Engineering Gene Vectors: Synthesis of Thioether-Lipid Polyethyleneimine Conjugates for pDNA and siRNA Delivery

by

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Abstract

Gene therapy offers a promising approach for treatment of a diverse array of diseases at their genetic root causes. The cationic polyethyleneimine (PEI) has been extensively used for successful non-viral nucleic acid delivery in experimental systems, but its clinical utility has been hampered by its severe cytotoxicity. Thus, hydrophobic modification of low molecular weight (LMW) PEIs has been used to enhance transfection efficiency along with acceptable toxicity. This study conjugated PEI1.2k with aliphatic lipids lauric, palmitic and stearic acid via thioether linkages and probed their *in-vitro* performance for nucleic acid delivery in suspension cells. The ^{1}H-NMR and FTIR confirmed the desired structural characteristics of the synthesized lipid-substituted polymers. Particle sizes of the pDNA polyplexes varied from 140$\pm$1 to 330$\pm$10 nm, with positive ζ-potentials (+4 to +12 mV). The modified LMW polyplexes demonstrated negligible toxicity (cell viabilities ~97%) at a polymer-to-pDNA mass ratio of 10. The extent of stearic and lauric acid substitution improved the *in vitro* transfection, outperforming 25 kDa branched-PEI and matching the efficacy of the commercial transfection agent Lipofectamine™ 2000, with over 80% pDNA-positive cells. Additionally, the inclusion of the *Trans-Booster* additive in the polyplexes further enhanced the transfection efficiency. Likewise, the potential for delivering other anionic biomacromolecules, such as small interfering RNA (siRNA) for gene silencing, was explored. Lauric acid-grafted polyplexes with therapeutic siBCR-ABL achieved approximately 58% maximum cell growth inhibition in drug-resistant chronic myeloid leukemia (CML) cells and maintained this effect for 9 days, which is comparable to or exceeds the duration reported in previous studies. These findings underscore the versatility of lipid-PEI conjugates via thioether linkage as delivery vectors for pDNA and siRNA in suspension-growing CML K562 and Jurkat T-cells, a strategy not previously reported in the literature and thus, merit further investigation.

Preface

Chapter 1 provides an extensive literature review focusing on the current advancements and challenges in gene therapy, particularly those hindering its clinical implementation. It emphasizes non-viral vectors, with a special focus on cationic polymers and the mechanism of action of polyethyleneimine (PEI). The chapter explores fundamental aspects of cellular delivery, covering uptake mechanisms and endosomal escape strategies. It also discusses potential approaches to enhance these crucial stages of gene delivery. Specifically, attention is drawn to non-cytotoxic, low molecular weight PEIs (1.2 kDa), highlighting opportunities for optimizing their transfection efficiency.

Chapter 2 details the design, synthesis, and characterization of lipid-modified PEI1.2k with thioether linkages (Lipid-S-PEI1.2k) specifically tailored for plasmid DNA (pDNA) delivery. This chapter, which has been submitted for journal publication under the title "Engineering Gene Vectors: Synthesis of Thioether-Lipid Polyethyleneimine Conjugates for pDNA and siRNA Delivery," encompasses experiments, document writing, and illustration creation performed by myself, with review by H. Uludag. The ^{1}H-NMR spectra were obtained by Dr. Vishwa Somayaji, Nuclear Magnetic Resonance Operator at the Pharmaceutical Analysis Laboratory (PAL). Additionally, FTIR spectra were acquired with technical assistance from Dr. Meng Wu at Dr. Hong Bo's laboratory, University of Alberta. Analysis and interpretation of these spectra were performed by me.

Chapter 3 explores the application of the synthesized Lipid-S-PEI1.2k polymers in delivering small interfering RNA (siRNA) for gene silencing, targeting the BCR-ABL(+) oncogene in chronic myeloid leukemic K562 cells. This application is also part of the journal submission titled "Engineering Gene Vectors: Synthesis of Thioether-Lipid Polyethyleneimine Conjugates for pDNA and siRNA Delivery." The experimental work, document writing, and illustrations were carried out by myself, with review by H. Uludag.

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Table of contents

List of Tables

List of Figures

Scope

The primary objective of this thesis is to advance gene therapy for targeted modifications and treatment of genetic disorders by optimizing non-viral delivery systems, particularly focusing on cationic polyethyleneimine (PEI) as an alternative to viral vectors. While high molecular weight (HMW) PEI and its derivatives are widely utilized in basic research and as benchmarks in polymer-based gene delivery, their clinical applications are limited. This thesis addresses this gap by enhancing the molecular properties of low molecular weight (LMW) PEI to improve transfection efficiency while maintaining safety standards.

Chapter 1 provides a comprehensive literature review essential for understanding gene delivery applications. It covers fundamental aspects of cellular delivery, including uptake mechanisms and barriers that hinder the clinical use of non-viral vectors. The chapter concludes with a forward-looking perspective, addressing current challenges and proposing solutions utilizing LMW PEIs in gene delivery.

Chapters 2 and 3 detail experimental investigations involving the design, synthesis, and characterization of lipid-modified PEI1.2k with thioether linkages (Lipid-S-PEI1.2k). Chapter 2 focuses on formulating polyplexes with plasmid DNA (pDNA), examining their physicochemical properties and assessing the impact of incorporating the polyanionic additive *Trans-Booster*. The role of hydrophobic substituents and thioether linker in enhancing functional materials for gene delivery is investigated. Chapter 3 explores the application of Lipid-S-PEI1.2k polymers for delivering small interfering RNA (siRNA) targeting the BCR-ABL(+) oncogene in K562 cells, evaluating their efficacy in long-term gene silencing.

Chapter 4 consolidates the thesis findings from Chapters 2 and 3. It provides conclusions drawn from the experimental results and discusses future perspectives for applying the synthesized lipopolymers. Recommendations for further studies aimed at enhancing gene delivery efficiency based on the insights gained in this thesis are also proposed.

Chapter 1. Polymeric Vectors for Gene Therapy

1. Introduction

The human genome provides a vast reservoir of information critical for therapeutic applications, as genetic factors often underlie many human diseases. The advent of next-generation personalized medicines based on gene therapy is driven by the need to effectively treat various inherited and acquired disorders [1]. The therapeutic potential of gene therapy is contingent on its ability to transfer specific nucleic acids directly into affected cells. This process enables the substitution of damaged genes and modulation of gene expression patterns [2], thus, averting the onset or progression of various diseases [3]. It entails precisely delivering exogenous genetic material—like DNA or mRNA for gene replacement, siRNA or miRNA for gene silencing, and CRISPR-Cas9 for gene editing [1,4,5,6].

A significant barrier in transitioning therapeutic nucleic acids to clinical use lies in their physical properties, which hinder cellular uptake and biodistribution within the body [7]. Factors such as large molecular mass, low hydrophobicity, and the anionic charge of their phosphate backbone impede interaction with cell membranes composed of anionic phospholipids, complicating effective delivery [8]. Additionally, the prolonged circulation time of these molecules poses a degradation risk due to endogenous enzymes [6].

To enable effective gene therapy, it is essential to develop carriers capable of targeted delivery of genetic material to cells, ensuring safety and efficacy. These carriers must perform essential yet conflicting functions; protect and maintain stability while outside the target cell but degrade or disassemble after cell internalization to release the nucleic acid [2,9,10]. This dual requirement presents significant challenges due to the intricate engineering required for precise delivery systems, impacting both efficacy and cost-effectiveness of treatments.

This study hypothesizes that engineered delivery vehicles can enhance *in vitro* delivery, thereby optimizing the therapeutic potential of nucleic acids [5,7]. Despite varying designs of carriers, the primary objective remains consistent: overcoming the inherent limitations of naked nucleic acids [8]. Efficient intracellular trafficking and successful release from carriers are crucial for maximizing nucleic acid functionality in gene therapy, particularly at target sites.

This section provides relevant background information for the current work. The succeeding sections focus on polyethyleneimine (PEI)-based gene delivery vectors, detailing their properties and mechanisms of action. It covers fundamental aspects of cellular delivery, including uptake mechanisms and endosomal escape, and explores potential strategies for enhancing the effectiveness of PEI-based delivery vectors.

1.1 Non-Viral vs Viral-based Carriers

Advanced materials are essential for the therapeutic delivery of nucleic acid molecules using both viral and non-viral vectors to transfer genetic material into target cells. An ideal vector should have low antigenic potential, high capacity, and high transfection efficiency, while also allowing controlled and targeted transgene expression [11]. Additionally, it should be reasonably priced and safe for both the patient and the environment. The selection of gene delivery vehicles depends on the specific therapeutic goal. However, a perfect vector that meets all therapeutic and safety requirements does not yet exist, indicating the need for further research in this field.

