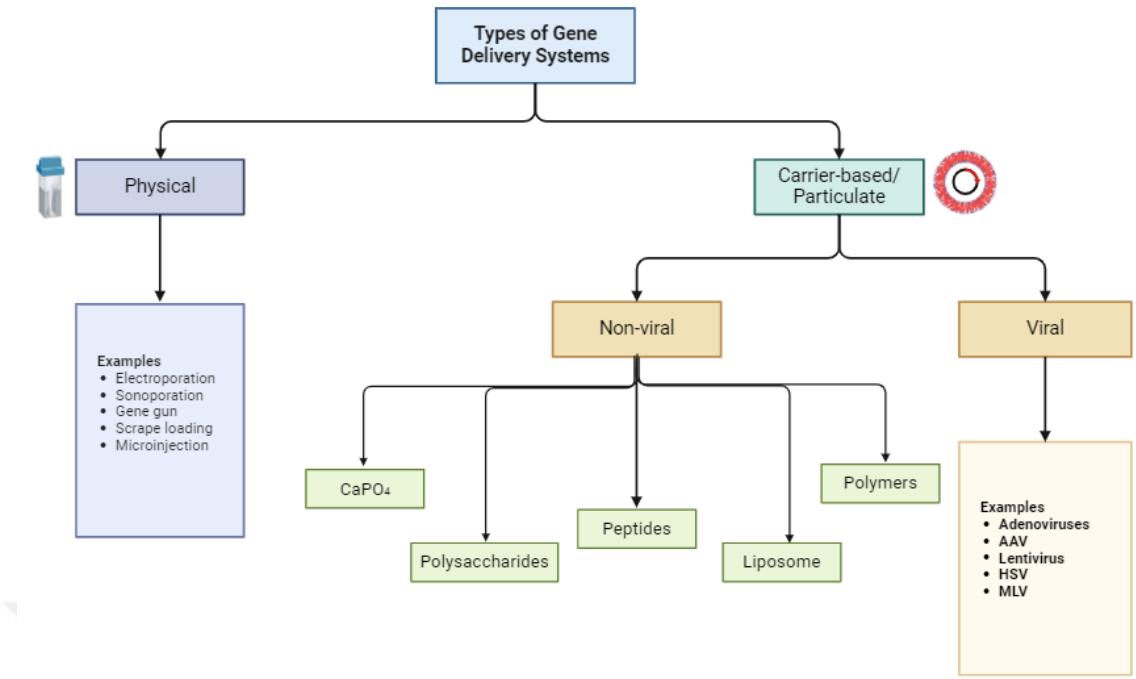


Figure 1.2. Types of Gene Delivery Systems

1.3.1 Viral Vectors

A virus is defined as a biological entity having the ability to penetrate the nucleus of a host cell and alter cellular machinery for expression of its genetic material, then infect other cells [20]. Therefore, viruses can be utilized to deliver foreign gene into target cells with high efficiency [19]. To use a virus as a gene carrier, its properties need to be modified through genetic engineering. The therapeutic gene replaces the pathogenic part of the virus, and the virus remains to be able to infect cells because it still possesses non-pathogenic components such as envelope proteins and fusogenic proteins [20]. These vectors are distinct from wild-type viruses since they are generated by removing genes responsible for replication, assembly, or infection and can transfer therapeutic genes into target cells. Viral vectors are considered as the most often utilized gene transfer vectors due to their high transfection efficiency [14]. At the same time, many viral vectors can express therapeutic genes in target cells for long periods of time, meaning that a single dose can provide long-term treatment and they might be naturally biased towards certain target cells. For example, they can bind to specific receptors on target cells, allowing therapeutic genes to be directed to specific cell types [19]. The drawbacks of viral vectors can be listed as follows: -The potentially lethal acute immune response that may be stimulated by viral vectors resulting in low effectiveness of the

therapy and a negative effect on the response to treatment. -It is exceedingly expensive and difficult to produce viral vectors in large quantities. -The viruses have a restricted ability to transfer large genes. -Viral genes might be integrated into the genome of the host cell leading to undesirable mutations or altered gene functions [16]. Clinical and commercial applications of viral vectors are strictly controlled by regulatory agencies to ensure the safety, efficiency, and quality of vectors [20]. Severe Combination Immunodeficiency (ADA-SCID), Leber Congenital Amaurosis (LCA), hemophilia, cystic fibrosis, and cancer are the main diseases in which viral vectors are used for helping to improve patients' health or relieve symptoms. The functionality of viral vectors is considered an important application in the field of gene therapy and allows promising results in the treatment of these diseases [21].

Adenoviruses, retroviruses, lentiviruses, adeno-associated viruses (AAV), and simple herpes virus are used most as carrier vectors. Table 1.1 summarizes the main viral gene transfer systems with their advantages and disadvantages [16].

Table 1.1. The main viral gene delivery systems, with their advantages and disadvantages

Vector	Advantages	Disadvantages
Adenovirus	Very high titers (1012 pfu/mL) High transduction efficiency ex vivo and in vivo Transduces many cell types Transduces proliferating and nonproliferating cells Production easy at high titers	Remains episomal Transient expression Requires packaging cell line Immune-related toxicity with repeated administration Potential replication competence No targeting Limited insert size: 4–5 kb
Adeno-associated virus	Integration on human chromosome 19 (wild-type only) to establish latent infection Prolonged expression Transduction does not require cell division Small genome, no viral genes	Not well characterized No targeting Requires packaging cell line Potential insertional mutagenesis High titers (1010 pfu/mL) but production difficult Limited insert size: 5 kb
Herpes simplex virus	Large insert size: 40–50 kb Neuronal tropism Latency expression Efficient transduction in vivo Replicative vectors available	Cytotoxic No targeting Requires packaging cell line Transient expression, does not integrate into genome Moderate titers (104–108 pfu/mL)
Lentivirus	Transduces proliferating and nonproliferating cells Transduces hematopoietic stem cells Prolonged expression Relatively high titers (106–107 pfu/mL)	Safety concerns: from human immunodeficiency virus origin Difficult to manufacture and store Limited insert size: 8 kb Clinical experience limited
Retrovirus	Integration into cellular genome Broad cell tropism Prolonged stable expression Requires cell division for transduction Relatively high titers (106–107 pfu/mL) Larger insert size: 9–12 kb	Inefficient transduction Insertional mutagenesis Requires cell division for transfection Requires packaging cell line No targeting Potential replication competence

1.3.2 Non-Viral Vectors

Non-viral vectors are synthetic or natural delivery systems that can transfer genetic materials (DNA, RNA, or other nucleic acids) to target cells without requiring the use of a virus physiology in a controlled, efficient manner. Non-viral vectors function by inserting genetic material into cells through a series of fundamental phases [15]. The initial step is packing the genetic material which is achieved by conjugating the genetic material to or loading it into the vector to supply appropriate conditions for protected transportation of the genetic material. In the second step, non-viral vectors

deliver genetic material to target cells. This process includes the stages of passage through the circulatory system, penetration into tissue, and reaching the cell surface. Biocompatibility and biodegradability of vectors have a crucial role at this stage. Non-viral vectors bind to cell surface receptors to enter the cell through mechanisms such as fusion or endocytosis. Genetic material is released by the vectors once they enter the cell. This may occur through mechanisms including endosomal escape or direct release into the cytoplasm. This stage ensures that the genetic material reaches target sites within the cell. The released genetic material is transcribed within the cell and initiates protein synthesis which enables therapeutic genes to be translated into functional proteins. Therefore, therapeutic proteins are produced by diseased cells, and the symptoms decrease [17], [18]. The most widely used non-viral delivery systems can be categorized as liposomes, peptides, and polymeric gene carriers [15]. Liposomes are microscopic vesicles comprised of phospholipid bilayers that can efficiently interact with target cells through their resemblance to the cell membrane structure. These molecules spontaneously assemble a double-layered arrangement in aqueous environments, creating a vesicle with hydrophilic heads on the external side and hydrophobic tails on the inner side The structure of the liposome allows to the creation of an internal environment that is segregated from the external environment and covers the loaded substances. Liposomes are an adjustable and flexible delivery technology considering their many advantages, including low toxicity, biocompatibility and biodegradability, manageable cellular interaction, and the capability to modify the surface characteristics and carrying capacity of the particle. However, they have certain disadvantages, such as high production costs, and stability problems, specifically in large-scale manufacture [18]. Peptides are short polymers made of amino acids that are beneficial for delivering pharmaceuticals or genetic materials due to their potential to target cells and their ability to cross cell membranes. In targeted therapies, peptides can be modified as ligands to function and bind to cell surface receptors with high specificity. On the other hand, synthesizing and purifying high-quality peptides can be expensive, and they can be rapidly degraded by proteases, reducing their transport capacity and efficiency [22]. Although utilizing liposomes and peptides provides a wide range of applications and significant potential in the fields of gene therapy including vaccine development, drug delivery systems, and biotechnology, some of the drawbacks of these carriers reveal the requirement for the use of polymeric carriers that offer more benefits.

1.4 Polymeric Carriers in Gene Delivery

The earliest chemical strategy for DNA transfer was to complex negatively charged DNA molecules with polycationic proteins in the presence of high salt concentrations to improve the DNA intake of cells. Since then, expanded knowledge of the molecular mechanisms of gene transfer in cells has increased the enthusiasm of polymer scientists to synthesize potentially versatile, custom-made polymers that can deliver genetic materials [23]. The physical stability of polymeric gene delivery systems must be maintained in the presence of serum proteins and high ionic strength, and the vectors must shield genetic materials from nucleases that are present in the extracellular spaces. Advances in polymer science have resulted in the evolution of polymeric nanoparticles produced by condensing and covering DNA to polymer, packaging DNA without condensing it and complexing DNA to NPs grafted with cationic surfactants. Three basic classes of polymeric NPs are under investigation for gene delivery studies (Figure 1.3) [23], [24].

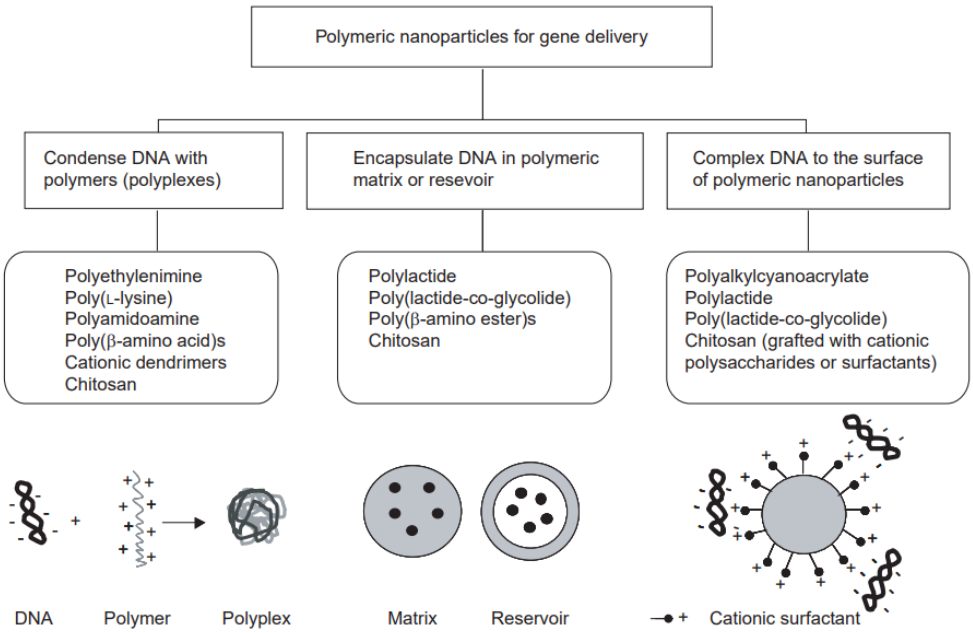
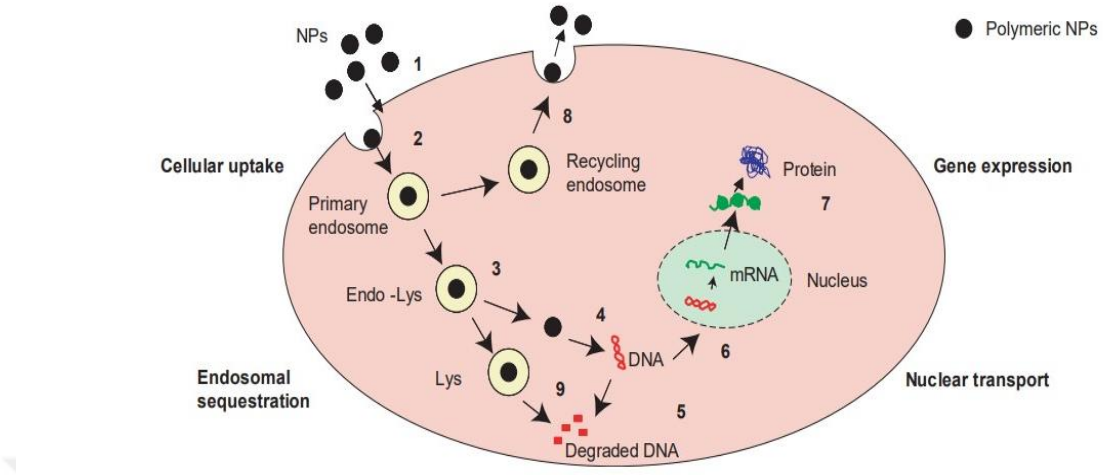


Figure 1.3. A schematic showing the different types of polymeric systems for gene delivery [24]

The NPs produced either by condensation, encapsulation or complexation of DNA have specific features and variable transfection impacts, making them appropriate for various gene delivery applications [25], [26]. To achieve successful gene delivery,

polymeric vectors must overcome several physical and biological obstacles before the gene is transferred to the desired site, a cell nucleus. Physical barriers are defined as the formulation of polymeric gene delivery vectors, which should be able to condense DNA to a proper size that can efficiently enter cells and maintain the stability and biological function of DNA. The physicochemical properties of polymers display a significant role in their biodistribution and pharmacokinetics and thus control the therapeutic effectiveness [27], [28]. The advantage of local gene delivery is that it can avoid unwanted exposure of gene delivery vectors to systemic circulation. This can be accomplished through intratumoral injections, direct injections into the tissue, intra-arterial injections for the liver, or direct instillation of gene-carrying vectors in the lungs. This also prevents the interaction with the blood elements and circumvents the phagocytosis of the vectors by the reticulo-endothelial system (RES) [24]. However, this type of local gene delivery needs that the polymeric NPs can diffuse within the tissue. Systemic administration of NPs is most advantageous for reaching the disseminated target tissues throughout the organism (e.g., in case of tumor metastasis) or tissues that are inaccessible for direct injections of NPs. Systemic administration of NPs is particularly advantageous for addressing the dispersed target tissues throughout the organism (e.g., in case of tumor metastasis) or tissues that are unavailable for direct injections of NPs. For systemically administered NPs, the foremost hindrance is to cross the vascular endothelium and the layers of a specialized extracellular matrix to reach the target tissues. Also, the phagocytosis by plasma proteins and their subsequent clearance by the RES are the other factors that alter the biodistribution of NPs. To deliver therapeutic genes effectively to target tissues, making improvements to the long-circulating properties of NPs is imperative. After being injected intravenously, NPs can be rescued from rapid opsonization by coating their surface with hydrophilic polymers like Polyethylene glycol (PEG) [24], [27], [28]. Tissue-specific ligands binding to the surface of polymeric NPs can also facilitate the active targeting of NPs to the specific tissue. Thus, the design of an efficient targeted gene expression system requires to comprehend the correlation between NP properties and the pharmacokinetics of their biodistribution. Furthermore, gene delivery vectors need to overcome several obstacles after being introduced in vivo to enter the nucleus. At each successive step, they lose a substantial portion of the genetic material (Figure 1.4) [24].



1) Cellular association of NPs; 2) internalization of NPs into the cells by endocytosis; 3) endosomal escape of NPs; 4) release of DNA in the cytoplasm; 5) cytosolic transport of DNA; 6) nuclear uptake of DNA; 7) expression of the gene; 8) exocytosis of NPs; 9) degradation of DNA either in Lys or in the cytoplasm. Major barriers include: cellular uptake of NPs, endosomal sequestration of NPs, nuclear transport of DNA/NPs and low levels of gene expression.

Figure 1.4. Schematic drawing of steps involved in gene delivery using polymeric carriers [24]

The chemical and physical stability in the extracellular space or systemic circulation, the association and internalization into cells through endocytosis, the intracellular release of DNA into the cytoplasm, the translocation of DNA into the nucleus in the cytoplasm, and the nuclear uptake of DNA are considered as substantial barriers [27]. The efficacy of vectors for DNA delivery relies on their association with cell membranes and the endosomal release of vectors. The ability of vectors to associate with negatively charged cell surfaces and the vector's internalization through endocytic mechanisms determine the efficiency of gene delivery systems [29]. Targeting ligands, which are specific to receptors on cell membranes can be utilized to enhance cell recognition and the association of NPs with the cells and facilitate receptor-mediated endocytosis. The primary hindrance to polymeric gene delivery is the possibility of the polymeric vectors becoming entrapped in the endosomal compartment after endocytosis. However, the efficiency of the endosomal escape of the polymeric vector extremely controls the transfection efficiency of the delivered genetic material. This has

galvanized continuous research to design methods for non-viral polymeric vectors to improve their endosomal escape and increase gene transfection [27], [28], [29].

1.4.1 Advantages And Challenges of Polymeric Carriers

Polymeric gene carriers are commonly utilized transfection agents in gene therapy and biotechnological applications due to offering numerous advantages to provide safe and efficient gene delivery to target cells. The most significant advantage of polymeric systems is their biocompatibility and low immunogenicity characteristics leading to minimal toxicity in living organisms. As compared to viral vectors, polymeric carriers do not induce robust immune reactions and enable repeated treatments to be administered more safely which is critical for the long-term efficacy and safety of gene therapy [30]. Polymeric gene carriers can be functionalized by the addition of varying functional groups and modification of surface characteristics due to their high tunability in their chemical structure. These alterations provide enhanced targeting capacity to carriers, improved cellular uptake, and optimal intracellular gene distribution. In addition, grafted ligands on the surface increase the specificity of targeting certain cell types or tissues [23]. Since polymeric carriers provide transient gene expression, they minimize the risks of insertional mutagenesis and potentially carcinogenic mutations caused by genomic disintegration. This feature raises the reliability of gene therapy applications and the controllability of the treatment process. The large-scale manufacture of polymeric gene delivery systems is less complex in terms of sterility and quality control, and more manageable and cost-effective due to easy achievement by chemical methods [24]. Another significant benefit of polymeric gene carriers is their gene loading capacity capable of delivering large DNA molecules or multiple genetic cargoes such as plasmid DNA, siRNA, miRNA, etc [15], [16]. The simultaneous transportation of genetic materials enables the implementation of multiple gene targeting and complicated gene therapy strategies. The polymeric vectors can achieve controlled and gradual release of therapeutic genes which ensures that gene expression is maintained for the expected period and increased therapeutic efficacy.

Despite the several benefits, some considerable challenges are possible to be encountered in the utilization of polymeric gene carriers [28]. One of the main barriers for the efficient delivery of genes into target cells is cell uptake and internalization of polymeric gene carriers which is impeded by the cell membrane's lipophilic properties.

Consequently, various approaches have been devised to promote the cellular uptake of polymers including surface charge modification, ligand incorporation to the surface, and improvements in the carrier's ability to adhere to the cell membrane [24], [28]. To achieve successful gene therapy, polymeric vectors are required to deliver their genetic cargo to intracellular organelles, such as the nucleus or mitochondria. However, polymeric vectors do not have the ability to target specific regions within the cell resulting in improper localization and diminished gene expression. Different strategies such as attaching a targeting molecule or altering the physical and chemical properties of the material have been developed to address this issue [30]. The major challenge of utilizing polymeric gene carriers is the controlled release of therapeutic genes which ensures the time-dependent optimal level of expression and efficient gene therapy. Excessively rapid gene release causes reduced therapeutic efficacy, potential toxicity, and adverse effects, while slow release may result in inadequate therapeutic efficacy. To overcome this obstacle, it is essential to optimize material properties and precisely adjust the release kinetics. Recently, the design of polymeric gene delivery systems that are sensitive to biological factors such as environmental pH, enzymatic activities, temperature, and redox balance facilitates the adjustment of the degradation rates and increases clinical efficacy and safety [30], [31].

1.4.2 Functional Polymer/DNA Conjugates

The advancements in new polymerization techniques have led to reduced polymer length dispersity and enabled copolymer synthesis for many controlled nanostructures. The incorporation of the hydrophobic/hydrophilic features of the synthetic polymer with DNA may enhance the functional properties of the complex. DNA is a highly programmable molecule with a wide variety of structures that offer the ability to regulate both the synthesis and conjugation of polymers. To date, several polymers including PPO, PCL, PS, and pNIPAM have been utilized to conjugate with DNA to form DNA-polymer conjugates. The polymer can alter the functionality of the corresponding conjugate due to its different physical and chemical properties. According to the progress documented, these polymer functions are mainly expressed by the following two aspects: (1) due to the hydrophobic core of DNA-polymer micelles, small-molecule drugs can be delivered by DNA-polymer conjugates, and (2) polymers can be attached to DNA to increase the stability of DNA-polymer

conjugates [32]. Each microenvironment in a biological system has distinctive properties which can be identified as biosignals. The chemical design for responsiveness to a specific biosignal (bioresponsiveness) provides site-specific functionalities with associated materials, for example, the cleavage of covalent bonds [33], [34]. Functional polymers display a role as gene carriers through the construction of DNA-polymer conjugates. In these systems, a covalent bond is formed between the terminus of DNA and a polymer, usually a polyanion or a nonionic polymer to evade self-aggregation [35]. The covalent attachment of the functional polymer to the therapeutic gene has the potential to augment its existing bioactivity and provide entirely new functions, including active targeting and cellular regulation of gene silencing processes [36]. The carrier system must deliver an intact gene into the cytosol to induce its therapeutic effect. Thus, the regulation of intracellular distribution (endosomal escape) and gene release are two properties that should be achieved in the polymer for intracellular delivery. In the DNA-polymer conjugate systems, an installed chemical linkage can be utilized for intracellular gene release. The cleavability of the covalent bond should correspond to the biosignals in the cell to prevent unexpected gene release and endosomal escape [37].

1.4.3 Smart Delivery Systems

In recent years, stimuli-responsive polymers have emerged in gene therapy studies due to their potential as effective targeted delivery systems. These polymers have significantly administered pharmaceutical research by being applied to tissue engineering, bioengineering, drug/gene delivery, biosensors, and textile applications [38]. These types of polymers also referred to as "smart" or "environmentally-sensitive, have the unique ability to adjust their physical structure in response to negligible environmental changes [39]. Smart systems that are sensitive to both the cellular and tissue levels are now employed in the treatment of medical conditions including infections and inflammatory diseases [40]. The stimuli-responsive polymers are classified based on the type of stimulus influencing the polymer to acquire the desired effect, identified as physical, chemical, or biological stimulus [41]. Also, the chemical structure of stimuli-responsive polymers can be dramatically altered by temperature, light, pH, electricity, magnetic fields, ion concentration, and enzyme degradation. In addition to graft copolymers and statistical/block copolymers, they can be integrated

into surfaces or utilized as cross-linked hydrogels [42], [43]. An added benefit of such polymers is their ability to complex, chemically conjugate, or physically combine with bioactive molecules such as nucleic acids, proteins, and organic molecules [44]. Carriers such as vesicles, micelles, and nanoparticles, incorporated with stimuli-responsive polymers, have been widely employed in tissue regeneration, wound healing, and cancer chemotherapeutics, as well as in gene therapy research [41]. Internal physiological conditions are targeted in a large percentage of delivery systems for advanced transport of bioactive molecules. These internally responsive stimuli systems offer several advantages such as their advanced predictability and reproducibility in vivo and feasibility in large-scale production [45]. Internal conditions are classified as, but are not limited to, physiological pH changes, physiological temperature, and biological elements. The properties of normal and pathological tissues are represented in Table 1.2. This study focuses on intracellular glutathione (GSH) level-dependent gene delivery systems, which are classified as redox-responsive polymeric systems.

Table 1.2. Biological parameters of normal and pathological tissues

Biological parameters	Normal tissues	Pathological tissues
pH	~7.4	~6.5 in tumor extracellular sites and inflammatory tissues; ~5–6 in endosomes; ~4–5 in lysosomes; ~6.4 (Golgi apparatus)
Specific enzyme	Low expression in intracellular compartments	Enhanced expression in intracellular compartments
Temperature	~37°C	~40–42°C
Glutathione (GSH)	Low expression with the same level between the intracellular environment and external matrix	~2–10mM in intracellular environment; ~2–10μM in extracellular matrix

1.4.3.1 Glutathione-Sensitive Release

Glutathione-sensitive polymeric systems have been considered highly effective as vectors in intracellular delivery of therapeutic agents, particularly genetic materials, due to the natural redox gradients within the human body [46]. The concentration of intracellular glutathione (GSH) has been identified to be about 2–10mM which is significantly higher than the extracellular GSH levels of 2–10μM [46]. Therefore, redox-responsive polymers such as polyanhydrides, poly(lactic/glycolic acid) (PLGA), and poly(b-amino esters) (PbAEs) can efficiently utilize the GSH gradient [41]. Disulfide bonds on polymer chains have also been used to induce redox response due to their increased stability at low GSH concentrations (in the extracellular environment) and instability at high GSH levels (in the intracellular environment) leading to cleavage of disulfide groups and triggering drug/gene release [41], [46]. Fig. 1.5 describes the intracellular drug delivery mechanism of these Glutathione-sensitive nanocarriers [46].

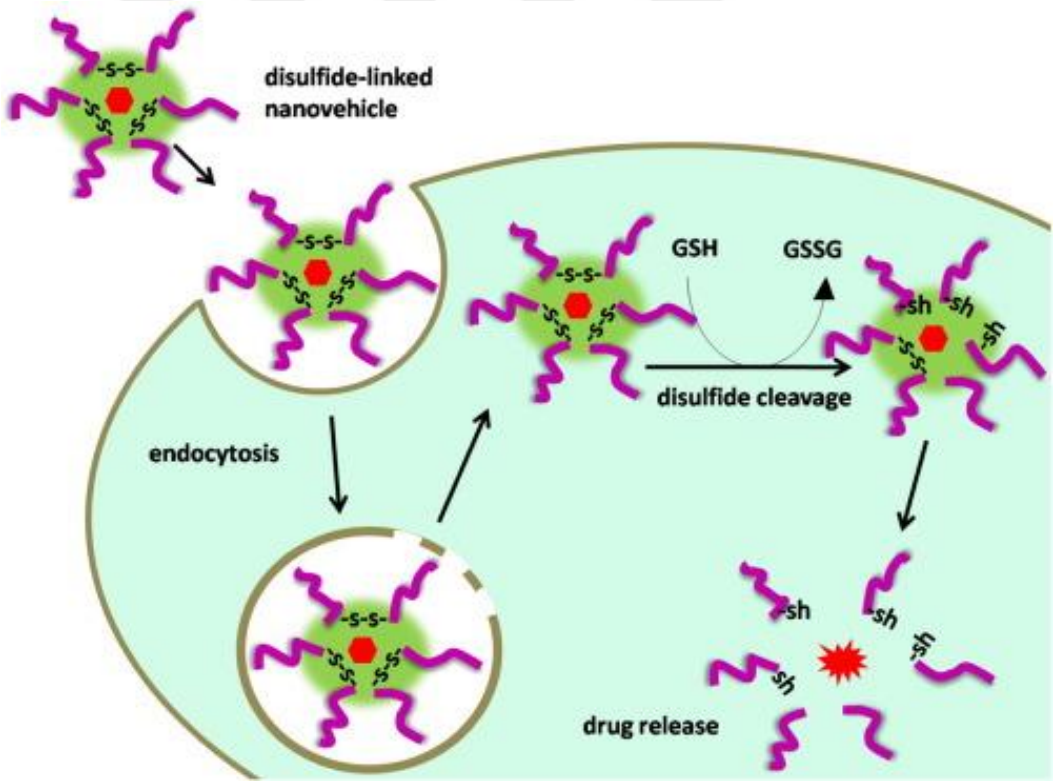


Figure 1.5. The mechanism of redox-sensitive nanocarriers for intracellular drug delivery [46]

The primary redox couple in animal cells is GSH/glutathione disulfide (GSSG) which controls the antioxidative capacity of cells [47]. The reduced form of GSH/GSSG

is preserved by NADPH and glutathione reductase. Also, different redox couples including NADH/NAD+, NADPH/NADP+, and thioredoxin red/thioredoxin ox have influence on the intracellular GSH level [47], [48]. Due to this substantial difference in GSH levels, GSH-responsive nanocarriers are employed as the most appealing targeted intracellular delivery systems [49]. Furthermore, glutathione has emerged as an excellent and prevalent internal stimulus to destabilize redox-sensitive nanocarriers, particularly in the endo/lysosome, cytosol, and cell nucleus to conduct effective intracellular drug and gene release [41].

Based on the publications, there are several strategies to introduce disulfide bonds to construct Glutathione-sensitive nanocarriers. For example, Jia et al. conjugated low molecular weight PEI to CS backbone using 3,3′-dithiodipropionic anhydrides (DTDPA) as a redox-cleavable linker which resulted in the efficient gene carriers having excellent gene condensation ability and redox-responsive characteristic [50]. In another study, Zhao et al. synthesized thiolated CS and PEI subsequently, and further, the oxidation of thiols in air conditions led to the formation of the disulfide bond [51]. Evidently, the oxidation of thiols might induce intramolecular disulfide bond formation as well as intermolecular disulfide bonds. This cross-linking mechanism could prevent the degradation of encapsulated therapeutics in blood circulation by improving the stability of nanoparticles in vitro and in vivo. Recently, cystamine with disulfide bonds was utilized to synthesize grafted copolymers of amphiphilic PEI and poly(ε-caprolactone) (PCL) as the nanocarriers for DOX and p53 plasmid DNA delivery. As the concentration of DTT increased from 0 to 10 mM, the cumulative release of DOX exhibited a significant increase, rising from 60% to about 100% within 48 h. Moreover, DOX/p53-DNA loaded nanoparticles showed to have a slightly enhanced cellular uptake than DOX loaded nanoparticles; after four hours, both nanoparticles had a significant uptake efficiency as compared to the free DOX solution. Given the electrostatic interactions between cell membranes and complexes, DNA internalization was highly affected by the surface charge density. This might be attributed to the "proton-sponge" effect and the transfection delay [52]. Using the thiols exchange technique, Lei et al. constructed a new gene delivery vector with disulfide bonds named RGD-PEG-SS-PEI. In the first step, c(RGDyk)-PEG-PDP was synthesized by the reaction of c(RGDyk)-PEG-NH2 with 3-(2-pyridyldithio) propionate (SPDP) having disulfide bonds. The thiol groups were conjugated to the PEI resulting in PEI-SH. The final product was acquired employing PEI-SH and c(RGDyk)-PEG-PDP. The disulfide

bonds were able to stimulate DNA release from PEI for improved cellular uptake and transfection efficiency. RGD-PEG-SS-PEI/pDNA displayed as an excellent gene transfer system in intracranial glioblastoma treatment since it could specifically interact with intracranial glioblastoma multiform receptors via RGD sequence [53].

Another approach to assembling GSH-responsive gene delivery systems is the conjugation of nucleic acids such as siRNA and antisense oligodeoxynucleotide (asODN) to polymers including PEG and hyaluronic acid via a disulfide bond [54], [55]. In contrast to the parent nucleic acids, these nucleic acid conjugates construct more stable complexes with polycations. However, the efficient intracellular release of active siRNA or asODN are accomplished by the hemolytic dissociation of disulfide linkages in the cytoplasm. A study conducted by Park et al. presented that compared with monomeric siRNA, more compact and stable polyelectrolyte complexes with polycations could be produced by the properties of increased charge densities and the presence of flexible chemical linkers belonging to multimerized siRNA linked with cleavable disulfide bonds [56], [57]. According to in vitro and in vivo results, these reductively cleavable multimerized siRNAs revealed remarkably improved gene-silencing efficiency [56]. Research in the literature substantiates that designing GSH-sensitive delivery systems is a promising approach for targeted gene release studies.

1.5 Poly(Ethylene Glycol) in Gene Delivery Applications

The ability of poly(ethylene glycol) to affect the properties of delivery systems is currently employed in a broad range of established and emerging therapeutic applications including polymeric gene carriers [58]. One of the excellent characteristics offered by PEG-based nanocarriers is stealth behavior in which structural parameters influence the biological and stabilizing effects [59]. In many applications, the molar mass of the polymer has been demonstrated to be significant for biocompatibility and stealth behavior. PEG is utilized with a molar mass ranging from 400 Da to 50 kDa for many pharmacological and medical applications. PEG having 20 kDa to 50 kDa molar mass is mainly used to conjugate small molecules such as low-molar-mass drug components, oligonucleotides, and siRNA. Increasing the size of the conjugates above

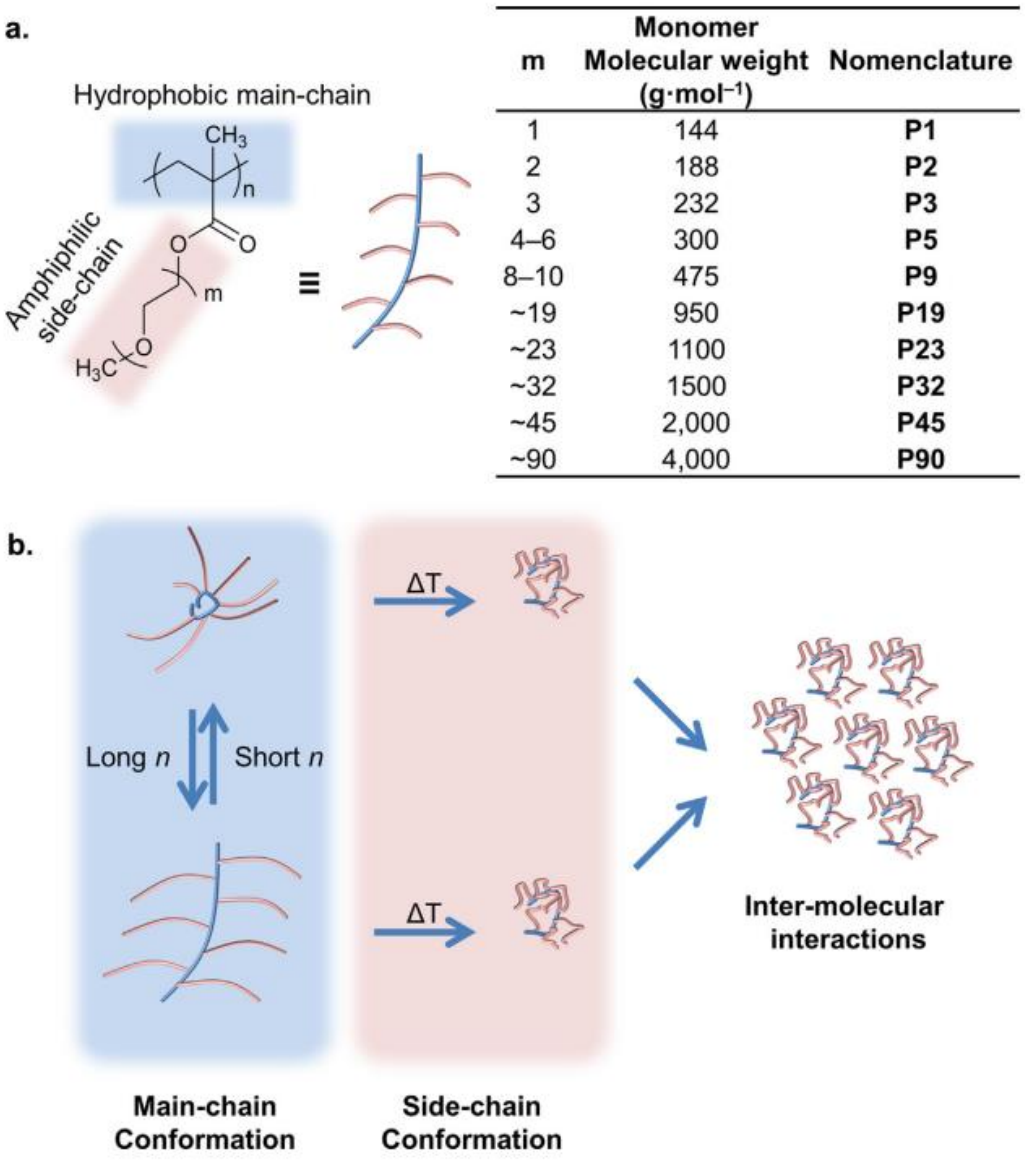
the renal clearance threshold prevents fast renal clearance resulting in evading subsequent elimination by the RES, reduced enzymatic degradation, and hidden cationic charges. In general, a low polydispersity index (PDI) is a fundamental requirement for the feasibility of a polymer in pharmaceutical applications. Polymers with a PDI value below 1.1 possess acceptable homogeneity and reproducibility that is critical in terms of body-residence time and immunogenicity of the delivery system [58], [59]. This prerequisite is fulfilled by PEG as well-defined polymers with PDIs around 1.01 are easily achievable by ethylene oxide polymerization. Moreover, PEG displays great solubility in organic solvents facilitating end-group modifications. Also, the water solubility of PEG leads to low intrinsic toxicity making it highly suitable for biological applications. In aqueous media, the solubility of PEG attached-hydrophobic drugs or carriers is enhanced due to its hydrophilicity. It provides genetic materials with increased physical and thermal stability and diminished aggregation of therapeutics in vivo caused by the formation of a “conformational cloud” which is the result of steric hindrance and/or masking of charges. The reason for "conformational cloud" formation is highly flexible polymer chains having a wide range of possible conformations. The higher transition rate from one conformation to another is correlated to the existence of statistically more polymer as a “conformational cloud” which inhibits protein interactions including enzymatic degradation or opsonization followed by (RES) uptake [60]. The decrease in the interactions with the body leads to less immunogenicity and antigenicity of PEGylated products and reduced risk of hemolysis, and aggregation of erythrocytes. The steric hindrance offers an additional benefit shielding the charge in charged delivery systems which consequently results in decreased zeta-potential and charge-induced interactions within the body. Such favorable properties named stealth effect provide the suppression of immune system recognition through opsonization and utilization of PEG in treatment of several diseases such as cystic fibrosis, severe combined immune deficiency, and hemophilia [60], [61].