Among various gene carriers, viral-based delivery systems remain the predominant option due to their natural ability to insert genetic material into host cells, offering high transfection efficiency and biocompatibility [4,26,12]. Viral vectors, such as retroviruses, adenovirus, adeno-associated viruses (AAVs), herpes simplex virus (HSV), and lentiviruses, have been modified to eliminate their toxicity while maintaining high delivery efficacy [1,6,11]. Despite significant clinical advances in gene delivery technology using viral vectors, they have several inherent shortcomings [1,2]. These include complex production processes, broad tropism, tumorigenicity, and limitations on the amount of genomic information they can carry (vector capacity) [1,7]. Additionally, concerns about immunogenicity and the potential for integrating with the host genome, causing permanent alterations, have driven researchers to explore safer alternative delivery agents [11, 34].

Developments in material science, synthetic chemistry, and nanobiotechnology have enabled non-viral vectors to potentially resolve many issues associated with viral vectors [5,7]. These vectors aim to mimic viral-like delivery by employing biomolecules to condense, protect, and deliver genetic material [5,8], especially emphasizing improvements in safety. Although non-viral vectors are generally less efficient than viral ones, they are simpler to prepare, offer manufacturing advantages, especially at the scale-up step and have a high gene encapsulation capability [10]. To become clinically relevant, non-viral vectors must better withstand the challenges of a physiological environment. Research and development in this area have focused on altering the vector's material composition or formulation to improve *in vitro* gene transfer efficiency and prevent premature degradation during *in vivo* circulation [5].

Non-viral delivery systems are classified into physical and chemical approaches. Physical methods such as electroporation, microinjection, sonoporation, and gene gun enable direct delivery of genetic or non-genetic material into target cells [8,13]. However, certain physical methods, such as electroporation, may induce significant cell death [15], restricting their suitability for *in vivo* applications [36,43]. Therefore, this study will primarily review chemical vectors, with a particular focus on polymer-based techniques. Other chemical vectors include inorganic materials and lipid-based systems [2,6,7,13,34]. **Figure 1.1** shows the frequently employed viral and non-viral vectors, emphasizing significant differences between these two categories [7,11,49].

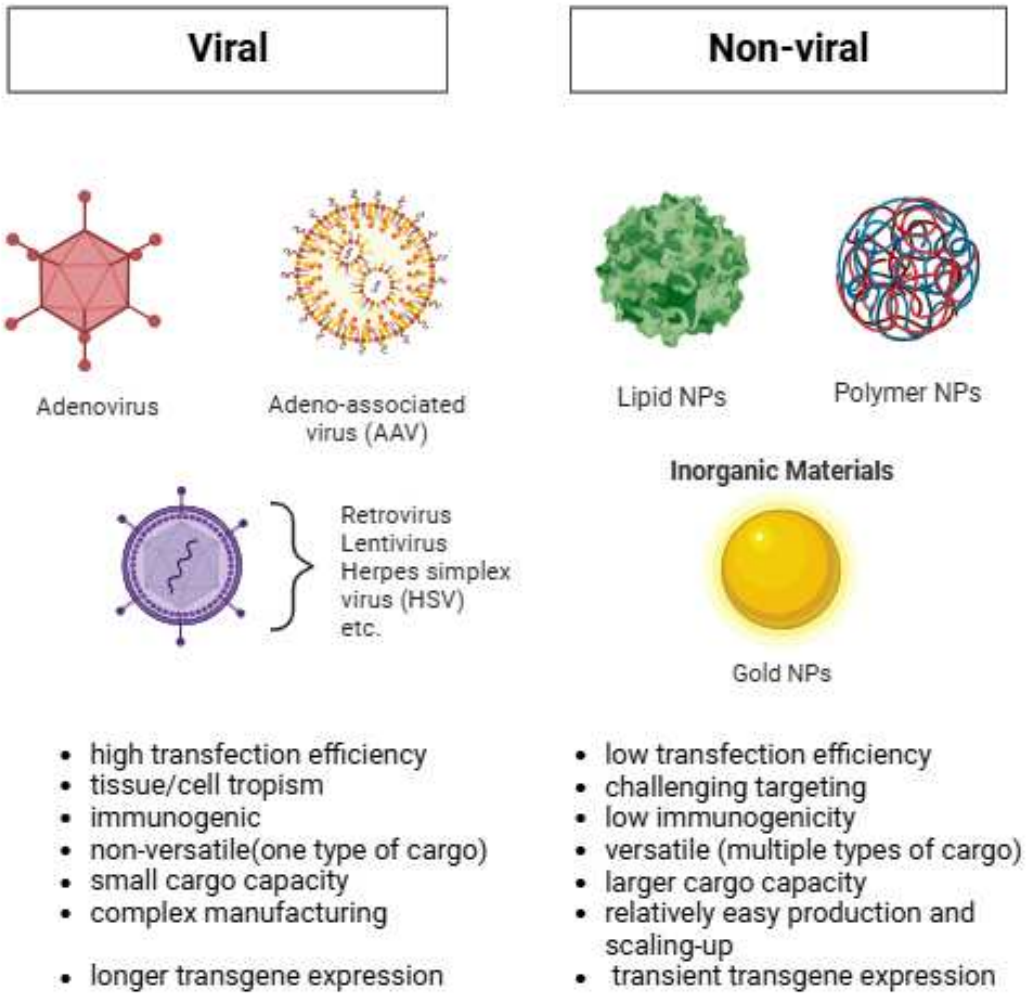

Figure 1.1 Representation of commonly used viral and non-viral vectors, highlighting key differences between the two types. NPs:Nanoparticles **(adapted from [7,49]).**

1.2 Cationic polymers and nucleic acid complexes (Polyplexes)

Cationic polymers designed for nucleic acid delivery must meet several essential criteria to ensure effective targeting of cells and sustained expression of genetic material. These criteria are influenced by factors such as the specific types of target cells, the required duration of gene expression, and the properties of the nucleic acids being delivered, including their size, molecular weight, and susceptibility to degradation. The versatility of polymer chemistry and their physical characteristics are crucial in enhancing delivery strategies. Polymers can be precisely engineered to control the release of cargo, facilitate transfection processes, and influence biodistribution, thereby providing a range of options to optimize delivery effectiveness [8]. These polymers show great promise as non-viral carriers for nucleic acids due to their ability to achieve high cellular uptake, excellent water solubility, and ease of synthesis. Customization with various chemical structures and functional groups further enhances their ability to enhance transfection efficiency and overall performance [23]. Their adaptability allows for precise tailoring based on specific physicochemical properties such as molecular weight, charge density, solubility, and hydrophobicity. Size and zeta potential are critical factors that influence their delivery effectiveness [18]. Cationic polymers have gained popularity as vehicles for nucleic acid transfer due to their simple formulation as gene carriers. Nucleic acids exhibit a strong attraction to cationic polymers primarily due to electrostatic interactions. Specifically, the negatively charged phosphates within the nucleic acid backbone interact with the positively charged amino groups of the polymer backbone or side chains, facilitating the spontaneous formation of polyplexes, which are polymer-nucleic acid complexes[10,11,23,34]. For gene delivery purposes, an excess of polymer is typically used (N/P ratio > 1) to condense nucleic acid cargo, generating vehicles with a positive surface charge [2,18]. This effective complexation, especially condensing large nucleic acids, creates nanoscale structures that facilitate cellular uptake and gene expression. Additionally, this self-assembly process neutralizes anionic charges, aiding absorption onto cell membranes and subsequent internalization, thus shielding the nucleic acids from enzymatic degradation [19,51]. Furthermore, cationic polymers can be modified to enhance cellular delivery. Adding small hydrophobic or bulky lipid groups can improve delivery due to the lipophilic nature of cell membranes. While cationic groups are primarily responsible for nucleic acid condensation, they can also be functionalized by covalently binding targeting ligands, drugs, or pH-sensitive groups to improve delivery efficacy [19,23]. Both naturally derived and synthetic cationic polymers offer flexibility in incorporating desired chemical functionalities and manipulating structural properties to meet various requirements in nucleic acid delivery applications [2,10,19]. Synthetic polymers have an advantage in their ability to precisely incorporate versatile chemistries, allowing for a broader range of formulation options. Examples include natural polymers such as chitosan, and synthetic ones like polyethylenimine (PEI), poly L-lysine (PLL), poly(amidoamine) (PAMAM) dendrimers and polydimethylaminoethyl methacrylate (pDMAEMA) [1,2,13,23]. **Figure 1.2** illustrates various cationic molecules utilized as non-viral carriers forming polyplexes with nucleic acids, enhancing cellular uptake and trafficking [23].