However, the constraints of the linear-chain structure of PEG have led to the utilization of polymers with distinct architectures. One significant derivative of PEG is POEGMA which is polymerized from OEGMA, a monomer with oligo(ethylene glycol) side chains, and has a brush-like structure. POEGMA is a "stealth" polymer due to "non-fouling" properties providing resistance to proteins and cells since POEGMA exhibits a high density of oligo ethylene glycol (EG) moieties in its three-dimensional hyperbranched structure [62]. The particular structure offers easy copolymerization and

formation of linear and hyperbranched structures by free or controlled radical polymerization as well as functional ability. The structure of POEGMA provides several opportunities for modifications and functional group integrations which enable the development of more tunable and effective systems in gene delivery applications by increasing specific interactions with target cells and improving the efficiency of the transport system [63]. In addition to structural properties, POEGMA demonstrated a well-defined structure with high bioactivity, and increased pharmacokinetics (PK) compared to its PEG counterparts preventing the induction of anti-POEGMA antibodies and recognition by anti-PEG antibodies [64]. POEGMA's broad range of functionality, customizability, and less immunogenicity display roles in its preferability in gene therapy and other biomedical applications.

1.5.1 Structural Characteristics of POEGMA

Poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) exhibits high versatility relying on its comb-type structure in which manipulation of the dimensions affects main-chain and side-chains allowing them to extend or collapse together or independently. This controlling ability and unique physical-chemical characteristics of POEGMA are key to having tunable thermo-sensitive and supramolecular assembly properties, and efficient protein repellency. Due to its impressive advantages, POEGMA has emerged as a widely-utilized polymer for functional coatings, biosensors, biomaterials, gene delivery systems, etc [65]. From a physical-chemical perspective, this comb-shaped polymer is considered distinctive because of its hydrophobic backbone and amphiphilic side chains. The chemical structure of POEGMA, nomenclature, and some commercially-available monomers are represented in Figure 1.7a. Modifications of side-chain length adjust hydrophilicity thereby affecting the hydration state and conformation, at a specific temperature. This phenomenon has been the subject of several comprehensive studies based on the impacts of molecular dimensions of POEGMA on thermoresponsive properties within (bio)materials [66], [67]. Furthermore, due to steric repulsion between the potentially long side chains, the main chain conformation is influenced by the relative lengths of the main versus side chains (Fig. 1.7b) [68].



m	Monomer Molecular weight ($g \cdot mol^{-1}$)	Nomenclature
1	144	**P1**
2	188	**P2**
3	232	**P3**
4–6	300	**P5**
8–10	475	**P9**
~19	950	**P19**
~23	1100	**P23**
~32	1500	**P32**
~45	2,000	**P45**
~90	4,000	**P90**

Figure 1.6. Structure of POEGMA and illustration of its extended and collapsed conformations [68]

1.6 Controlled Polymerization Techniques

The current studies about novel polymers focus on more advanced biomedical applications to address patients' problems with increased efficacy and minimal discomfort. For instance, the gene therapy field conducts numerous research to provide a more reliable method for gene delivery than viruses as vectors [23], [69]. The biocompatibility of a polymer which is a highly significant property in biomedical applications is determined by the protein adsorption on the polymer surface and

successive cellular interactions. Such interactions with the biological environment are affected mostly by the functional group's distribution on the biomaterial surface. Fermentation methods are used to produce many valuable biocompatible polymers of microbial origin from natural sources which are truly biodegradable and nontoxic [70]. Enzymes involved in both hydrolysis and oxidation are usually responsible for biodegradation. The flexibility of aliphatic chains is higher than aromatic ones which results in fit into the active sites of enzymes and, thus, easier biodegradation. Irregularities in chain morphology prevent crystallization which hinders polymer degradation and favors disassociation [71]. Given the importance and relevance of polymers in the field of medical science, there are different polymerization techniques and synthetic methods that are utilized to prepare polymers comprising both natural and synthetic ones and their properties. Polymerizations are categorized based on the types of reactions involved in the synthesis process [70], [71]. The three main types of polymerizations can be listed as addition polymerization, condensation polymerization, and novel polymerization techniques.

The addition polymerization process involves the synthesis of polymers from monomers without losing small molecules. The compounds that undergo addition polymerization are usually unsaturated monomers such as olefins, acetylenes, and aldehydes. This technique is also known as chain-growth polymerization due to proceeding stepwise through reactive intermediates. The addition reactions are the most common and thermodynamically preferred chemical transformations of olefins. In general, bulk, solution, suspension, and emulsion polymerization methods are utilized to prepare polymers [72].

In condensation polymerization which is also known as step-growth polymerization, the elimination of small molecules like water, ammonia, methanol, and HCl induces the joining of two different monomers. The number of reactive functional end groups identifies the type of final product resulting from condensation polymerization. The monomers utilized in addition polymerization and condensation polymerization have distinctive characteristics. The main features of monomers in the condensation polymerization process are comprising at least two reactive sites and functional groups like —OH, —NH_2, or —COOH instead of double bonds. Monomers having one reactive group yield end products with a lower molecular weight due to terminating a growing chain. Typically, the reaction involves the utilization of two or more different monomers [72].

In novel polymerization techniques, ATRP (Atom Transfer Radical Polymerization), click polymerization, and RAFT (Reversible Addition-Fragmentation Chain Transfer) display significant roles in providing the synthesis of complex high molecular-weight polymers and their homogeneous distribution. ATRP is an effective living polymerization technique for achieving precise molecular weight, similar chain length distribution, and chain end functionality in well-defined polymers or copolymers. ATRP is favorable in the construction of different polymer topologies (linear, comb, hyperbranched, star/multiarmed, and network polymers) and compositions (homopolymers, graft copolymers, statistical copolymers, and block copolymers) [73].

ATRP offers several advantages including its ability to control molecular weights, produce homogenous polymers with desired features, optimize mechanical and physical properties and introduce functional groups to polymer chains providing versatility. Furthermore, the mechanism of ATRP enables the production of block copolymers by sequentially polymerizing different monomers and combining the features of individual blocks. The utilization of novel polymerization techniques offers innovative approaches to polymer science, allowing advanced control and customizability in future biomaterial development processes [74].

1.6.1 Raft Polymerization and Applications in Gene Delivery

In the gene therapy field, new synthesis methods have been inherently required to acquire polymers with well-tailored and well-defined characteristics including shapes, molecular weights, compositions, and architectures, since there is limited control over the architecture of cationic polymers like PEI polymers. Recent advances in polymer chemistry have enabled the development of cationic vector libraries categorized according to shapes, compositions, and architectures, and their efficacies in gene delivery were assessed to determine the optimal structures [75], [76], [77]. Living radical polymerization (LRP) techniques comprising reversible addition-fragmentation chain transfer polymerization (RAFT) emerged as a breakthrough in the field of gene delivery in the 1990s and facilitated the construction of polymer libraries [75], [78]. As a versatile and suitable method, RAFT polymerization enables the production of well-defined telechelic (reactive) polymers offering tolerance to various solvents, reaction condition changes, and functionalities [78]. The synthesis of RAFT polymer occurs

through a chain transfer process relying on the utilization of chain transfer agents (CTAs) which are organic compounds with thiocarbonyl thiol moieties and a radical initiator such as 4,4'-azobis(4-cyanovaleric acid) (ACVA), also known as 4,4'-azobis(4-cyanopentanoic acid), and azobisisobutyronitrile (AIBN). In Figure 1.7, the scheme of the RAFT polymerization mechanism is illustrated [79].

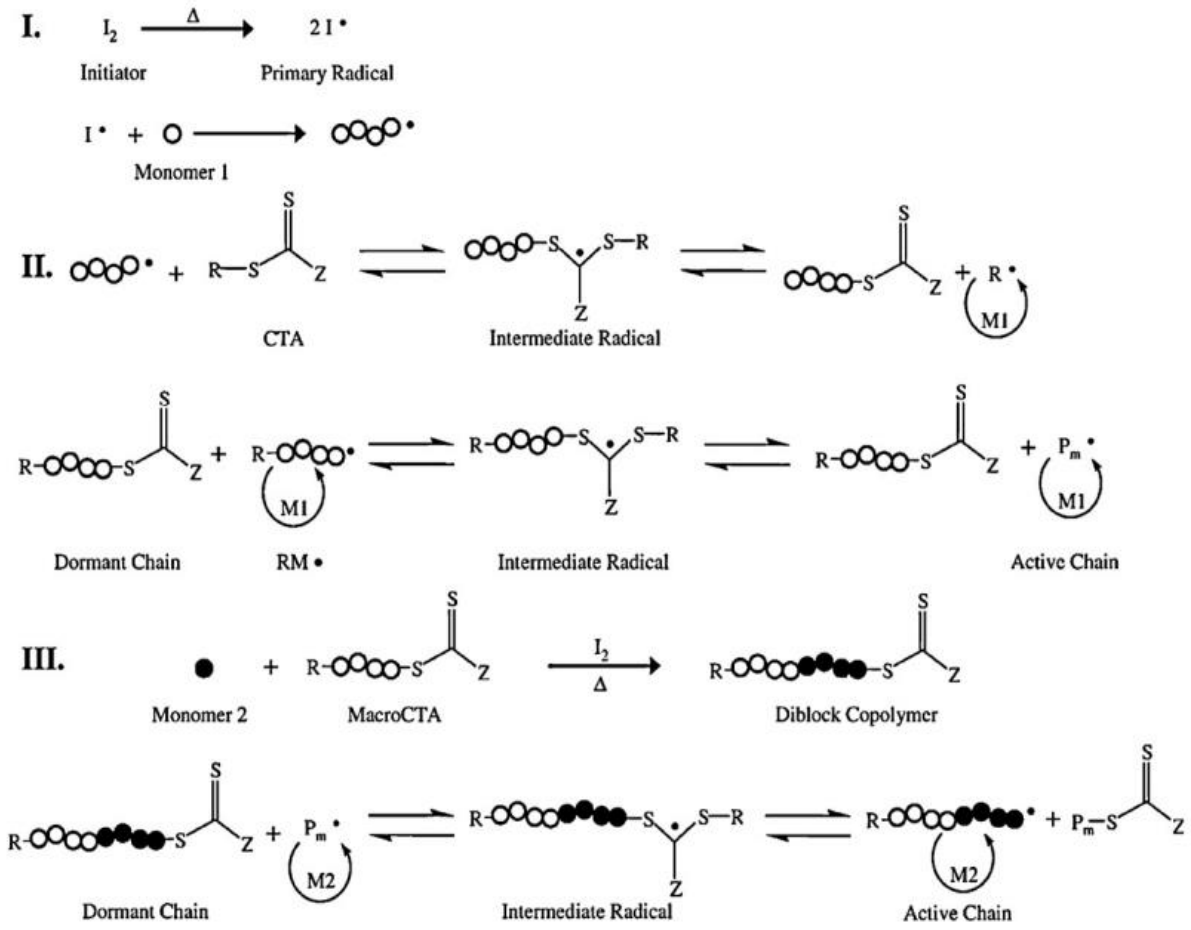


Figure 1.71. Schematics of RAFT polymerization [79]

The RAFT polymerization results in a linear polymer with an R-group and a dithiocarbonate moiety in different ends. The final product demonstrates low polydispersity and precise molecular weight critical to attaining particular mechanical, thermal, and solubility characteristics. It is typically challenging to polymerize some of the functional monomers including acrylamides, styrenes, and methacrylates, and the RAFT mechanism enables the synthesis of polymers constructed from various monomers. Such polymers can be assembled in specific molecular architectures such as

block, gradient, statistical, comb, brush, star, hyperbranched, and network copolymers (Figure 1.8) due to the sequential addition of different monomers provided by the nature of the RAFT process [80]. Furthermore, due to improved functional group tolerance, polymer functionalities such as responsiveness to chemical and thermal stimuli are increased by integrating different moieties into the polymer chain. Consequently, since the first utilization of the RAFT polymerization technique in 1998, there has been a remarkable increase in water-soluble polymer synthesis for gene delivery applications [78].

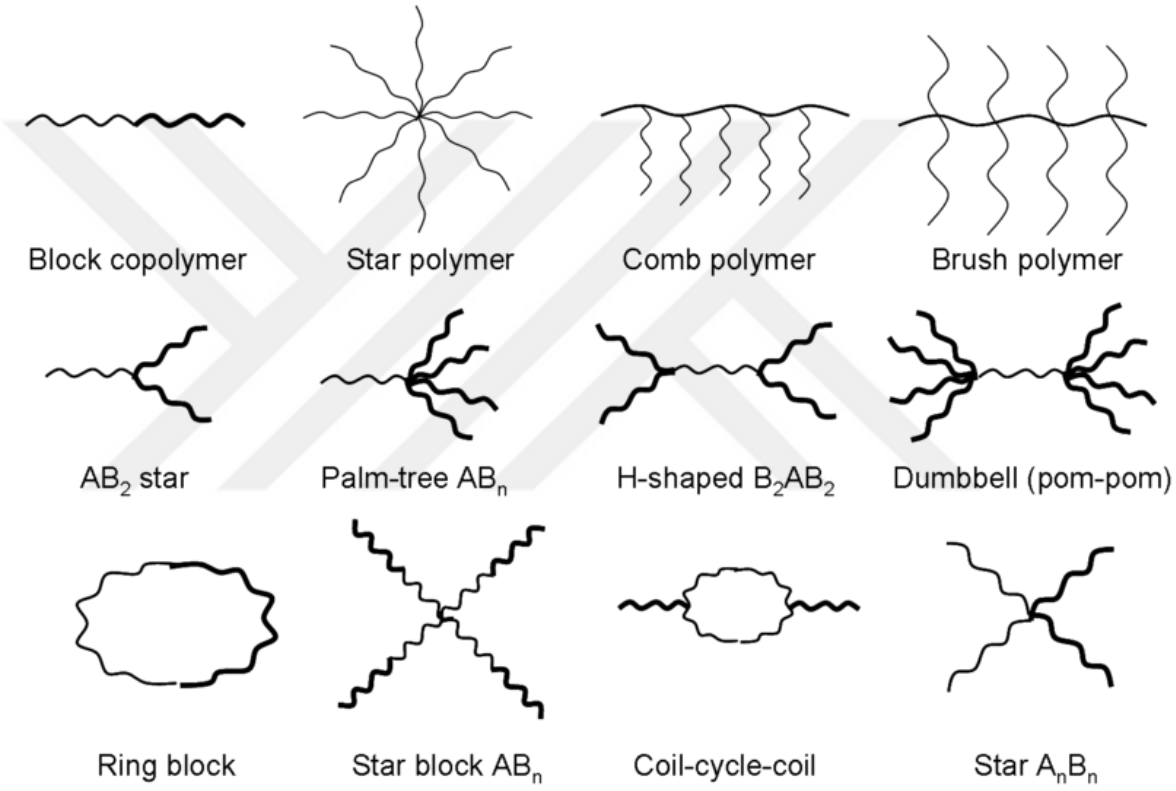


Figure 1.8. Complex architectures accessible via the RAFT process [80]

Stimuli-responsive copolymer synthesis by RAFT polymerization is a significant step in producing successful gene delivery vectors. As exposed to external stimuli, stimuli-responsive copolymers undergo a conformational change resulting in self-assembling micelles with separate hydrophobic and hydrophilic regions in an aqueous environment [76]. It was established that such modifications in the structure of cationic polymers have a significant influence on the efficacy of gene delivery. For instance, gene delivery vectors can have improved physiochemical and biological characteristics such as increased gene compaction, steric stability, and reduced toxicity

provided by their hydrophobic components [81]. Poly(ethylene glycol) methacrylate) (POEGMA), a type of temperature-sensitive polymer synthesized via RAFT, is utilized due to DNA compaction efficiency in gene delivery applications and its reported biocompatibility and suitability for biomedical applications [82].

1.6.2 Functionalization of Polymers

The chemical structure design and functionalization techniques are currently investigated in biomedical applications. To produce novel redox-sensitive polymer therapeutics, a variety of redox-sensitive functional groups such as sulfides, disulfides, selenides, and boronic esters are utilized [83]. The disulfide group is one of the extensively applied redox-responsive groups to functionalize polymeric vectors due to its significant advantages. The chemistry of disulfide bond (S—S) has the ability to overcome intracellular and extracellular barriers offering versatile designs to polymeric nanocarriers. The nature of S—S displays reducible and cleavable characteristics under the reductive conditions caused by GSH, nucleophiles, electrophiles, and even photons [84]. Also, the relevance of thiol-disulfide exchange reaction with several physiological events including enzymatic activity, protein stabilization, and redox cycles in the body increases the feasibility of disulfide groups [85]. From a synthesis perspective, there are advanced methods to incorporate the S—S bond into polymeric delivery systems.

1.6.2.1 PDS Modification in Gene Delivery

Recently, the utilization of pyridyl disulfide (PDS) in the synthesis of polymeric architectures of acrylamide and acrylic monomers has emerged as a significant research subject in polymer science [86]. Several crosslinking polymeric structures have been produced by incorporating PDS and employed as delivery vectors such as shell-cross-linked micelles, and interlayer-cross-linked micelles/nanogels. As compared to other S—S-comprising systems, PDS-based polymeric nanocarriers supply reactive pendants or terminal groups due to pyridylthio moiety enabling substitution of any thiol-containing compounds by being itself is an excellent leaving group as 2-pyridine-thione molecular form [87]. Such features have led to extra attention to PDS in the fabrication of biodegradable therapeutic systems.

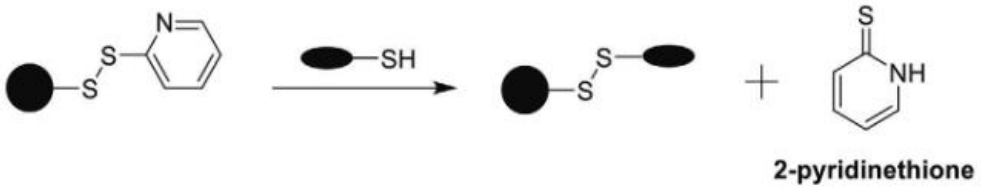


Figure 1.9. Schematic illustration for the replacement of the PD group by thiol-contained molecules [88]

As illustrated in Figure 1.9 [88], the thiol-disulfide exchange of PDS results in the formation of asymmetric disulfides and the elimination of the 2-pyridine-thione leaving group. The most important point is that the thiol-disulfide exchange reaction involving PDS can be achieved with desired yields in both organic and inorganic solvents under mild conditions, and while not interfering with other functional groups including hydroxyl groups, amines, and carboxylic acids [89]. PDS which is located as a backbone terminal group or in the end groups of polymer chain provides reactivity against thiolated molecules and formation of free thiol groups in the presence of S─S reducing agents such as 1,4-dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) [90]. The defined advantages of PDS-attached polymers have been successfully used in redox-responsive DNA/RNA delivery systems by conjugating thiol-modified nucleotides with polymers. The published literature confirmed that PDS-involved polymer derivatives exhibit strong DNA binding ability, can assemble polymer-DNA complexes with optimum size and low cellular toxicity, and mediate transfection. Moreover, the DNA unpackaging ability of PDS-based polymers was increased with the high concentration of GSH, which makes the potential of the PDS to be exploited in the glutathione-sensitive polymeric gene delivery systems [88].

1.7 Significance of The Study and Potential Applications

The primary objective of this study is to develop poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA) synthesized via Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerization and modified with Pyridyl Disulfide (PDS) to provide a conjugation with thiolated plasmid DNAs (pDNAs) in order to construct covalently-bound glutathione-sensitive gene delivery system. It was aimed to investigate the potential of this new system in gene transfer to human breast adenocarcinoma cells.

It is achievable to transfer therapeutic genes to cancer cells utilizing the newly established glutathione-sensitive gene transfer system. The high concentration of glutathione in tumor regions provides the activity of the system in these cells and increases the effectiveness of gene therapy in the treatment of cancer by the transfection of therapeutic genes to target cells. The gene delivery system stated in this study facilitates to conduct of genetic research and disease modeling in genetic pathways by examining gene function. Preliminary research may assess the safety and efficacy of gene therapy approaches, which can yield valuable insights as moving toward practical implementation. This glutathione-sensitive system can offer an approach to the pathophysiology of oxidative stress-related disorders due to intracellular redox sensitivity. Furthermore, it can assist in drug delivery enabling controlled drug release in specific target cells.

The synthesis of POEGMA by the RAFT polymerization technique and modification of polymer chain with PDS to integrate a disulfide bond and conjugation of —SH with thiolated pDNAs, is a significant contribution to the literature in terms of its applications in the field of polymer functionalization and gene therapy. This methodology demonstrates high feasibility in polymer science and biomedical applications. The findings of this study could serve as the basis for further research on the several diseases that can be treated with gene therapy including cancer, hemophilia, hereditary blindness muscular dystrophy, severe combined immunodeficiency, sickle cell disease, etc.

Chapter 2

Experimental Section

2.1 Materials

OEGMA (Mn = 500 g mol^{-1}), 1,4-dithiothreitol (DTT), 2,2-dithiodipyridine (DTDP), ethanolamine (ETA), 4-cyano-4-(thiobenzoylthio)pentanoic acid (CTA), 4,4-azobis(4-cyanovaleric acid) (ACVA), N-(3 dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), 2,2′-Diaminodiethyl disulfide dihydrochloride (Cystamine dihydrochloride), Imidazole, 2,2-dithiodipyridine, Ethylenediaminetetraacetic acid (EDTA), were purchased from Sigma Aldrich and used as received. N, N-Dimethylformamide, methanol dried (Max. 0,005 %H2O) were purchased from Merck and used as received. Plasmid DNAs (dpy10 and pAD67) were obtained from Kaplan Laboratory, Abdullah Gul University. All other chemicals were used of analytical grade. MDA-MB-231 (human breast adenocarcinoma) cell lines were provided by Assoc. Prof. Ömer Aydın, ERFARMA, Erciyes University.

Obtention of Plasmid DNAs

In this study, two different plasmid DNAs, pAD67 and dpy10, were utilized. These plasmids, specifically designed for CRISPR applications in C. elegans, were generously donated by AGÜ-Kaplan Lab. Purified dpy10 and pAD67 were used to evaluate the gene transfer capacity of a newly synthesized polymeric carrier into MDA-MB-231 cells. The plasmids served as models to assess the efficiency of gene delivery.

2.2 Methods

2.2.1 Synthesis of POEGMA via RAFT Polymerization

First of all, macro chain transfer agent (macroCTA, Poly(OEGMA)) was synthesized by the RAFT polymerization of OEGMA according to the Topuzogulları et al [91]. Reaction scheme is illustrated in Figure 2.1. Briefly, 2.375 mL OEGMA (Mn: 500) was dissolved in 6.625 mL DMF with (14 mg, 5.0 x 10^{-5} mol) CTA while the solution was purged with N_2 for 30 min. Meanwhile, (12.6 mg, 4.5 x 10^{-5} mol) ACVA was dissolved for 30 min, and 1 mL mixture was extracted from the total volume and added to the OEGMA/DMF solution under N_2. The mixture was heated to 70 °C for 2.5 hours at 250 rpm. The polymer was precipitated with cold diethyl ether three times and centrifuged at 8000 relative centrifugal force (rcf) for 10 minutes at 24 °C. The final product was dried under the vacuum at room temperature. The proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was used to analyze the chemical compositions of purified homopolymer.

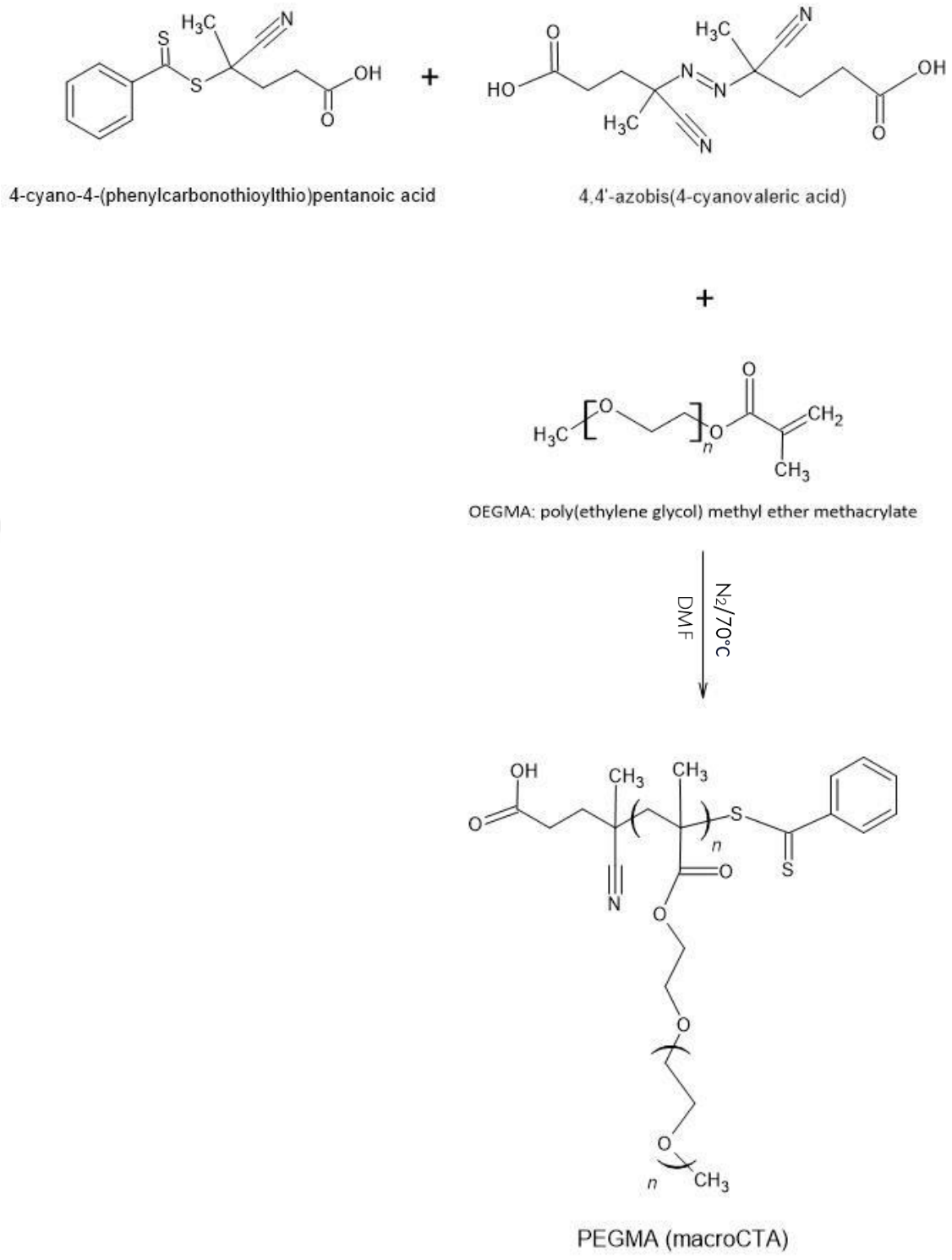


Figure 2.1. Reaction Scheme for RAFT Polymerization of POEGMA

2.2.2 Pyridyl Disulfide Modification of POEGMA

Pyridyl disulfide (PDS) modification of the POEGMA was carried out according to Boyer et al. [92] in order to exchange the -SH group and form a disulfide bond and the chemical scheme of the modification is presented Figure 2.2. Briefly, 100 mg of macroCTA and 2,2-dithiodipyridine (15.4 mg, 7.0×10^{-5} mol) were dissolved in DMF (425 µL). The vial was sealed by rubber septum and purged with N_2 for 10 min. Ethanolamine (36.37 mg, 6.0×10^{-4} mol) was dissolved in DMF (1 mL) and 31.94 µL of this solution was added to the vial under N_2. The mixture was stirred at room temperature for 4 h. The yellow-colored mixture was precipitated from cold diethyl ether and dried under a vacuum. The product was dissolved in water and then dialyzed (Fisherbrand 21-152-9 and 21-152-18 dialysis tubing-3500 to 14000 Dalton) against water/methanol (50:50) and only water, respectively. The final product was lyophilized and examined by ^{1}H-NMR spectroscopy.

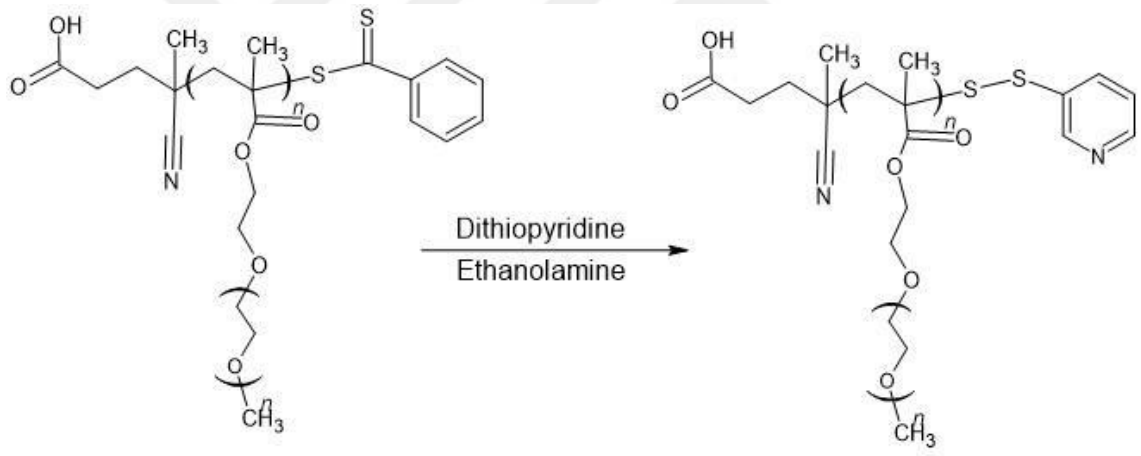


Figure 2.2. The Chemical Scheme of PDS Modification of POEGMA

Characterization

The Viscotek TDA302 GPC equipment (AGU MERLAB) was used to perform Gel Permeation Chromatography (GPC) studies on polymers. The separation was carried out using an Eprogen CatSEC300 column at a flow rate of 0.4 mL/min. The mobile phase was 0.1 M acetate buffer (0.15 M NaCl). The GPC system was calibrated with a single standard of linear PEO (Mw = 21 kDa, PDI = 1.07).

^{1}H-NMR spectra of the synthesized POEGMA and PDS Modified-POEGMA were analyzed from Bruker 400 MHz (Germany) liquid NMR spectrometers using deuterated DMSO as the solvent (ERU TAUM).

Fourier Transform Infrared Spectroscopy (FT-IR, Thermo Scientific, Nicolet 6700) was used to obtain infrared absorption or emission spectra and to perform qualitative analysis of POEGMA and PDS Modified-POEGMA in the range of 400-4,000 cm^{-1} (AGU MERLAB).

Zeta potential measurement was used to determine the charge shift of POEGMA, PDS-modified POEGMA, dpy10 and pAD67, POEGMA conjugated dpy10 and POEGMA conjugated pAD67 using water as the solvent by Malvern Zetasizer NanoZS (United Kingdom (AGU MERLAB).

2.2.3 Cystamine Modification and DTT Reduction of *dpy10* and *pAD67* Plasmids

Modification and DTT Reduction of pDNAs

Using a carbodiimide reaction, cystamine can be added to DNA or RNA to modify the 5′ phosphate group which is described in Figure 2.3 [93]. The plasmids (dpy10 and pAD67) obtained from AGU, Kaplan Lab were modified by the addition of cystamine. In this step, 1.25 mg of the 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was weighed into an Eppendorf tube. 7.5-15 nmol (60-120µg) plasmid dissolved in 100 µl reaction buffer (phosphate-buffered saline (PBS) with EDTA: 10 mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2) and 75 µl of the solution was added to the tube. Then, 50 µl of 0.25 M cystamine in 0.1 M imidazole, pH 6.0 was added immediately because EDC is labile in aqueous solutions. The mixture was vortexed, and then the tube was placed in a microcentrifuge and spun for 5 min at 18000 rpm. After the centrifuge, an additional 200 µl of 0.1 M imidazole, pH 6.0 was added. The reaction was mixed and incubated at 37°C, for 4 h. After incubation, for reduction of the cystamine disulfides, 200 µl of 1.0 M DTT was added and incubated at room temperature for 15 min. This will release 2-mercaptoethylamine from the cystamine modification site and create the free sulfhydryl on the 5′ terminus of the oligonucleotide.

SH-labeled plasmids were purified by dialysis using 10-mM sodium phosphate, 0.15 M NaCl, 10 mM EDTA, pH 7.2, and cystamine modification was confirmed with free thiol detection method.