Despite these advantages, cationic polymer-mediated gene delivery remains less efficient than viral vectors [10]. Cationic charges play dual roles in non-viral gene delivery: essential for condensing genetic materials into nanoparticles yet potentially hindering polyplex dissociation, thus impacting gene expression efficiency. The following section discusses major challenges faced by cationic polymeric vehicles for gene delivery and explores strategies to overcome them.

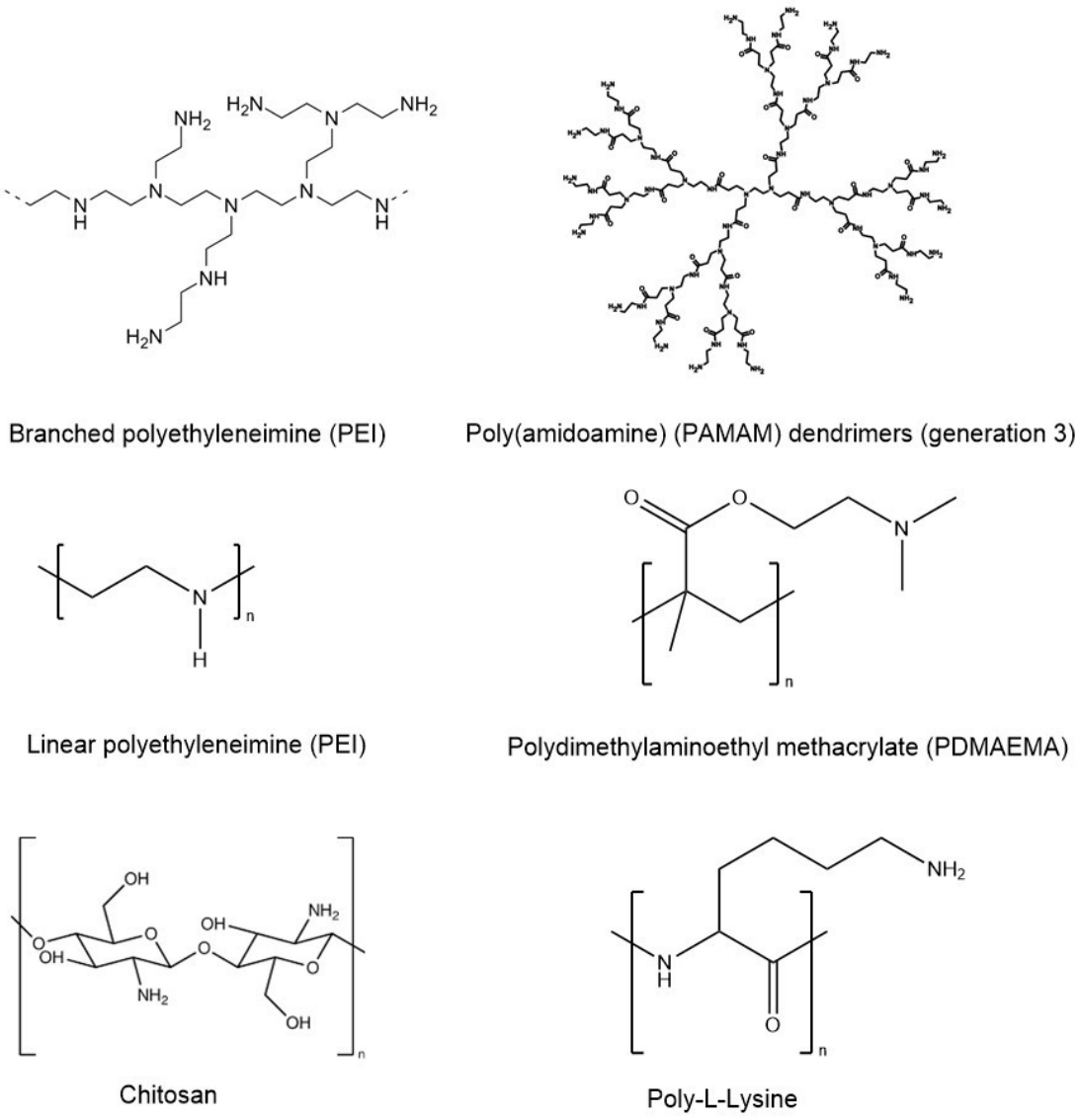

Fig. 1.2. Structure of cationic polymers employed as carriers of nucleic acids.

1.3 Challenges in cellular delivery and internalization of polyplexes

Polyplexes, used for gene delivery, encounter a complex array of extracellular and intracellular barriers that pose significant challenges for their clinical translation [18]. When administered intravenously, these vectors can interact with blood components such as serum proteins, leading to the formation of a "protein corona" that alters their surface properties and impedes their effectiveness in interacting with target cells and tissues [12]. Furthermore, larger carriers often undergo rapid accumulation in the liver due to phagocytosis by Kupffer cells, which limits their circulation time and reduces their availability for targeting other tissues. Achieving prolonged circulation and efficient targeting of specific tissues depends critically on the size and surface properties of polyplexes [18].

Once within target organs, they face additional intracellular barriers that hinder efficient transfection. These barriers include crossing the cellular membrane, escaping from endosomes if trapped, and effectively releasing genetic material into the cytoplasm or nucleus [10]. The interaction with the plasma membrane is crucial for overcoming thermodynamic barriers to membrane poration, influenced by membrane lipid composition and dynamics, which affect internalization efficiency and subsequent silencing efficacy across different cell types[8].Successful gene delivery systems must navigate challenges such as enzyme degradation, interactions with serum proteins, electrostatic repulsion, innate immune responses, endosomal entrapment, and precise mechanisms for cargo release [1, 6]. To address these hurdles, polyplexes must exhibit characteristics like efficient nucleic acid condensation, stability in serum, targeting specificity, effective cytoplasmic transport, and nuclear localization [1,6,21]. Overcoming these barriers is crucial for advancing gene therapy into a clinically viable option, requiring the development of systems that ensure optimal gene packaging, extended circulation, systemic delivery, cellular uptake, endosomal escape, intracellular release, and nuclear entry [21].

Gene therapy, first conceptualized nearly 50 years ago, aims to normalize gene expression using the cell's machinery to regulate protein production, offering a versatile approach for treating various conditions through modulation of gene expression [6, 52]. Up-regulation involves introducing molecules such as DNA and synthetic messenger RNA (mRNA) in forms like plasmids, minicircles, or minivectors [8, 47, 48]. This process requires protection against cellular defenses, efficient escape from endosomes, and unpacking to enable transcription within the nucleus via the nuclear pore complex. Conversely, down-regulation primarily occurs post-transcriptionally in the cytoplasm using molecules such as small interfering RNA (siRNA), antisense oligonucleotides (ASOs), short hairpin RNA (shRNA), and microRNA (miRNA) [12,52]. These RNA molecules inhibit translation into proteins by base pairing with target messenger RNA (mRNA) with the aid of the RNA-induced silencing complex (RISC). While siRNA targets specific sequences with full complementarity, miRNA exhibits partial complementarity and can target multiple mRNAs. Because translation predominantly occurs in the cytoplasm, RNA delivery technologies do not necessarily require nuclear entry, necessitating different carrier properties [10,12].

It is essential to note that gene therapeutics tailored for one type of nucleic acid, such as siRNA or mRNA, cannot be directly applied to another type like DNA due to differences in physical properties and the need for delivery to distinct intracellular sites of action (e.g., cytosol for RNA versus nucleus for DNA) [5,10]. Plasmid DNA (pDNA) is a well-studied form of DNA, characterized by its circular double-stranded structure capable of autonomous replication within a cellular host [6,52]. These molecules typically range in size from 1 to 200 kilobase pairs (kbp) and possess an anionic phosphodiester backbone and stable deoxyribose structure. Due to their size and structure, pDNAs engage in strong electrostatic interactions and efficiently condense when complexed with cationic carriers [6]. Successful transfection of pDNA depends on its delivery to the nucleus of host cells, where it utilizes cellular machinery for transcription and translation to produce proteins. Once inside the nucleus, pDNA can exert long-lasting effects, particularly if its components integrate into the host genome [18].

Synthetic, small (20-30 bp), double-stranded siRNAs are utilized to inhibit mRNA translation from targeted genes by directing RNA-induced silencing complex (RISC) activity, thereby activating the RNA interference (RNAi) mechanism for effective silencing or downregulation of specific protein expression [5,52]. RNAi is extensively studied for its ability to selectively knock down disease-causing genes post-transcriptionally. These engineered exogenous nucleic acids can be introduced into cells to disrupt disease-related gene expression and regulate the production of targeted proteins [6,10]. Upon delivery into the cytosol, one of the two 20–30 base-long RNA strands in the siRNA duplex is degraded [2]. The remaining strand, known as the guide strand, is selectively retained in the multiprotein RNA-induced silencing complex (RISC). The siRNA guide strand directs the siRISC to the target mRNA through complementary base pairing, resulting in the knockdown of the target RNA. Importantly, unlike expression-based systems, siRNA-mediated gene modulation by RISC circumvents the need for nuclear translocation, overcoming a significant limitation in alternative gene regulation approaches [5]. Despite differences, many challenges and carrier requirements remain similar for both DNA and siRNA delivery [21]. **Figure 1.3** provides a schematic representation of some of the intracellular barriers [19,23].