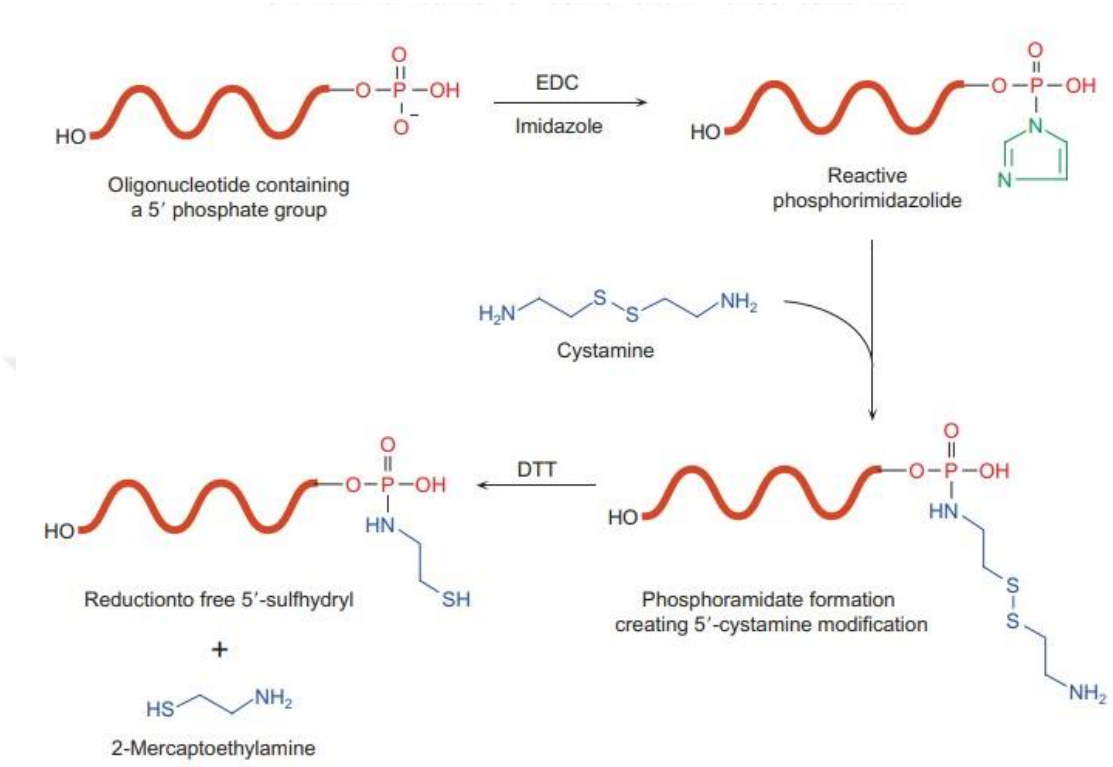


Figure 2.3. The Chemical Scheme for DNA Modification with Cystamine Using the EDC/Imidazole Reaction [93]

Cystamine-calibrated Free Thiol Detection Method with 2,2-dithiodipyridine

2,2-dithiodipyridine (DTDP) reacts with free thiol groups to initiate disulfide bond formations, and the by-products formed as a result of this reaction induce a color shift in the solution. This change reveals the presence of thiol groups in the environment. Pyridine-2-thione formed when DTDP reacts with free thiol groups is evaluated by absorbance at a wavelength of 324 nm. Within the scope of the experimental procedure, 1 mM stock solution of cystamine dihydrochloride and concentrations of 0 µM, 10 µM, 20 µM, 30 µM and 40 µM were prepared. By adding 100 µL of 1 mM DTT to cystamine solutions, mixtures were prepared, and the solutions were stirred for 2 h. Then, 100 µL of 1 mM DTDP was added to each 1 mL of cystamine-DTT mixture. The solution was

stirred for 15 minutes. The obtained solutions were measured at 324 nm in a spectrophotometer and absorbance values were recorded. Finally, a linear regression analysis was performed between cystamine concentrations and absorbance values to create a calibration curve. The followed reaction of free thiol detection is represented in Figure 2.4.

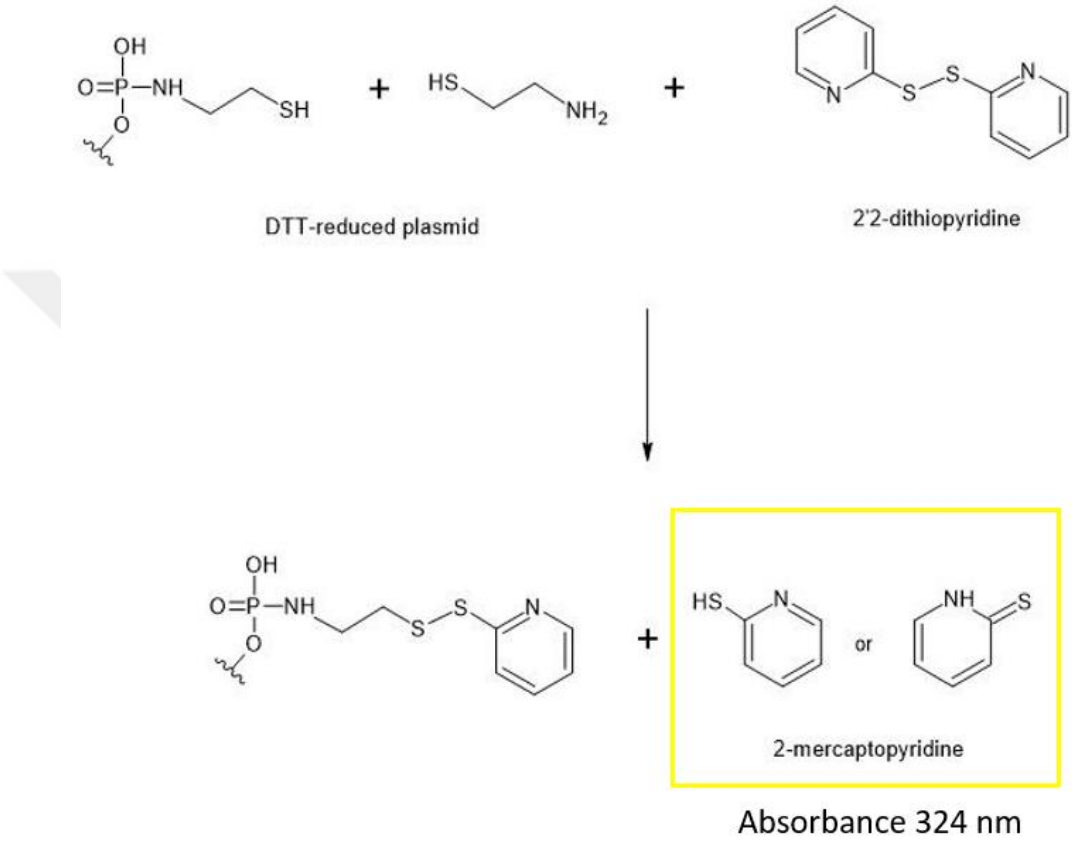


Figure 2.4. Schematic Representation of Free Thiol Detection Method

2.2.4 Conjugation of Modified Polymer And DTT-Reduced pDNAs

Dithiothreitol (DTT), which functions similarly to TCEP, reduces any existing disulfide bonds and allows free thiols to oxidize under mild conditions to form disulfide bonds between two free thiols. Following the experimental protocol stated by Bayram, et al. [91], DTT reducing agent was used to conjugate POEGMA containing the PDS group to the modified plasmids. To perform conjugation reaction, in Figure 2.5, briefly, 2 mL of dry methanol was used to dissolve 15 mg of polymer, dpy10 (800 ng, 75×10^{-9} mol), and pAD67 (640 ng, 75×10^{-9} mol) in separate reactions. DTT (1.5425 mg, 5 ×

10^{-6} mol) was added dropwise to the reaction solution after being dissolved in 2 mL of dry methanol. Subsequently, the mixtures were stirred at 250 rpm overnight in the dark and dialyzed against dry methanol for 2 days. Then, the resultant products were precipitated three times with diethyl ether and vacuum sealed. Gel electrophoresis was performed to confirm the formation of the disulfide bond and observe the efficiency of the conjugation process by analyzing the free plasmid remains. Furthermore, the products of conjugation reaction were purified using FavorPrep™ Plasmid DNA Extraction Mini Kit, FAPDE001-1, (AGU, Fidan Lab) and the disulfide bond formation between POEGMA and pDNAs was confirmed by 1H-NMR spectroscopy.

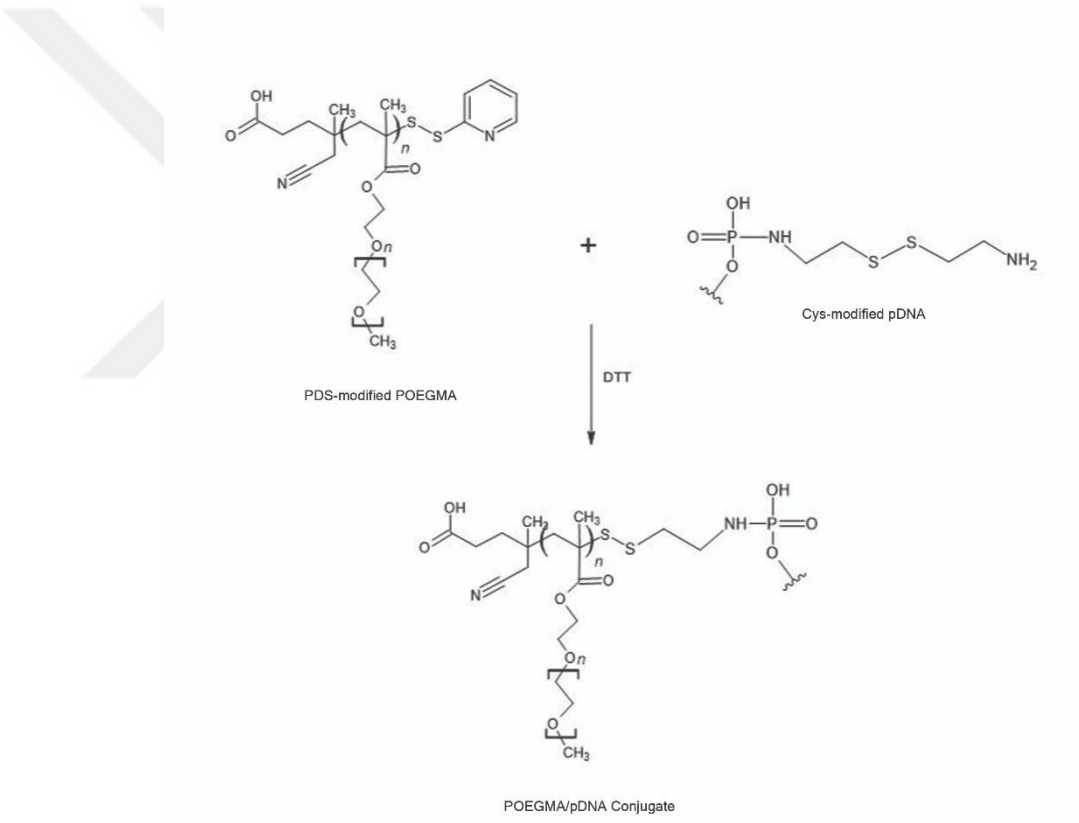


Figure 2.5. The Chemical Scheme for POEGMA and Plasmid Conjugation

2.2.5 Gel Electrophoresis Analysis

While free plasmids can migrate within the gel, POEGMA-bound plasmids are prevented because the weight and the size of plasmids increases, and their negative charges shift towards positive after conjugation. A gel electrophoresis assay was performed to confirm the ability of plasmids dpy10 and pAD67 to bind to POEGMA. In this context, dpy10 and pAD67 plasmids and POEGMA-plasmid DNA conjugates were dissolved separately at a concentration of 10 μM using RNAse-free water. Each conjugate was analyzed with free pDNAs by gel electrophoresis on a 1% agarose gel in 1M Tris-acetate EDTA buffer solution by applying a current of 150 V for 30 min.

The ability of various concentrations of glutathione to break disulfide bonds leads to the dissociation of polymer-plasmid conjugates at different rates at different glutathione levels and, consequently, to gene release and GSSG byproduct (Figure 2.6). This release was visualized by gel electrophoresis assay according to experimental procedure stated by Wang, et al. [94]. Briefly, glutathione was added to the conjugation products whose synthesis was described above at concentrations of 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM, respectively. Samples were incubated at 37°C and 150 rpm for 3 hours. After incubation, the products were analyzed by gel electrophoresis on a 1% agarose gel in 1M Tris-acetate EDTA buffer solution by applying a current of 150 V and 400 Amps for 35 minutes. During gel loading, each sample was loaded with 2 μl loading dye and results were visualized with the Molecular Imager® Gel Doc™ XR+ System.

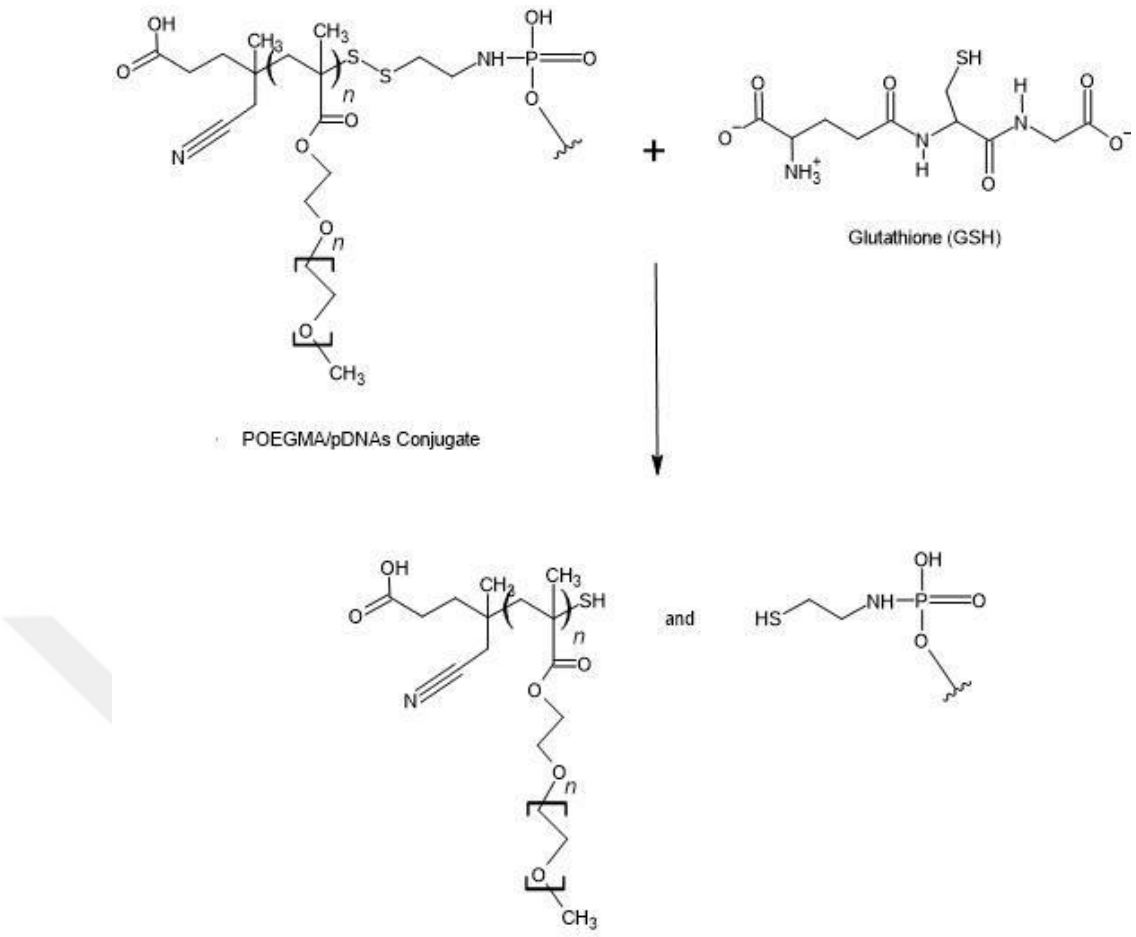


Figure 2.6. The Chemical Scheme for GSH Release of POEGMA/pDNAs Conjugate

2.2.6 Intracellular Uptake Assay in MDA-MB-231 cells

Plasmid Staining

After the optimization process within house-method, the concentration, volume, and incubation duration of propidium iodide (PI) for interaction with plasmid DNA were determined. The plasmids were incubated with 200 µL of 100 µg/mL PI at room temperature for 30 minutes. During this incubation period, the plasmids are vortexed for 5 seconds every 5 minutes. After the incubation period, the plasmids are ready to be administered to MDA-MB-231 cells.

Intracellular Uptake Assay of Plasmids

The internalization of plasmids was monitored and compared using a Guava EasyCite flow cytometer within a 6-hour period. MDA-MB-231 cells, which are triple-negative breast cancer cells, were counted and 300,000 cells were seeded into each well of a 6-well plate. The cells were incubated with PI-stained plasmids for a duration of six hours. Following the incubation, the cells were gathered through centrifugation at 2500 rpm for five minutes and subsequently washed twice with PBS. The cells were analyzed using a red filter. Untreated control cells were used as a baseline, and the percentage of cells treated with plasmids was determined in comparison to the controls [95]

Chapter 3

Results

3.1 POEGMA Synthesis and Characterization

RAFT (Reversible Addition-Fragmentation Chain Transfer) polymerization technique, which allows restraining the length of the polymer chain and enables the polymer to have the desired properties, was utilized to deliver a plasmid DNA comprising the relevant gene to MDA-MB-231 cells. This was achieved by reacting OEGMA with ACVA, producing free radicals, and CTA, a polymer chain growth regulator, to acquire the charged polymer. ^{1}H NMR spectroscopy was performed to characterize the structure of POEGMA which has pink gel form (Figure 3.1.).