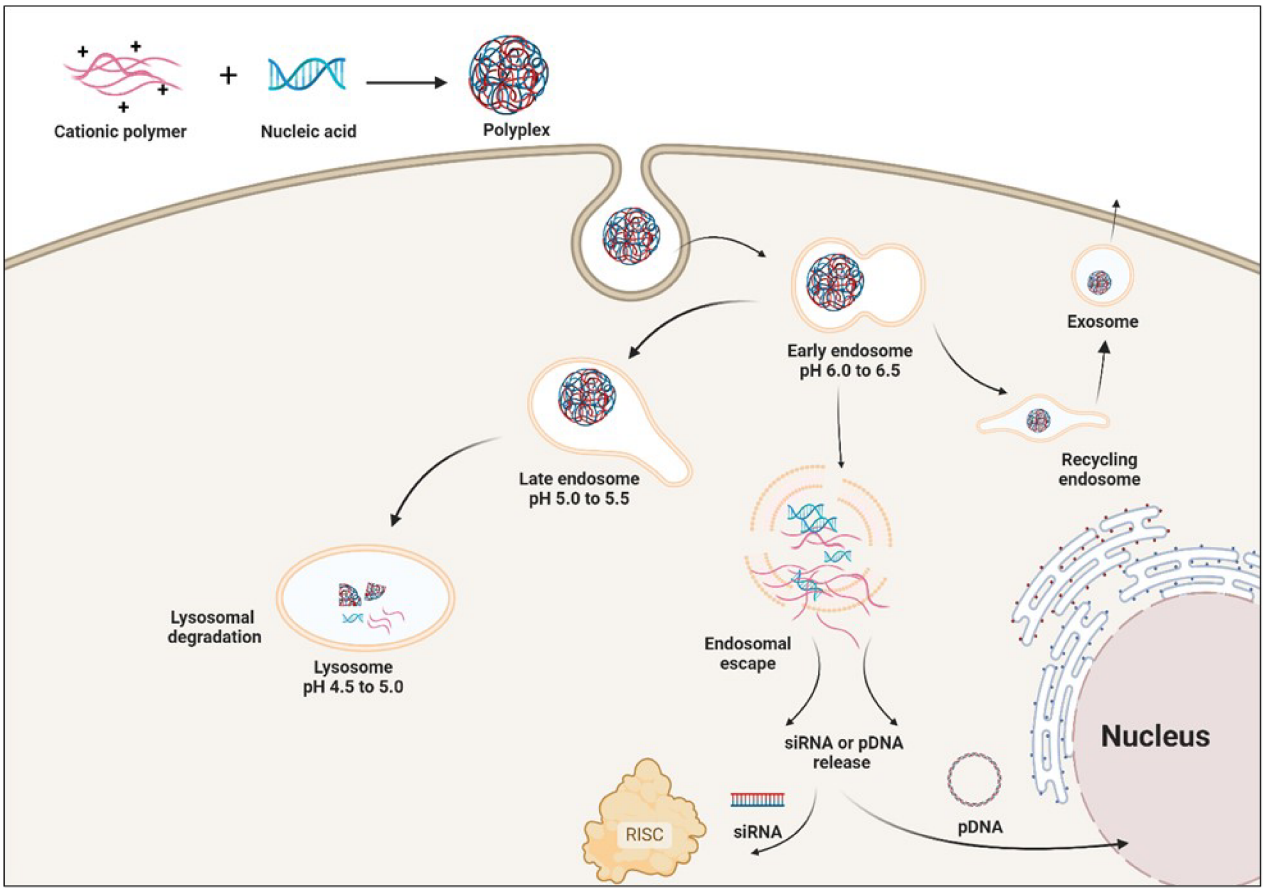

Figure 1.3. Schematic diagram of the possible mechanism in cellular uptake and trafficking of polyplexes with intracellular barriers (adapted from [19]).

1.3.1 Cellular Uptake of Polyplexes

A critical requirement for effective cell entry of the polyplex is its surface interactions with cell components. Upon interaction with the cell membrane, polyplexes present an opportunity for internalization [12]. The pathways through which these polyplexes are taken up by cells play a crucial role in determining the efficiency of gene delivery vectors, impacting intracellular processing, trafficking, and recycling of the internalized polyplexes [12,39]. The cellular uptake of cationic PEI-polyplexes is triggered by binding to negatively charged cell membranes and primarily occurs via endocytosis [12,18,23,26 ,34,52]. This induces adsorptive-mediated endocytosis leading to accumulation within the endosomal compartment [12,34, 39]. Endocytotic pathways can be broadly categorized into two main pathways: phagocytosis and pinocytosis. [12,23,34,39,54]. Phagocytosis is a specialized process primarily executed by certain cell types such as lymphocytes, dendritic cells, neutrophils and macrophages [12,34,54]. It serves a critical role in host defense mechanisms and the clearance of dead cells and debris. This process begins with receptor-ligand interactions on the cell surface, prompting extensions that engulf and internalize particles, forming a phagosome. Subsequently, the phagosome merges with lysosomes, culminating in the formation of a

phagolysosome. Integral to phagocytosis is opsonization, where opsonins such as immunoglobulins and complement proteins coat target materials. This coating signals phagocytes to recognize the presence of particles and initiates phagocytic activity. Professional phagocytes, particularly macrophages, excel in engulfing these large, opsonized particles (>1 μm), thereby significantly contributing to their rapid clearance from the bloodstream. Organs like the liver and spleen play key roles in this process, actively engaging in the degradation and elimination of opsonized particles. Pinocytosis is further sub-divided into clathrin-mediated endocytosis (CME) and non-clathrin-mediated endocytosis that includes macropinocytosis, caveolae-mediated endocytosis(CvME), and clathrin- and caveolae-independent endocytosis [12,34,39]. Relevant uptake pathways for cationic polyplexes in general are CvME, CME, phagocytosis as well as direct membrane penetration [19].

CvME was initially considered the most favorable pathway for transfection due to its non-acidic and non-degradative environment, which preserved the integrity of the internalized nucleic acid cargo. Studies demonstrated that CvME led to efficient transgene expression in COS-7 and HeLa cells during PEI-mediated transfection [39]. CvME provides a route for cellular uptake, particularly for particles smaller than 60 nm or specific ligands such as folic acid, albumin, or cholesterol [23,34]. Within this pathway, ligands are routed to the Golgi apparatus or endoplasmic reticulum, offering an intracellular route that bypasses lysosomal degradation [34]. However, contrasting views emerged, suggesting that CME might be the most efficient pathway. CME not only provided an acidic environment essential for PEI complex-mediated endosome disruption through the proton-sponge effect but also facilitated the movement of transgene cargo toward the perinuclear region, enhancing the likelihood of nuclear import [39]. CME is initiated by ligand binding to cell membrane receptors, enabling uptake of particles smaller than 200 nm and directing nanoparticles to cells expressing specific receptors. Particles internalized via CME are typically routed to degradative endosomal/lysosomal compartments. pH-sensitive polyplexes exploit this acidic milieu to escape from the endosome and release their gene cargo [34,54]. While it is evident that a universally optimal pathway for all gene delivery vectors may not exist, many specific uptake mechanisms remain unclear and require further investigation. Transfection pathways vary among different cell types, influenced by the biochemical nature and physicochemical properties of the vectors and resulting polyplexes, especially concerning PEI derivatives with variations in structure, molecular weight, or chemical modifications [34,39].

1.3.1.1 Factors Affecting Cellular Uptake

There are several factors in cellular uptake of polyplexes that should be considered including polymer structure and molecular weight, as they can significantly influence the polyplex' physicochemical properties and consequently, the cellular uptake mechanism. Morphological characteristics such as size and shape, as well as the surface chemistry are responsible for controlling surface charge and hydrophobicity/hydrophilicity [12,34]. Since polyplexes are usually a group of heterogeneous particles with diverse properties, several uptake pathways may be involved in the internalization of one kind of polyplexes

into a single cell type [54]. These traits collectively dictate the mechanism of cell internalization [12,23,32,41]. Additionally, the characteristics of the cell membrane, which vary among different cell types, also play a significant role in determining the entry route [34,54]. **Figure 1.4** depicts these physicochemical properties that impact the cellular uptake of polyplexes [12,34].

Size and shape

Size profoundly influences the internalization and intracellular fate of polyplexes. Studies consistently indicate that nanoparticles with diameters below 200 nm are optimal for cellular uptake via CME, contrasting with larger particles which are more prone to phagocytosis by macrophages due to opsonization and subsequent clearance [12]. For instance, PEI/pDNA polyplexes larger than 500 nm primarily enter cells through macropinocytosis [54]. This variation in size significantly affects biodistribution, with smaller particles often achieving superior tumor accumulation owing to prolonged circulation times [12]. Furthermore, polyplex size dictates both the pathway of entry into cells and their subsequent intracellular trafficking. Experimental evidence using fluorescent latex beads ranging from 50 to 1000 nm has shown that different particle sizes can engage distinct uptake mechanisms [55]. For example, particles up to 500 nm are internalized by B16 cells, with smaller particles distributed throughout the cell interior and larger particles localized predominantly at the cell periphery. The choice between CME and CvME uptake pathways depends on particle size; larger particles may require specific electrostatic interactions for caveolae-mediated entry. This size-dependent mechanism also impacts transfection efficiency, as larger PEI polyplexes (1000 nm) tend to transfect cells more efficiently than smaller pDMAEMA polyplexes (200 nm), potentially due to their faster sedimentation rates. Interestingly, inhibiting caveolae-mediated uptake reduces PEI polyplex uptake but has a lesser effect on pDMAEMA polyplexes, highlighting their differential reliance on caveolae-mediated pathways for effective gene expression. Importantly, relying solely on clathrin-mediated pathways may not ensure efficient delivery of genetic material to the cell nucleus [23]. Beyond cellular uptake, polyplex size significantly influences their circulation and transport in the bloodstream. Recent studies comparing PAMAM dendrimers with slight size variations (4.3 nm vs 6.5 nm) have demonstrated substantial differences in circulation time and enhanced accumulation in the brain with larger polymers. Similarly, reducing the size of PEI/pDNA polyplexes helps evade normal clearance mechanisms, potentially extending their circulation time. Moreover, the size of the carrier critically determines its ability to exploit the enhanced permeability and retention (EPR) effect observed in tumors and other tissues with leaky vasculature. However, it's essential to consider that this strategy may not universally apply to all tissue types and could lead to unintended accumulation in non-targeted areas [18].

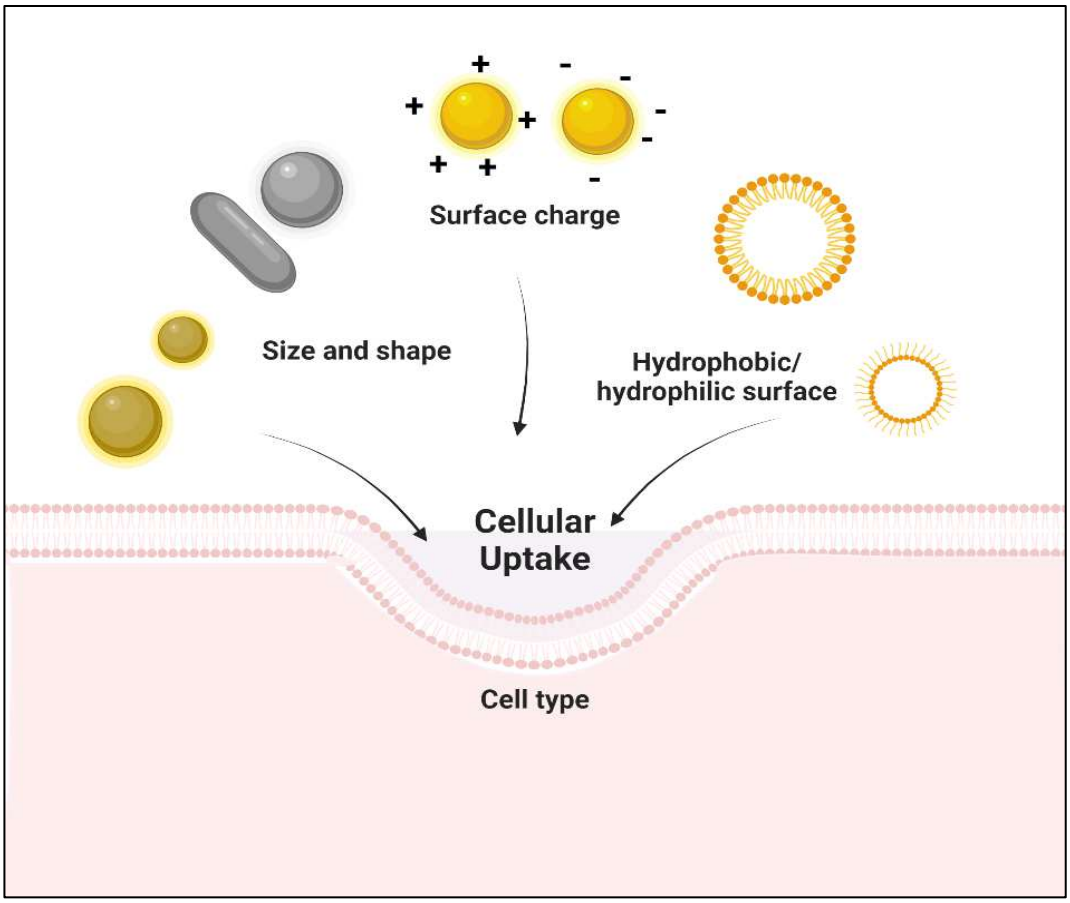

Figure 1.4. Some of the physicochemical properties that influence cellular uptake of Polyplexes (adapted from [12]).

Considerable efforts have been dedicated to understanding how the shape of polyplexes influences cellular uptake efficiency and pathways, as well as toxicity and biodistribution [12]. However, drawing definitive conclusions about the optimal shape for uptake remains challenging, encompassing various materials and even within studies of the same material. For example, there is inconsistency in the literature regarding the cellular uptake of elongated nanoparticles such as filaments or rods compared to their spherical counterparts. Rod-like nanocarriers have shown potential advantages *in vivo* due to reduced uptake by macrophages, which inhibits their endocytosis by both macrophages and cancer cells, potentially enhancing tumor accumulation [12,41]. Studies involving rod-like hydrogels have observed lesser uptake by HeLa cells compared to their spherical counterparts. Additionally, protein-coated spherical gold nanoparticles exhibit more efficient uptake than rod-shaped ones in HeLa cells, SNB19 cells, and STO cells [54]. Conversely, enhanced uptake of rod-like micelles compared to spherical micelles has been frequently reported [41].

Surface chemistry

Surface chemistry is crucial in determining cellular uptake efficiency, influencing factors such as polyplex surface charge, hydrophobicity, and the addition of molecules and targeting ligands. These aspects directly impact interactions with cell membranes [56]. Specifically, surface charge and hydrophobicity govern non-specific binding forces with cell membranes, while functionalities capable of receptor-ligand interactions facilitate receptor-mediated endocytosis [12,56]. Cationic surface charge typically correlates with significantly higher cellular uptake and toxicity in non-phagocytic cell lines compared to anionic particles [12]. The cell membrane, composed of a lipid bilayer and anionic membrane proteins, facilitates the uptake of cationic polyplexes. The zeta potential of assembled polyplexes, rather than the charge of carriers, dictates membrane interactions [8]. Charged moieties in carriers and carrier-to-nucleic acid ratios determine the zeta potential. However, uptake efficiency significantly decreases when the net positive charge approaches neutral. A neutral surface charge weakens polyplex interactions with membrane proteins, leading to increased aggregation that hinders internalization. This effect is influenced by anionic proteins in blood circulation in vivo and serum components in in vitro transfection media [13]. Maintaining a weak positive charge (<+5 mV) is suggested to balance necessary cell surface interactions while minimizing nonspecific binding, provided complex aggregation near neutral charge is addressed [8].

Due to their strong attraction to anionic cell membranes, cationic particles can utilize non-endocytic pathways such as membrane disruption and hole creation, which are less accessible to negatively charged particles. Nevertheless, some studies suggest that particles with a slight negative zeta potential exhibit increased circulation time and enhanced tumor accumulation by reducing non-specific interactions with cells [12]. Incorporating polyethylene glycol (PEG) into polyplexes addresses these issues by reducing aggregation, enhancing stability, minimizing clearance, and prolonging systemic circulation time in vivo. This is due to PEG's high hydrophilicity, electrical neutrality, and steric-repulsive properties [12,30,34,44,45,63]. However, the improved stability and steric hindrance introduced by PEG may sometimes inhibit the intended cellular uptake and reduce transfection efficiency. Despite these considerations, adding PEG to polymeric vectors remains a widely adopted strategy in numerous in vivo applications [18,56].