Figure 3.1. The appearance of synthesized POEGMA

Yu et al. confirmed the presence of POEGMA block by ^{1}H-NMR analyses [96]. The chemical shifts of the pendant poly(ethylene glycol) (PEG) chains' methoxyl group

(-OCH_3) protons were observed at 3.40 ppm in the ^{1}H-NMR spectra obtained from the precipitated polymer. Furthermore, protons observed at 7.0 and 8.5 ppm in the ^{1}H-NMR spectra and minor peaks within the 7.5-8 ppm range belonging to the ring protons of the RAFT agent, according to a study by Bayram et al., pointed out the efficient synthesis of POEGMA [91]. These integrations confirmed the success of polymer synthesis and structural properties and evaluated the efficiency of polymerization. As a result of GPC analysis, the weight average molecular weight (Mw) of the polymer was determined as 30,400 Da, and the polydispersity index (PDI) was calculated as 1.076 which indicates that the molecular weight distribution of the polymer is quite narrow. The numerical value of the polydispersity index closes to 1 implies the homogeneity of the polymer chains and the efficiency of the synthesis reaction. Thus, a PDI value of 1.076 indicated that the polymerization process was carried out successfully, and the properties of the resultant polymer are consistent. According to Size Exclusion Chromatography analysis stated by Yu et al., PDI values were in the range of 1.11–1.30, indicating that the polymerization was controllable [96]. The analysis in our study acquired a PDI value of 1.076, which signifies a more desirable molecular weight distribution in controlled synthetic polymers. As compared to the precursors, shorter elution times and a slight increase in PDI values implied the success of POEGMA polymerization in both studies. In addition, the small shoulder was observed in the SEC traces on the side with the larger molecular weight and considered as a combination of the PEGMA monomer's molecular weight dispersion. Following that, the zeta potential of the POEGMA was measured as -1.39 ± 0.09 mV which is the demonstration of a slightly negative charge.

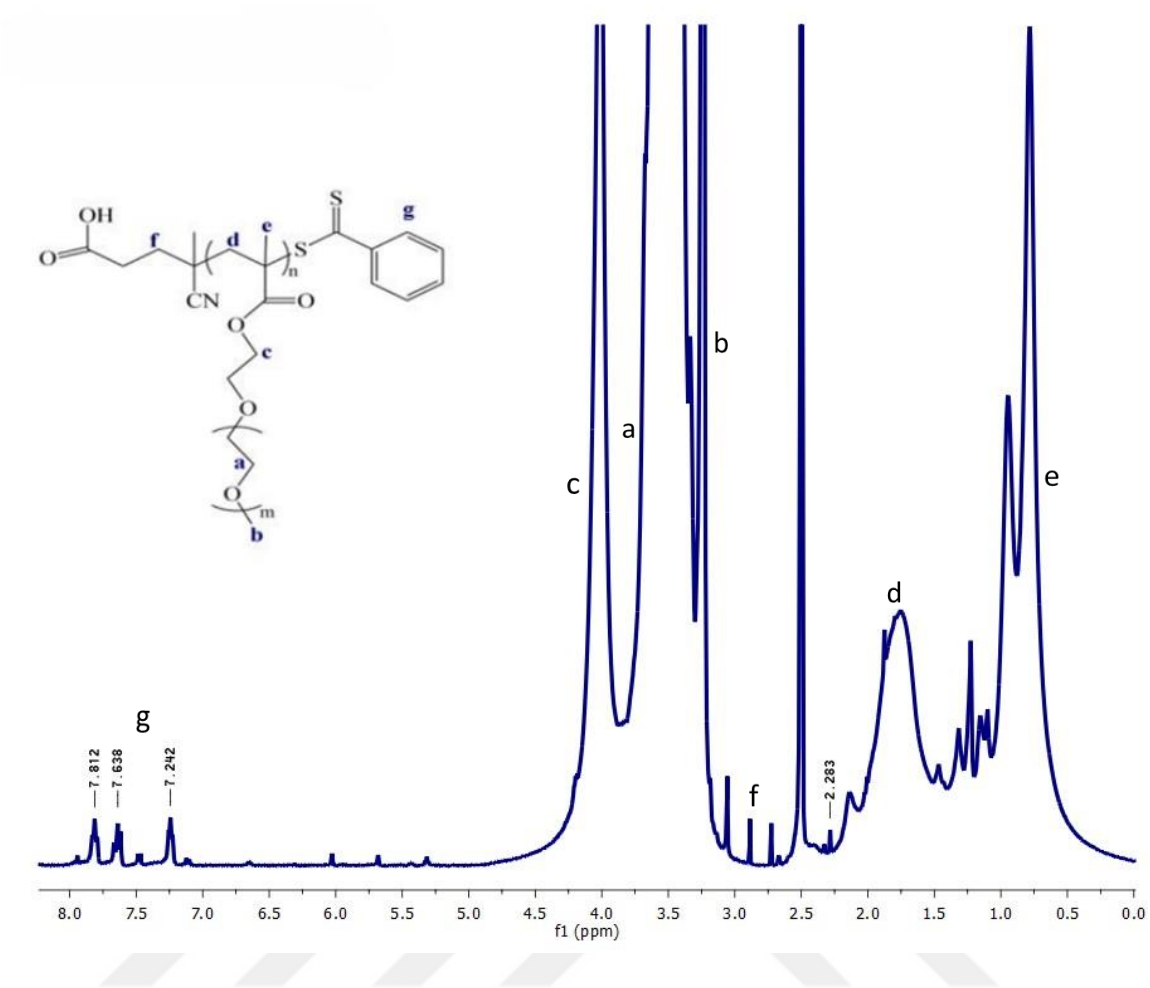


Figure 3.2. ^{1}H NMR spectra of POEGMA

The previous findings demonstrate the success of our study and the consistency of RAFT polymerization with the literature. The PDI value and particular peaks on NMR spectra in our results reveal that POEGMA polymerization is highly controlled which further enhances functionalization.

3.2 Pyridyl Disulfide-Modified POEGMA Preparation and Characterization

Pyridyl disulfide (PDS) was used for altering the -SH groups in the POEGMA polymer in order to generate a stable disulfide bond and reactive pyridyl ring. Since disulfide bonds are known for their reversible characteristics under reducing circumstances, this alteration is essential to improve the polymer's functionality and

stability, particularly for applications demanding disulfide bonds. The reaction yielded pyridine-2-thione leading to a color change to yellow (Figure 3.3.) that was employed as a qualitative indicator to understand the success of modification.

Figure 3.3 The appearance of PDS-modified polymer solution due to pyridine-2-thione formation

In addition, the PDS-modified functionality of RAFT-polymerized POEGMA was evaluated according to research by Boyer et al. who stated that pyridyl ring was observed at 7.1, 7.3-7.4, and 8.4 ppm, indicating that PDS binding to the POEGMA [115]. The chemical shifts of ring protons (7.4–7.5–7.8 ppm) attributed to the RAFT agent were detected to change to 7.1–7.6–8.4 ppm in our study, consistent with the literature. Given the insoluble nature of DTP in water, binding to a water-soluble molecule is necessary to observe the pyridyl group in deuterium oxide. The disappearance of Z-fragment's methylene signals (S-CH_2 and CH_2–CO_2H) at 3.55, and 2.6 ppm and the –CH signal adjacent to thiocarbonylthio at 4.6 ppm, and the appearance of a broad signal at 3.4 ppm belonging to the thiol group confirmed that the reduction and PDS binding were successful.

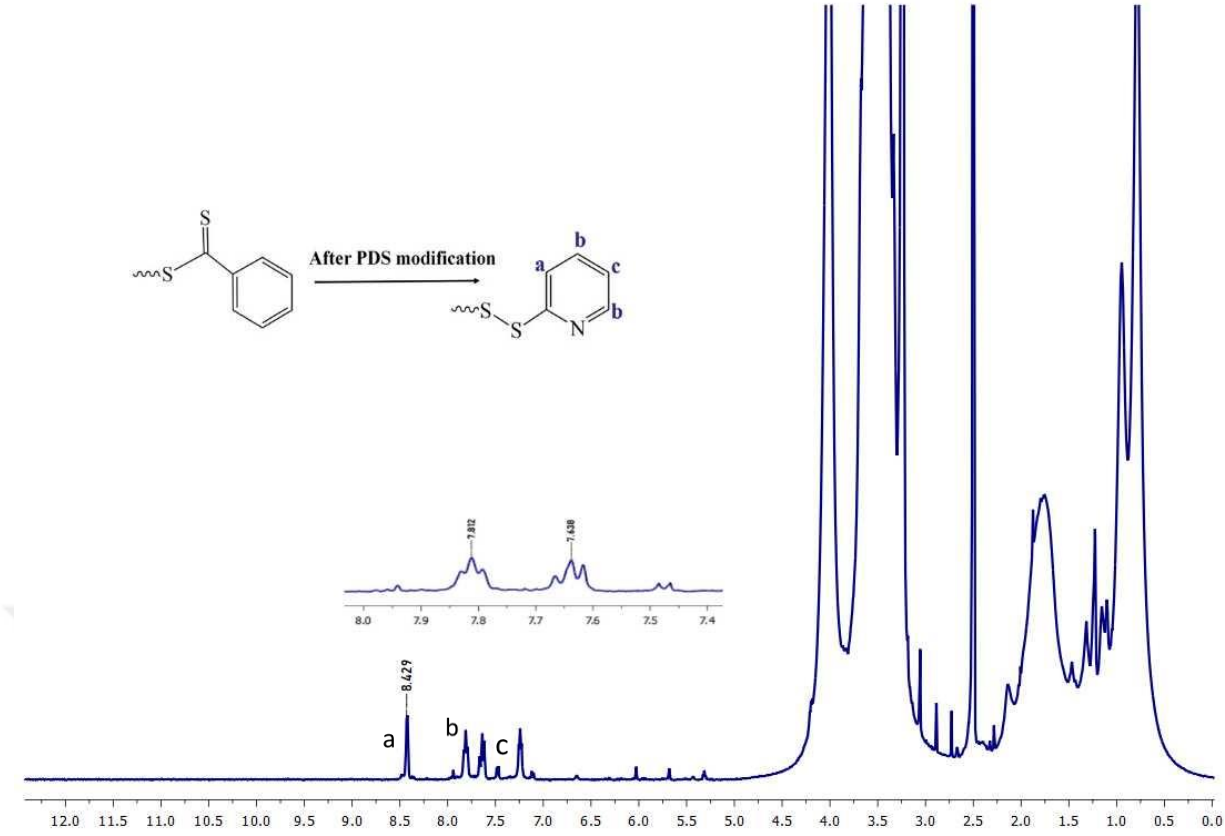


Figure 3.4. ^{1}H NMR spectra of PDS Modification of POEGMA

Moreover, Fourier Transform Infrared Spectroscopy (FTIR) was employed to characterize POEGMA and PDS-modified POEGMA structure as a comparative analysis. Bayram, et al. [91]assigned the band at 1720, 2870, and 1100–1245 cm^{-1} to the groups of C═O, –CH2– (–CH3), and C–O–C, respectively to confirm the structure of POEGMA. In our findings, the bands observed in 1726, 2866, 1096-1246 revealed the success of the synthesis reaction. The newly appeared band at 1674 and 3566 demonstrated the presence of the pyridine which was attained from pyridyl disulfide as predicated in Cox, et al. [97]

Zeta potential values determined after modification as -1.13 ± 0.06 mV indicated no considerable change in the charge of the polymer. The functionality of the PDS end group was also confirmed by reacting it with DL-dithiothreitol (DTT), a strong reducing agent, to yield thiol-terminated POEGMA and pyridine-2-thione which are detectable with a UV-Vis spectrophotometer. This study offered an uncomplicated strategy to end group (bio)functionalization of RAFT-polymerized molecules eliminating the possible stability problems. This modification displays a significant role in the chemical conjugation of thiolated-plasmid DNA with PDS-bound POEGMA, and successful

implementation of the modification is a crucial step to increase the DNA binding and gene transfer efficiency. Furthermore, this procedure has the ability to modify dithio- and trithiocarbonate RAFT end groups and ((meth)acrylamides and (meth)acrylates). Due to the increase in well-defined polymer production via the RAFT process, PDS modification is increasingly preferred over alternative functionalization techniques in biomedical and biotechnology applications.

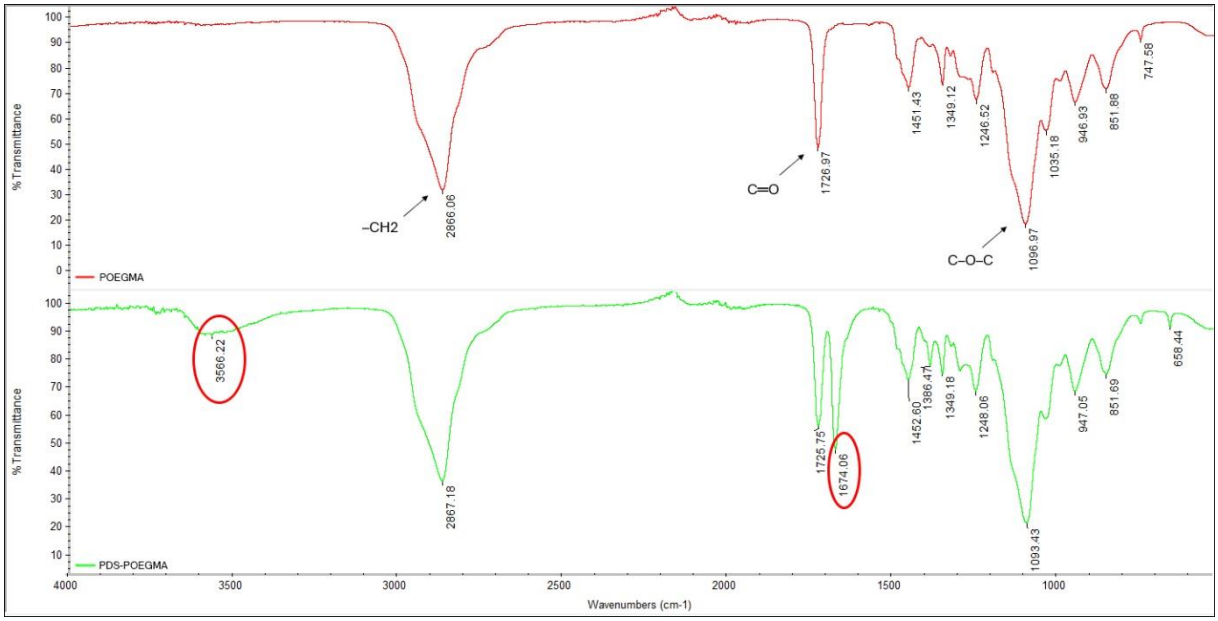


Figure 3.5. Comparative FT-IR analysis of POEGMA and PDS-modified POEGMA

3.3 Cystamine Modification of pDNAs and Free Thiol Detection with 2,2-Dithiodipyridine (DTDP)

Assembly of a sulfhydryl group to DNA or RNA probes enables conjugation processes involving sulfhydryl-reactive heterobifunctional crosslinkers to be performed in a controlled manner. A sulfhydryl group attached to the probe allows to maintain hybridization ability in the final conjugate by directing the coupling process to a specific site on the nucleotide strand. Furthermore, compared to homobifunctional reagents, heterobifunctional crosslinkers of this type facilitate two- or three-step conjugation procedures to be applied, yielding a better end-product of the desired conjugate. In this study, the method represented by Ghosh et al. [98], in which the oligonucleotide,

cystamine, and EDC were all reacted together in an imidazole buffer, was employed to modify plasmid DNAs (dpy10 and pAD67) with cystamine at the 5′ phosphate group using a carbodiimide reaction that was previously illustrated (Figure 2.3). Using disulfide-reducing agents like DTT to reduce the cystamine-labeled pDNAs releases 2-mercaptoethylamine and generates a free thiol group for conjugation. The procedure stated by Grasetti and Murray [99] was carried out to determine the presence of the cystamine group (—SH) linked to pDNAs. Within the scope of the analysis, 2,2-dithiodipyridine forms disulfide bonds reacting with free thiol groups, and the byproducts of this reaction cause the color of the solution to change. This color change indicates the presence of thiol groups. The reaction of DTDP with free thiol groups produced pyridine-2-thione, which was determined by measuring absorbance at 324 nm. As reported in previous studies, coupling —SH to pDNAs through cystamine addition and measuring the absorbance after reacting with 2,2'-dithiodipyridine is a reliable technique [99]. In the study by Hansen, et al. [100]4,4'-dithiodipyridine was used for the sensitive detection of thiols since 4-PDS, like DTNB, enables the thiol-disulfide exchange reaction followed by the formation of the chromogenic compound 4-thiopyridone (4-TP) which shows stoichiometric release. Moreover, Kurz et al.[101] used 4,4’-dithiodipyridine to quantify free thiols of protein samples. The described methods had the advantage of being used for separate consideration of thiol reactivity and the presence of disulfide bonds. The developed method determined thiol oxidation reactions more accurately by 4-PDS compared to the commonly used Ellman’s reagent.

Following the experiment, the attachment of the cystamine molecule to plasmids dpy10 and pAD67 was analyzed. The absorbance values of the DTDP-treated dpy10 and pAD67 were measured. The findings of the absorbance measurement indicated that the concentration of cystamine molecule in the reaction environment was 31,74 and 37,56 respectively (Figure 3.6). These results demonstrate the success of cystamine binding to dpy10 and pAD67.