Additionally, incorporating targeting ligands and other molecules onto the surface of vectors represents a promising strategy for achieving specific targeting and enhancing uptake. This approach is particularly advantageous when the target cell expresses specific receptors at elevated levels that are not as prevalent in other cell types, thereby reducing the risk of off-target effects. While this strategy does not actively guide the polymer vector to a specific site, it can significantly increase the concentration delivered to the target site if the vehicle naturally encounters this target. In the context of delivering nucleic acids to tumors, recent studies have demonstrated enhanced accumulation at tumor sites by attaching sugars, proteins, or drugs

to the vehicle's surface that bind to overexpressed receptors on tumor cells. For instance, hyaluronic acid (targeting CD44 receptor), lactoferrin, epidermal growth factor, folic acid , peptides, and antibodies have been employed for this purpose[12,18,56].

Cell type

The cellular uptake of polyplexes is significantly influenced by the type of cell, which exhibits distinct preferences for uptake mechanisms. Caveolae, essential for the caveolae-mediated endocytosis (CvME) pathway, are present in various cell types, particularly abundant in endothelial cells lining vessel walls. Hence, endothelial cells have been extensively studied in CvME pathway research. Conversely, studies on PEI/pDNA polyplex transfection pathways consistently identify COS-7 cells as primarily utilizing the clathrin-dependent pathway, irrespective of PEI structure (linear or branched) [54,56]. Hard-to-transfect cell lines, including primary, stem, and suspension cells, pose challenges for gene delivery due to their limited ability to internalize gene carriers. Physical methods such as electroporation or nucleofection are effective alternatives for these cell types, suggesting that the primary challenge lies in the initial attachment of vectors rather than the subsequent expression post-endocytosis. For instance, suspension cell lines like leukemic cells often lack sufficient surface area and extracellular matrix for effective attachment of conventional cationic carriers, thereby limiting uptake efficiency [26, 46]. The expression of proteoglycans plays a crucial role in determining transfection efficiency across different cell types. Stem cells, which generally lack proteoglycans, struggle with vector attachment and subsequent polyplex uptake [48]. Current research endeavors aim to modulate proteoglycan expression to enhance transfection efficacy using cationic methods. This variability in proteoglycan expression among tissues likely explains why certain cell types are more susceptible to transfection than others. For example, chronic myeloid leukemia (CML) K562 cells exhibit significantly lower siRNA uptake compared to attachment-dependent cell lines like breast cancer MDA-MB-231 cells, due to the absence of surface proteoglycans [36, 43, 46]. Therefore, the critical barrier in polyplex transfection of hard-to-transfect cells often lies in the initial attachment of polyplexes to the cell surface. Understanding the intricate interplay between cell type specificity and uptake mechanisms is crucial for developing effective gene delivery strategies tailored to diverse cellular environments.

1.3.2 Endosomal escape

To reach the intracellular environment, polyplexes must traverse lipid membranes and be internalized into cells. Typically, gene vectors are taken up via endocytosis, necessitating their escape from the endosomal compartment to access the cytosol for siRNA release or the nucleus for DNA delivery [5,6]. Polyplexes modified with targeting ligands can bind to specific cell-surface receptors, facilitating internalization predominantly through receptor-mediated endocytosis. Regardless of the internalization pathway, polyplexes end up localized within endocytic vesicles, which pose a hostile environment. Initially, they are directed to early endosomes that merge with sorting endosomes, offering a route for materials to be

transported back to the membrane and eventually expelled from the cell through exocytosis. However, the prevailing route leads polyplexes into late endosomes, which undergo rapid acidification (~pH 5) due to the ATPase proton-pump enzyme on their membrane. Subsequently, polyplexes progress into lysosomes with even lower pH (~4.5), harboring various degradative enzymes [19, 33].

High molecular weight (HMW) PEI-based polyplexes demonstrate significant transfection efficiency despite entering acidic endosomal/lysosomal compartments post-endocytosis [8, 29]. This efficacy is attributed to PEI's ability to induce endosomal escape through the proton sponge effect [19, 33, 52,54, 57]. The proton sponge effect relies on PEI's buffering capacity from its primary, secondary, and tertiary amine groups, which, upon protonation, can elevate the pH within endosomal/lysosomal compartments. When a PEI-based polyplex becomes trapped within acidic compartments, it absorbs protons pumped into these organelles, thereby buffering and delaying acidification within the vesicles. The amino functionalities remaining within PEI serve as a buffering agent or 'proton sponge'. As intramolecular positively charged amino groups repel each other more strongly, the polymer swells. To prevent the accumulation of a charge gradient due to proton influx, ATP-driven chloride ion influx also restores the acidic environment [19, 58]. This process creates an evolving osmotic imbalance, which is balanced by water entry. The resulting membrane tension, caused by continuous endosomal swelling and polymer expansion, destabilizes the endosome and facilitates the release of its contents into the cytoplasm [19, 57]. **Figure 1.5** illustrates the proposed mechanism of the proton sponge effect demonstrated by PEIs.

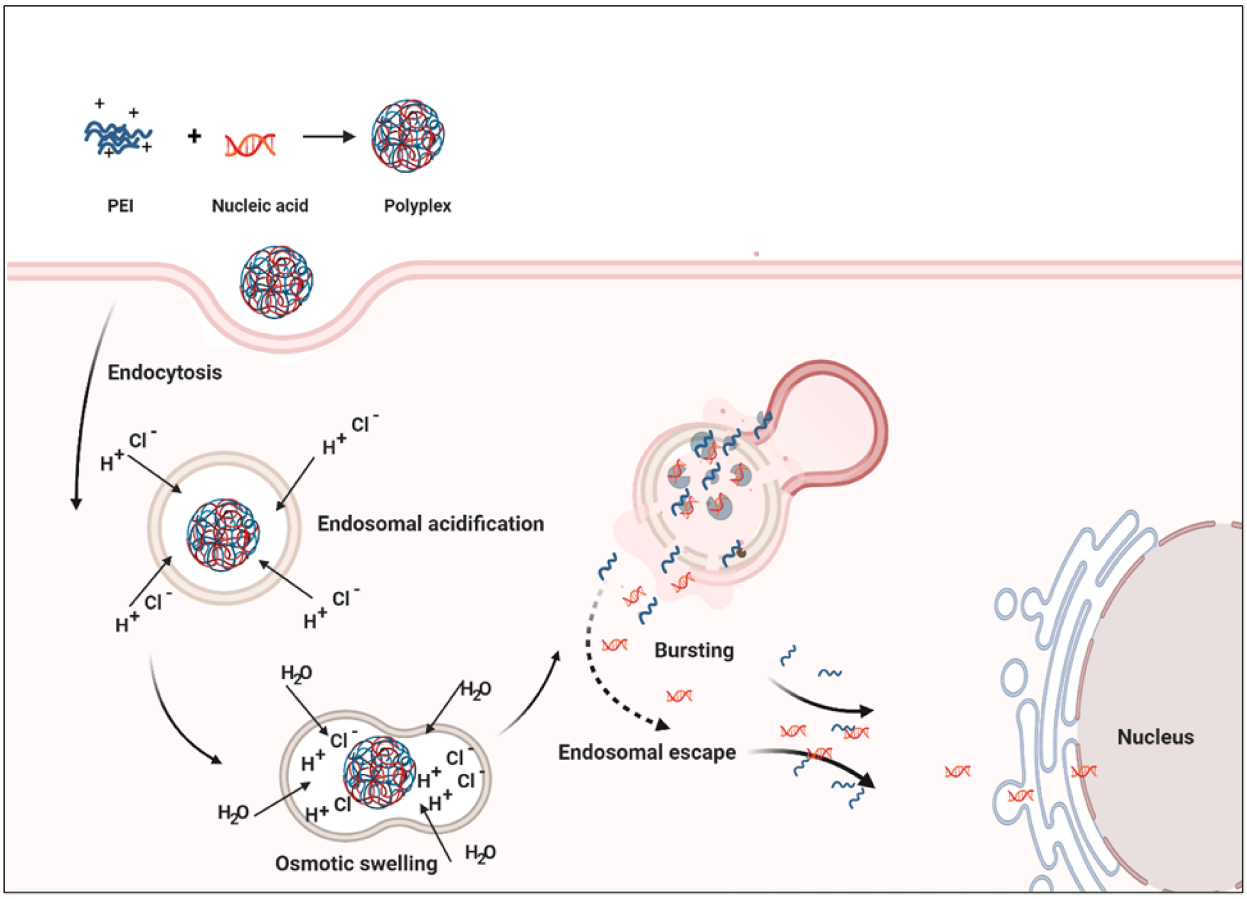

Figure 1.5. Proposed mechanism of proton sponge effect exhibited by PEIs (adapted from [2]).

Despite these mechanisms, only a small fraction (less than 10%) of PEI polyplexes escape the endosomal pathway before lysosomal entrapment, with the majority undergoing degradation within these compartments. Enhancing the proportion of PEI polyplexes that escape the endosome remains crucial for improving nucleic acid delivery [34]. It is important to note that much of the nucleic acid cargo, whether DNA or siRNA, may become trapped and degraded within these vesicles. Only the cargo that escapes into the cytoplasm can proceed to reach the nucleus [33]. Effective polymer design must achieve a balance between strong binding and efficient condensation of nucleic acids while ensuring their release. This release may involve competition with genomic DNA, cytosolic proteins, or anionic membrane lipids for binding. Therefore, polymer design should incorporate mechanisms for releasing genes at specific stages of delivery, ideally within the nucleus [33]. While certain carriers facilitate uptake efficiently, they may experience reduced transfection efficiency if they form tightly bound polyplexes. Designing carriers with labile linkages, such as disulfide bonds, can facilitate the controlled release of nucleic acids within the cytoplasm. For example, cross-linking low molecular weight PEI with agents like dithiobis(succinimidylpropionate) (DSP) and dimethyl-3,3'-dithiobispropionimidate (DTBP) [59], or 1,3-butanediol (or 1,6-hexanediol) diacrylates, can create carriers featuring disulfide and amide linkages that degrade efficiently. These smaller building blocks naturally clear from the body without adverse effects. Evaluating nucleic acid levels within cells post-delivery

and assessing target mRNA suppression can effectively gauge carrier efficacy. Minimizing cytotoxicity and stress-related factors in delivery methods is critical to maintain nucleic acid delivery system functionality and mitigate off-target effects [6].

1.4 Polyethylenimine (PEI)-based Polyplexes

Considered the ‘gold standard’ in non-viral gene delivery, high molecular-weight (HMW) PEI25k (25 kDa MW branched PEI) is a potent carrier due to its superior induction of transgene expression [4,52,60] which has been demonstrated to be more efficient than other commonly employed cationic polymers like chitosan or polyamidoamine (PAMAM) [13,19,34]. PEI possesses a high charge density owing to its polyamine structure composed of repeating units of two aliphatic carbons and an amino group, $–(CH_2–CH_2–NH)_n–$ (43 g mol^{-1})[19, 41,54]. This high cationic charge density is beneficial for nucleic acid condensation, cellular uptake and its high transfection performance is attributed to its ability to facilitate endosomal escape through the "proton sponge" effect” [4,19,52,54,57] which was discussed in the previous section.

There are two distinct chemical structures of PEI, the linear (lPEI) and the branched (bPEI). LPEI bears solely secondary amino groups in the backbone except for the primary amines at terminals [19,41,54], while bPEI contains primary, secondary, and tertiary amines at an approximate ratio of 1:2:1[34]. In general, branched PEI has been found superior in nucleic acid delivery compared to the linear structure [8,69]. The abundant presence of amine groups allows for versatile functionalization of PEI carriers which permits adaptation to different requirements for gene delivery [34]. Moreover, due to its structure, PEI acts as a weak-base buffering polyelectrolyte, exhibiting pH-dependent protonation behavior and charge-driven conformational changes. It has broad buffer capacity which extends from basic to acidic ranges, with a secondary maximum at pH 4 to 7 (pKa 4.5), explaining its effectiveness in acidic environments like endosomal/lysosomal compartments [52].

HMW PEI has been extensively studied *in vivo* using animal models, revealing its potential for nucleic acid condensation, cellular uptake, endosomal escape, and transgene expression. However, its clinical application is hindered by severe cellular toxicity arising from its strong cationic charge density. This characteristic not only promotes complex aggregation on cell surfaces but also induces cell necrosis [33,34,35]. The polymer's molecular weight, structure, and degree of branching directly influence this cationic charge density, which is essential for its biological activities but also contributes to cytotoxic effects. PEI, like other cationic polymers, is proposed to enhance genomic material uptake due to its surfactant-like properties, capable of disrupting lipid bilayers of cell membranes, creating nanoscale holes and causing membrane thinning and erosion. However, this membrane-destabilizing action has a dual effect—it enhances efficiency in delivering nucleic acids but also correlates with cytotoxicity. Thus, while promising for gene delivery, the balance between efficacy and safety remains crucial [40]. Moreover, HMW PEI's non-specific interactions with serum proteins and negatively charged components of non-target cells, coupled

with its non-biodegradable nature, contribute to its prolonged presence in tissues. This characteristic exacerbates its cellular toxicity [33,35,40]. Lower MW (LMW) PEIs present acceptable toxicity profiles but, unfortunately, these small PEI polymers do not display efficacious nucleic acid delivery into cells [36,37,17], since they are inadequate to complex and transport the nucleic acids across cell membranes [38].

1.5 Strategic Modification of PEIs for improved delivery

1.5.1 Hydrophobic modification of LMW PEIs

Hydrophobic modification of low molecular weight polyethyleneimine (LMW PEI) represents a key strategy to enhance their gene delivery capabilities. This modification promotes nucleic acid compaction through additional hydrophobic interactions, thereby increasing the lipophilicity of polyplexes. These changes are advantageous for improving cell membrane interactions and enhancing cellular uptake [37,16,26,28,32]. By modifying LMW PEIs, the charge density is reduced, altering their cytotoxicity profile. Key aspects of hydrophobic modification include varying the length and type of hydrophobic groups, adjusting the degree of modification, and selecting appropriate linkers between the charged backbone and hydrophobic groups.

Research has focused on understanding the structure-function relationships of hydrophobic groups and their substitution levels, aiming to optimize gene delivery efficiency while minimizing toxicity. Incorporating polysaccharides into LMW PEIs further improves biocompatibility and nucleic acid delivery [34]. Polysaccharides like dextran, cyclodextrin, starch, and chitosan derivatives (e.g., N-octyl-N-quaternary chitosan, O-carboxymethyl chitosan) have been successfully coupled to LMW PEIs, enhancing their performance as gene carriers. Furthermore, cross-linking LMW PEIs using bioreducible linkers offers a promising strategy to enhance transfection efficiency. This approach leads to the formation of HMW PEI carriers that are biodegradable while retaining sufficient positive charge essential for condensing nucleic acids, a critical requirement for effective gene delivery [7,18]. Bioreducible linkers, such as redox-sensitive esters, carbamates, and disulfides, as well as pH-sensitive linkers like polyaspartates, hydrazones, ketals, and glutardialdehydes, play a crucial role in facilitating the efficient unpackaging of vectors within the cytoplasm. This capability is pivotal in overcoming a significant challenge faced by non-biodegradable PEI carriers, thereby substantially improving overall transfection efficacy [34].

Studies on the modification of LMW PEIs with various hydrophobic groups have shown enhanced transfection efficiencies compared to unmodified PEIs, approaching or even surpassing those of higher molecular weight PEIs like PEI25k [34]. However, hydrophobic modifications can also increase cytotoxicity compared to unmodified LMW PEIs, although generally less than PEI25k [14,17]. Optimization efforts continue to focus on balancing transfection efficiency with cytotoxicity, exploring parameters such as hydrophobic group type, degree of substitution, and formulation conditions (e.g., N/P ratio, pH, ionic strength) to achieve desirable gene delivery outcomes.

Forrest et al. observed that acetylated PEI25k polyplexes exhibited increased cellular uptake, thereby enhancing transfection efficiency while preserving cell viability. Despite a reduction in charge density, these polyplexes maintained a high ζ-potential critical for membrane interaction. However, modifications affected the efficiency of endosomal release, ultimately influencing the overall efficacy of gene delivery. The additional acetyl groups on PEI led to decreased cellular uptake in certain cell types, and these polyplexes were more prone to dissociation compared to native PEI. This susceptibility to premature dissociation from charged cellular or endosomal membranes may result in some plasmid DNA being released into the cytosol [23].

Zheng et al. synthesized reduction-sensitive PEI1.8k conjugates with lipoic acid, demonstrating reduced transfection efficiency with increasing substitution degree (DS) while enhancing DNA condensation. These conjugates facilitated efficient DNA unpacking in HeLa cells, highlighting their potential as low-toxicity gene carriers compared to PEI25k [28].

Alshamsan et al.[61] explored oleoyl (C18:1) and C18-substituted PEI for siRNA delivery, highlighting enhanced complex stability against dissociation induced by heparin and serum nucleases. Despite high ζ-potential and improved siRNA binding, challenges in optimizing uptake efficiency with lower polymer concentrations were observed, aiming to balance transfection efficiency with reduced toxicity.

Teo et al.[62] examined the influence of alkyl (ethyl, octyl, dodecyl) and aromatic functional groups (benzyl, phenyl urea) on PEI1.8k, revealing that shorter hydrophobic groups (ethyl) enhanced transfection efficiency compared to longer ones (octyl, dodecyl). Additionally, aromatic groups like benzyl further improved efficiency, emphasizing the critical role of hydrophobic group type and length in optimizing gene delivery with LMW PEIs.