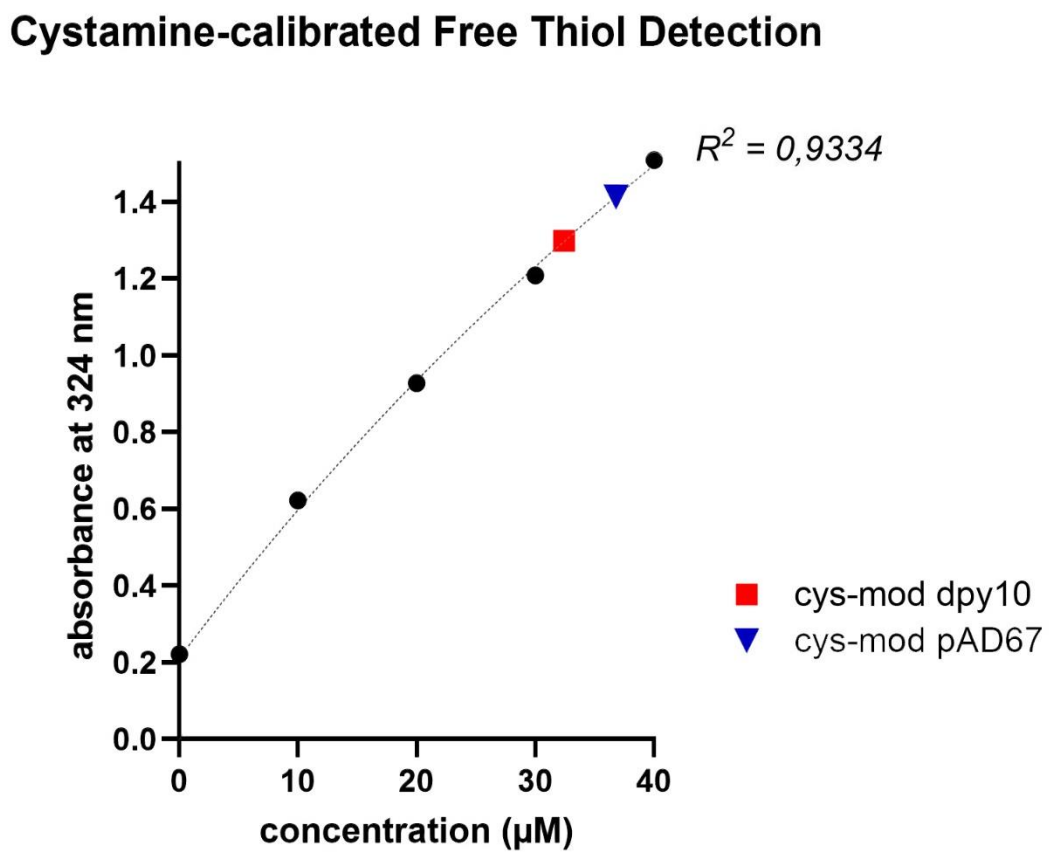


Figure 3.6. The Concentrations of cys-mod dpy10 and pAD67 in Free Thiol Detection Assay

These findings of this study allow for the formation of a covalent linkage between thiolated pDNAs (dpy10 and pAD67) with PDS-treated POEGMA polymer which is an essential step in the construction of our gene delivery system.

3.4 Conjugation of POEGMA and dpy10 and pAD67 Plasmids

The conjugation procedure used cystamine-modified plasmids dpy10 and pAD67 with DTT to form a covalent linkage with POEGMA containing the PDS group. DTT reduces existing disulfide bonds and enables free thiols to produce new disulfide bonds. It was achievable to conjugate POEGMA with modified plasmids successfully

by utilizing the advantage of this feature of DTT. The success of DNA-polymer attachment can be determined by agarose and SDS-PAGE assay through band shifts. In agarose gel, the emergence of a new band at a higher molecular weight indicating successful polymerization [102], [103], [104], or the higher molecular weight band depicting efficient conjugation [105], [106], [107], can be utilized to monitor conjugation. Due to the increase in size or the overall change in charge, a band shift can also be noted on noncovalent attachment. DNA coated in positively charged polymers is retarded or migrates toward the negative electrode. Furthermore, using PAGE, which detects a band shift of decreased migration with increased molecular weight, the formation of more complex nanostructures may also be examined [108]. Following the completion of the reaction, gel electrophoresis assay and zeta potential determination were used to confirm the conjugation process. In the gel electrophoresis results (Figure 3.7), migration of separately loaded dpy10, POEGMA, dpy10-POEGMA conjugate, pAD67 and pAD67-POEGMA conjugate on the gel was examined. The bands on the gel were found to contain fragments that are unique to dpy10 (2507 bp) and pAD67 (7271 bp). In contrast, despite being stained with ethidium bromide, the POEGMA-conjugated pDNAs were visible on the wells but were unable to migrate on the gel despite being stained with ethidium bromide. In previous studies, the synthesis of DNA–polymer conjugates display several limitations since the mixture of these two materials has contrasting properties in one reaction pot such as incompatibility in solvents, charged polymer repulsion, and the steric strain which results in low yields of DNA and polymer conjugation [106], [109]. On the other hand, our gel electrophoresis results indicate that conjugation was achieved with full efficiency for both dpy10 and pAD67 and no free plasmid remained. These results demonstrate consistency with previous studies. The investigation on polymeric DNA delivery by Souza et al. [110] presented that the increased size of the plasmid DNA/polymer complex led to altered migration on 1% agarose gel and even the retention of DNA near the loaded well. Moreover, the research conducted by Forcato, et al. [111] focused on the effect of particle size on transgene delivery. It documented that efficiently complex-forming polymer/DNA systems did not display free pDNA mobility, but complete retardation. The absence of pDNA retardation was associated with poor complexation efficiency.

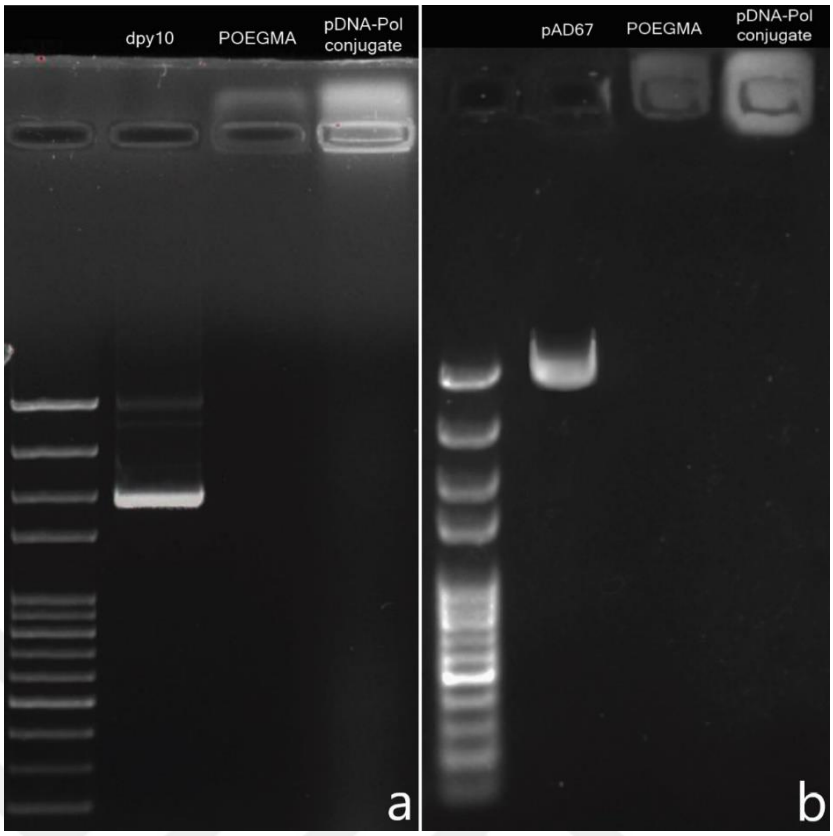


Figure 3.72. Confirmation of Conjugation by Gel Electrophoresis, a) dpy10 pDNA and b) pAD67 pDNA.

The overall physical characteristics of the DNA-polymer product, such as zeta potential can be examined to determine the conjugation interactions. Zeta potential measurements also provided important findings confirming the conjugation process. The zeta potential of the POEGMA-dpy10 conjugate was determined as -3.397 ± 0.0217 mV, whereas the zeta potential of dpy10 alone was -9.85 ± 1.318 mV. Similarly, the POEGMA-pAD67 conjugate's zeta potential was measured at -4.41 ± 0.296 mV, while the zeta potential of pAD67 alone was -14.4 ± 0.871 mV. The existence and efficiency of conjugation were verified by the statistically significant decrease in negative charge for both dpy10 and pAD67 (Figure 3.8). The findings of gel electrophoresis and zeta potential analyzes suggest that POEGMA can effectively conjugate with biomolecules via disulfide bonds and that DTT plays a critical role in this process. In addition, the zeta potential of POEGMA was determined to be -1.39 ± 0.09 mV in our study. This negative charge was diminished upon binding to pDNAs, which resulted in significant changes in the features of the final conjugate. The DNA-polymer complex may demonstrate enhanced solubility as a result of this charge decrease. In polymer-DNA conjugates, aggregation is a common issue, often caused by high charge densities that

result in strong intermolecular interactions. Such interactions can be reduced by POEGMA decreasing the proneness of the conjugate to aggregate. Furthermore, the decrease in overall charge can contribute to POEGMA-pDNAs conjugate stability which provides less possibility to precipitate in solution.

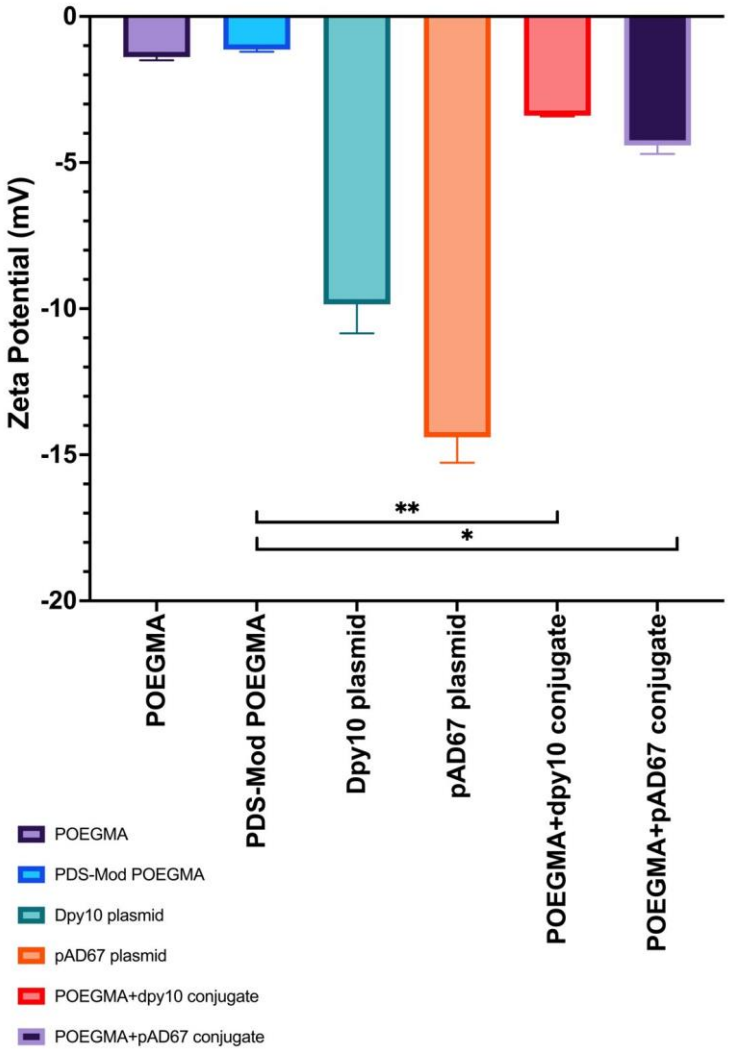


Figure 3.8. Zeta Potential (mV) Determination of POEGMA and pDNAs

Moreover, the disulfide bond formation between POEGMA and plasmid DNAs can be characterized by ^{1}H NMR spectroscopy (Figure 3.9). According to a study by Xu, et al. [112] focused on preparation of glutathione-sensitive nanoparticles, ^{1}H NMR spectrum of synthesized particles showed the characteristic resonance due to –CH bonds adjacent the new disulfide bond at 2.79 ppm. In our study, the peaks appeared in the 2,81 and 2,82 belonging to POEGMA/pAD67 and POEGMA/dpy10 conjugates

confirmed the presence of disulfide linkage. The newly constructed disulfide bond displays a significant role in the carriers' stimuli-responsive characteristics.

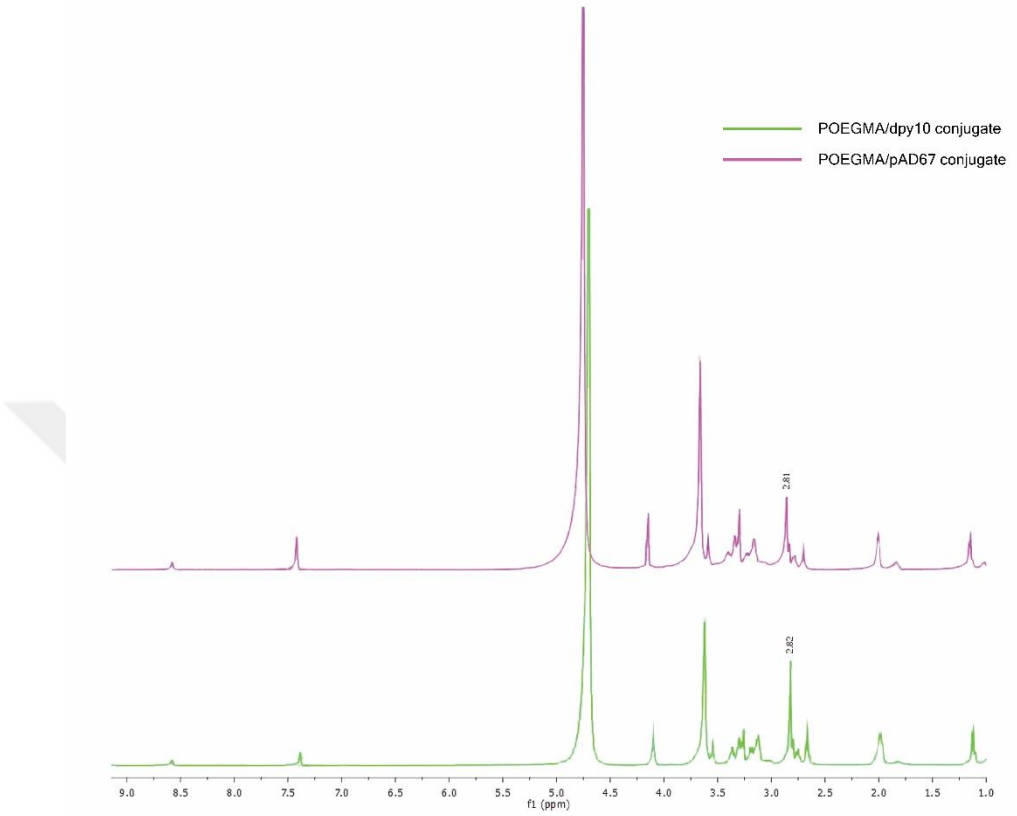


Figure 3.9. ^{1}H NMR spectrum of POEGMA/pDNA Conjugates

3.5 GSH Concentration-Dependent Release of dpy10 and pAD67

The ability of various glutathione (GSH) concentrations to break disulfide bonds and the dissociation rates of POEGMA-pDNAs conjugates resulted in gene release which was visualized by gel electrophoresis. Gel electrophoresis findings were evaluated to examine the effect of GSH on the formed disulfide bonds and its concentration-based effect when each of the dpy10 and pAD67 plasmids were treated with GSH at concentrations of 0.5 mM, 1 mM, 2 mM, 5 mM, and 10 mM, respectively. In the resulting agarose gel image (Figure 3.10), 0.5 mM GSH concentration did not

cause disulfide bond cleavage, and any visible free plasmid was not observed. However, the released pDNAs and breaking of disulfide bonds gradually increased as GSH concentrations rose from 1 mM to 10 mM. The highest concentration of 10 mM GSH caused POEGMA-dpy10 and POEGMA-pAD67 conjugates to be completely depleted in the well where they were loaded. This depletion demonstrates that 10mM is the most effective GSH concentration to disrupt disulfide bonds.

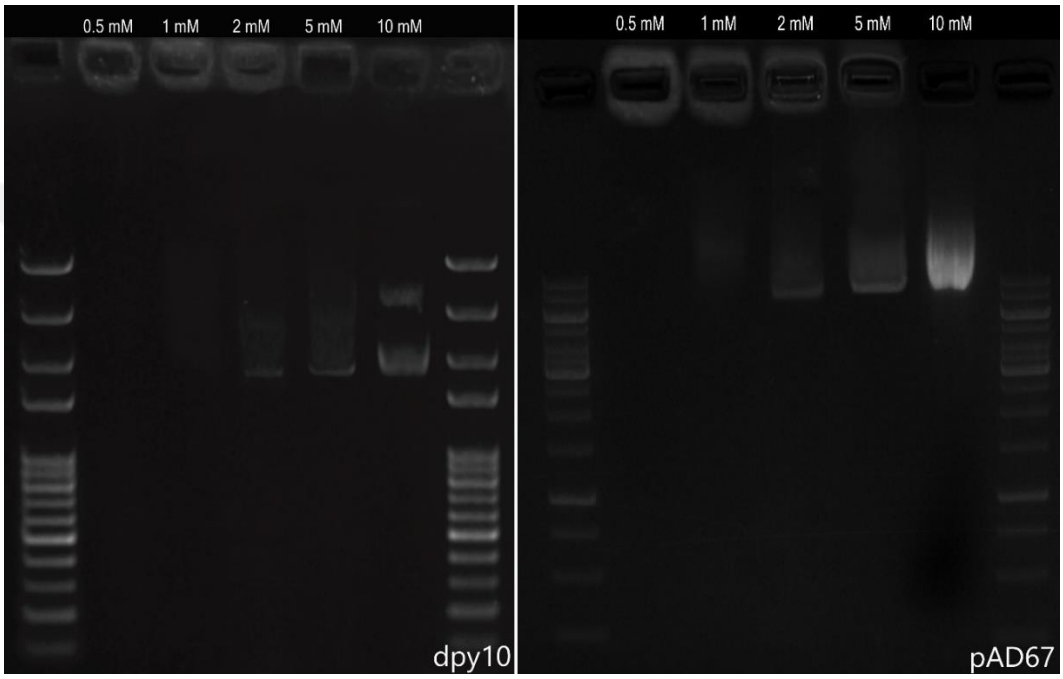


Figure 3.10. GSH-sensitive release of dpy10 and pAD67

Based on the findings of our study, glutathione-sensitive properties of POEGMA/pDNA conjugates display a significant contribution to the literature. It has been demonstrated that the release of pDNA and the dissociation of the disulfide bonds assembled between linear POEGMA and dpy10 and pAD67 take place in a GSH-dose-dependent manner. The ability of POEGMA/pDNA conjugates to be disrupted by glutathione suggests that these complexes may be utilized in biological systems to provide controlled gene release. Given that glutathione is a naturally occurring organic compound in the intracellular environment, such sensitivity can selectively activate gene carrier systems intracellularly and promote gene expression in the targeted cells [113]. The gradual increase of gene expression with increasing GSH concentrations allows for precise control of gene expression levels. This is a great advantage,

particularly in situations where precise expression is required, such as cancer treatment [47]. Controlled and dose-dependent release of therapeutic genes increases treatment success and minimizes potential side effects. The findings of this study achieved by breaking disulfide bonds in a redox-active environment have the potential to improve the efficacy and safety of our gene delivery system in therapeutic applications.

3.6 Cellular Uptake and Internalization of POEGMA-pDNAs Conjugates in MDA-MB-231

Flow cytometry is a technique used to determine the chemical and physical characteristics of particular cells. In this study, the primary objective of flow cytometry analysis is to quantify and qualify the amount of fluorescent material bound to dpy10 and pAD67 taken by MDA-MB-231 cells. The proportion of internalized POEGMA-pDNA conjugates within 6 h was determined in the context of a cellular uptake and internalization assay. Propidium iodide (PI), which exhibits red fluorescent emission, was bound to plasmid DNA to assess plasmid uptake in cells compared to the untreated control group. Untreated control cells were used as a baseline, and the percentage of cells that internalized PI-treated conjugates was determined using flow cytometry. When comparing the cellular uptake of two different conjugates (Figure 3.11-3.13), it was observed that the first conjugate POEGMA-pAD67 achieved an average internalization of 94.26% ± 0.31 after 6 hours, while the second conjugate POEGMA-dpy10 reached an average of 98.61% ± 0.31 (Figure 3.13).

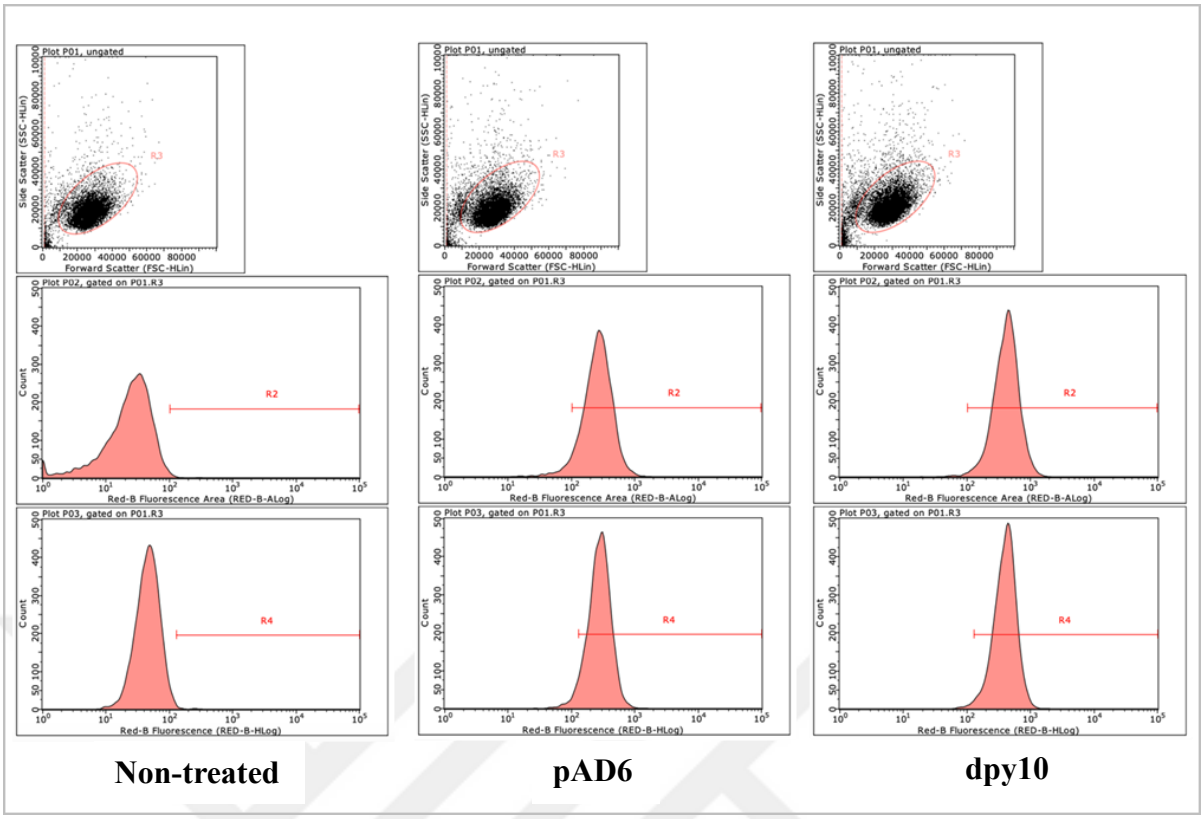


Figure 3.11. Analysis of conjugate uptake into MDA-MB-231 cells within 6 hours using flow cytometry.

The success of gene delivery by polymeric systems relies on their cellular uptake and internalization. As an example, chitosan, which is favored in gene delivery systems due to its positive charge and ability to condense DNA into nano-sized complexes, exhibits low to moderate cellular uptake as a biocompatible and biodegradable polymer. According to research conducted by Zhang et al., chitosan-DNA nanoparticles showed 60-70% uptake in A10 and K562 cells [114] . Despite the great biocompatibility of chitosan, newly established POEGMA-pDNA conjugates appear to have higher rates of cellular uptake. PEI (polyethyleneimine)-utilized delivery systems are noted for high internalization rates and cellular uptake. Boussif, et al. stated that 80–90% of cellular uptake was acquired by using PEI-DNA complexes in 3T3 and HepG2 cell lines [115]. Although known as the gold standard in nonviral delivery, PEI has several drawbacks that affect the effectiveness of therapeutics. The stability of PEI polyplexes has been determined inadequate at high concentrations, in the exposure of salt and serum, and during lyophilization [116], [117], [118]. Excipients can enhance the viscosity resulting in reduced aggregation risk, but may also cause adverse biological impacts or changes in mechanical characteristics of the carrier upon utilization in the field of tissue

engineering hydrogels or scaffolds. The rapid aggregation of PEI-pDNA polyplexes due to plasma protein adsorption has been reported in previous studies [118]. As another example, PDMAEMA (Poly(2-dimethylaminoethyl methacrylate))-based systems are significant cationic polymers to construct gene delivery vectors with high positive charge. However, the cytotoxicity of PDMAEMA leads to limitations in the applications. Yu, et al. grafted PDMAEMAs from hydrophobic hyperbranched PEHO cores (PEHO stands for poly(3-ethyl-3-(hydroxymethyl)-oxetane) yielding hyperbranched multibranched PEHO-g-PDMAEMA with higher cellular uptake rates varying 85-95% [119]. As compared to well-known polymer-based gene delivery systems, POEGMA-pDNA conjugates demonstrated higher cellular uptake rates (94.26 ± 0.31% and 98.61 ± 0.31%) in our findings. POEGMA-pAD67 and POEGMA-dpy10 conjugates had uptake rates comparable to PEI and PDMAEMA-based systems, which exhibit high internalization with toxicity problems. In conclusion, RAFT-polymerized, PDS-modified POEGMA has emerged as a reliable candidate for gene therapy applications.

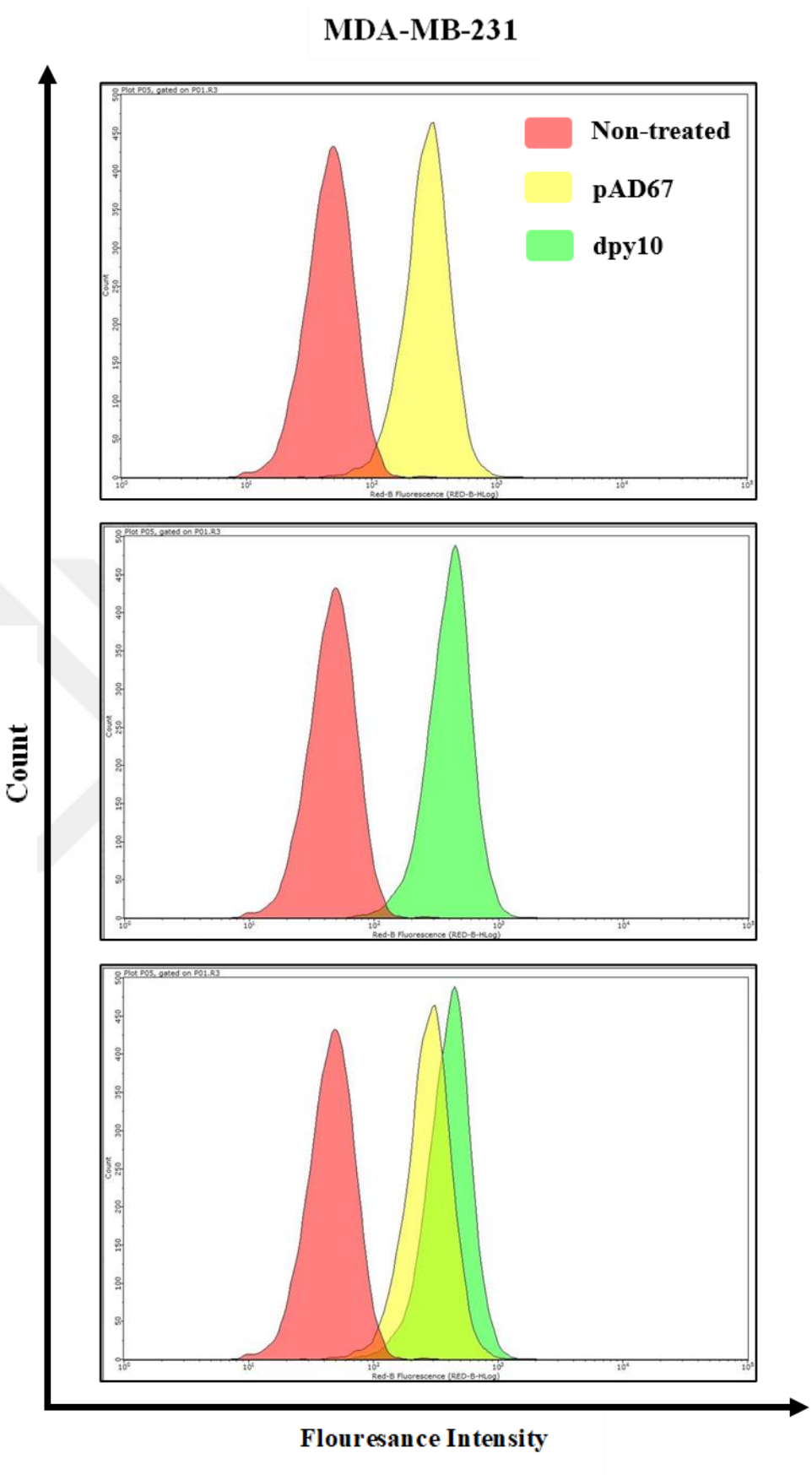


Figure 3.12. Comparative analysis of the uptake of two different plasmids into MDA-MB-231 cells within 6 hours using flow cytometry with red filter.

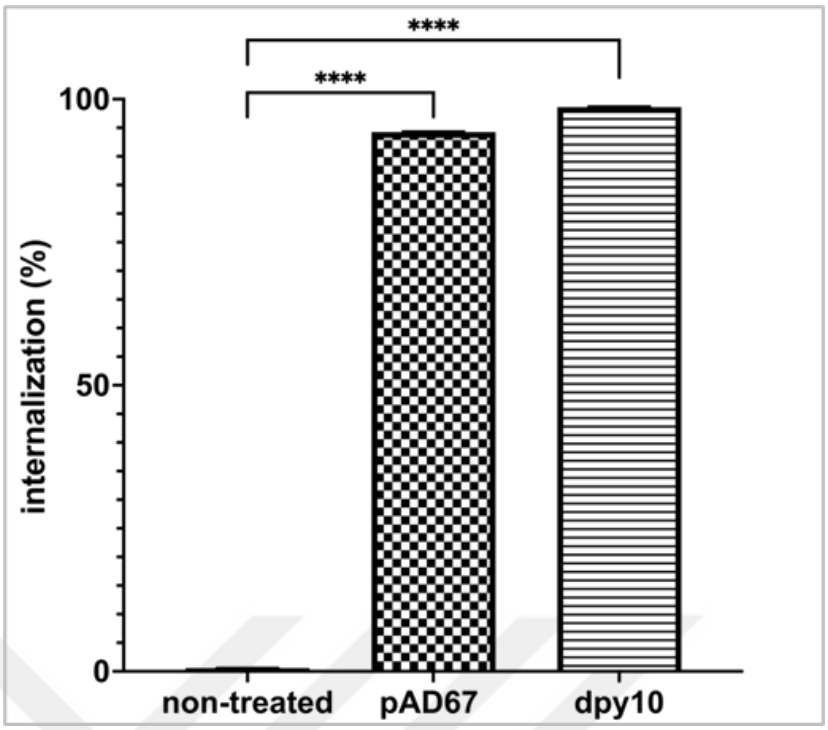


Figure 3.13. Quantitative analysis of the intracellular uptake of two POEGMA-pDNAs within 6 h in MDA-MB-231 cells. Bar graphs are presented as the mean of different experiments, expressed with ± standard deviation (SD). The P-value is indicated as ***p < 0.0001.

Chapter 4

Conclusions And Future Prospects

4.1 Conclusions

In this research, we developed and assessed a novel gene delivery system by Pyridyl Disulfide (PDS)-modified poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA). Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerization was used in the synthesis to precisely control the polymer chain length and characteristics, which are critical for its function in gene delivery. Characterization of the synthesis and functionalization strategies demonstrated the effective development of a gene delivery vector that can conjugate with cystamine-modified plasmid DNAs (pDNAs) to achieve glutathione-sensitive gene transfer.

The desired properties and polymer structure were accomplished by utilizing the RAFT polymerization technique in POEGMA synthesis. The chemical shifts on the 1H NMR spectra of POEGMA corresponded in the previous publications and confirmed the success of synthesis. GPC analysis revealed a polydispersity index (PDI) value of 1.076 and an average molecular weight (Mw) of 30,400 Da, implying a homogenous molecular weight distribution. Pyridyl disulfide (PDS) modification was employed for the functionalization of POEGMA by polymer chain integration of disulfide bonds. Zeta potential measurements and ^{1}H NMR spectroscopy confirmed the new bond formation with a slight negative charge. Thiolation of plasmid DNAs, dpy10, and pAD67, with cystamine modification enabled the formation of POEGMA-pDNAs conjugates via disulfide linkage, and the presence of free thiol groups on pDNAs was determined by DTDP assay. The efficiency of conjugation was presented by agarose gel electrophoresis and zeta potential values. The decreases in the zeta potential results were correlated to the negative charge reduction of dpy10 and pAD67 after binding to POEGMA possessing a slight negative charge. The formation of a disulfide bond between POEGMA-dpy10 and POEGMA-pAD67 displays glutathione-responsive

characteristics providing controlled gene release and transfection efficiency, thus enhancing the potential of the gene delivery system. Finally, cellular uptake studies indicated a high rate of internalization of POEGMA-dpy10 (98.61 ± 0.31%) and POEGMA-pAD67 (94.26 ± 0.31%) in MDA-MB-231 cells validating the more desirable findings compared to well-known polymer-based gene delivery systems.

4.2 Societal Impact and Contribution to Global

By assembling a novel gene delivery system, improving the redox-sensitive properties, and providing versatility and adaptability, this research exhibits substantial contributions to the field of polymer science, biomedical engineering, and gene therapy. Utilizing RAFT-polymerized POEGMA functionalized by PDS with the aim of cystamine-modified thiolated pDNA delivery offers a novel approach in gene delivery systems. Precise control over polymer structure resulting in well-defined conjugates provides the feasibility of the POEGMA carrier in biomedical applications. This new system sensitive to glutathione concentrations in biological systems enables both safe and efficient transfection, as it provides stability in circulation during delivery and promotes gene release in cells where a high level of glutathione is present. The employment of this newly established gene delivery system is suitable for several applications, such as the treatment of inherited disorders and cancer due to straightforward synthesis and modification processes providing easy adaptation and customization. Given the efficacy of PDS-modified POEGMA/cystamine-modified pDNA conjugates in preliminary observations, this study offers reliable findings for further investigations which should focus on structure optimization, disease-specific application, and performance assessment in more complex biological systems.

Several Sustainable Development Goals (SDGs) of the United Nations Development Programme (UNDP), particularly about health, innovation, and sustainability, are closely aligned with the development of the PDS-modified POEGMA gene delivery system in this study First, this study contributes to Goal 3: Good Health and Well-Being by offering a more effective and safe gene delivery strategy. This development might lead to more effective treatments with fewer side effects for cancer and genetic disorders, which is critical for improving global health outcomes. Second, the study supports Goal 9: Industry, Innovation, and Infrastructure by accurately controlling polymer properties using RAFT polymerization. By reducing waste and

improving resource usage, this material production strategy encourages sustainable industrial practices—a crucial step toward innovative biomedical developments. Furthermore, by reducing the requirement for repeated drug doses, this study addresses Goal 12: Responsible Consumption and Production. A more effective gene delivery technique could decrease the use of pharmaceuticals. Also, the use of environmentally friendly and sustainable materials in medical applications is highlighted by biocompatible materials like POEGMA. Finally, this gene delivery system's versatility and ease of customization ensure that the technology could be extensively used to treat a range of health concerns promoting effective resource usage. This also contributes to Goal 10: Reduced Inequality by increasing the affordability and accessibility of advanced therapies, particularly in regions with low incomes.

4.3 Future Prospects

This study presents that controlled molecular structure and functionalization strategies have the ability to display a significant role in the development of efficient polymer-based vectors. As highlighted by the key findings, the POEGMA-pDNAs system has great potential for more comprehensive applications, particularly gene-therapy-utilized disease treatments. Moreover, there are a few aspects requiring improvements and explorations such as the enhancement of synthesis yield, the safety and potential side effects in vivo, and the feasibility of combination therapies. As considered, all the findings, this study expands our knowledge of non-viral, polymeric gene delivery systems and contributes to the design of effective and precise treatment strategies.

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