Our group has made significant strides in developing lipid-modified LMW PEIs with molecular weights ranging from 0.6 to 2.0 kDa. Notably, these lipid modifications have greatly enhanced the uptake and delivery efficiency of siRNA and pDNA across various cell types compared to unmodified PEI [64]. Considerable attention has been dedicated to investigating how the lipid composition influences the efficacy of PEI derivatives.

Neamnark et al. [14] explored PEI2k modified with linear aliphatic lipids such as caprylic (CA), myristic (MA), palmitic (PA), stearic (StA), oleic (OA), and linoleic acid (LA). Their findings highlighted that longer-chain lipids, like oleic acid, demonstrate superior transfection efficiency compared to shorter-chain ones like caprylic acid. This study underscores the critical impact of lipid chain length and chemical structure on the delivery performance of LMW PEI.

Bahadur et al. [17] studied the effects of palmitic acid (PA) substitution on LMW PEIs (0.6, 1.2, 2 kDa) polyplex properties. They observed that PA substitution consistently increased zeta potentials (+35–40 mV) and cellular uptake, leading to enhanced transfection efficiency, especially with 1.2 kDa PEI containing approximately two PA moieties per PEI molecule, which proved effective for transgene expression. Although PA substitution initially increased PEI cytotoxicity, this effect was transient and was quickly overcome by cell proliferation, unlike with high molecular weight PEI25k. Furthermore, the presence of chloroquine further improved the efficiency of PA-substituted PEIs, suggesting persistent endosomal barriers affecting complex internalization. Thus, lipid-substituted LMW PEIs demonstrated comparable transfection efficacy to HMW PEI without its cytotoxic effects.

Meneksedag-Erol et al. [70], utilizing molecular dynamics (MD) and experimental methods, investigated LMW PEI modified with short propionic acid (PrA), specifically assessing the impact of substitution levels. They identified optimal surface hydrophobicity and surface charge density of PEI/siRNA polyplexes at an intermediate substitution ratio. Beyond this optimal value, excessive migration of PrA towards the polyplex core induced toxicity, affecting both uptake efficiency and silencing efficacy.

Aliabadi et al. [35] studied hydrophobic substitutions on PEI2k, revealing decreased binding affinity to siRNA due to interference with electrostatic interactions. However, increased ζ-potential promoted stronger interactions within polyplexes, enhancing cellular uptake with manageable transient toxicity. Moreover, the hydrophobic substitution on LMW PEI can significantly augment the cellular uptake of the polyplexes depending on the substitution level without an appreciable increase in the toxicity.

1.5.2 Incorporation of additives in Polyplexes

Previous studies have emphasized a critical issue where negatively charged serum components bind to positively charged polyplexes [34,45]. This interaction is driven by PEI's cationic charge, resulting in non-specific interactions with cellular components, opsonization, and clearance by the mononuclear phagocyte system (MPS). These factors collectively reduce transfection efficiency. To overcome these challenges, steric stabilization is commonly employed by introducing a protective layer on polyplex surfaces to prevent non-specific interactions with serum components [63]. Among various strategies, the use of hydrophilic polymers like polyethylene glycol (PEG) is extensively researched and utilized to modify polyplex surfaces [12, 30,34, 44, 45, 63]. For instance, PEG-PEI block copolymers provide stable covalent binding of PEG to PEI, reducing positive charges and cytotoxicity. This modification also lowers polyplex affinity for blood proteins, extending circulation time and improving targeting to desired sites [12,63]. However, PEGylation may weaken electrostatic interactions with nucleic acids, potentially limiting condensation and cellular uptake [34]. Alternatively, anionic polymers can electrostatically bind to cationic polyplexes, modifying their surfaces to enhance stability and minimize serum opsonization. These polyanionic additives are incorporated directly into delivery systems, forming ternary complexes (**Figure 1.6**) [36,44,45,63]. Ternary complexes have shown enhanced transfection efficiency compared to lipid-modified PEI-based polyplexes

alone [36,29,30,42,44,45]. Typical additives include polyanionic molecules such as polyaspartic acid, polyglutamic acid, poly(acrylic acid) (PA), poly(propylacrylic acid) (PPAA), dextran sulfate (DS), and hyaluronic acid (HA), each contributing to enhanced transfection through various mechanisms. They stabilize the polyplexes by coating or shielding them from external molecules [44,45], thereby reducing the associated cytotoxicity on target cells, and promoting effective self-assembly [30]. Additionally, these additives can improve the dissociation of polyplexes [36, 42], facilitate robust intracellular release [65], and potentially enhance the cellular uptake of nucleic acids [29, 63].

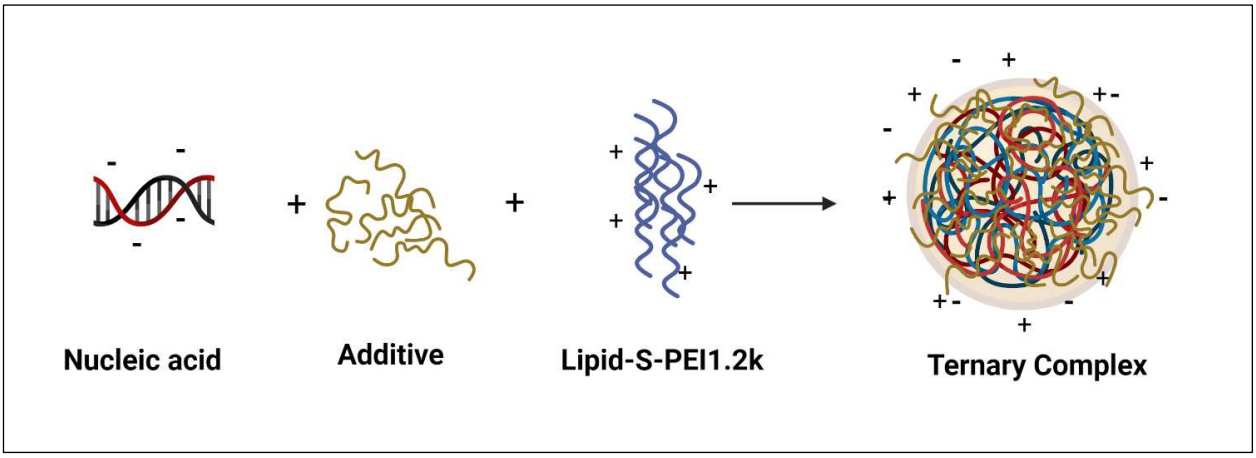

Figure 1.6 Formation of ternary complexes with incorporation of additives.

HA polymers are particularly noteworthy among these polyanionic additives and serve as an alternative to PEG-based stabilizers for particles. HA achieves similar particle stabilization outcomes to PEG while additionally targeting CD44 receptors [12]. Extensively studied due to its anionic polysaccharide nature, which is a key component of the extracellular matrix, HA has shown increased transfection efficiency for both pDNA and siRNA-based PEI polyplexes, suggesting broad potential in nucleic acid therapeutics [12,36, 42, 45, 67]. HA shields excessive cationic charges of PEI, protecting polyplexes from serum protein binding and facilitating timely loosening for access to gene transcription machinery [16]. Furthermore, HA alters the physicochemical properties of polyplexes, influencing parameters like ζ-potential and siRNA dissociation propensity [12,36, 42, 67].

Kurosaki et al. [44] employed γ-polyglutamic acid (γ -PGA) to electrostatically coat the pDNA/PEI polyplex, resulting in stable anionic particles. This coating markedly decreased the polyplex's toxicity. Additionally, the ternary complex was effectively taken up by cells via a γ-PGA-specific receptor-mediated pathway, resulting in remarkably high transgene efficiencies. In addition, Parmar [42] conducted further investigations into polyanionic polymers such as poly (acrylic acid) (PA), dextran sulfate (DS), and the neutral polymer methyl cellulose (MC) to better understand the mechanisms contributing to enhanced siRNA polyplex efficacy, comparing their performance with HA. The study revealed that both HA and PA significantly improved siRNA delivery by facilitating better polyplex dissociation. In contrast, MC was found to be

ineffective, highlighting the critical role of anionic charges in enhancing the effectiveness of additives in siRNA delivery.

Studies by Rajendran et al. [29] and Santadkha et al. [30] utilized the commercially-available additive *Trans-Booster*, resulting in enhanced cellular uptake in PEI-based polyplexes of pDNA and siRNA, respectively. This improvement in uptake can be attributed to enhanced polyplex stability, improved electrostatic complexation, shielding against nonspecific interactions with serum components, and efficient self-assembly of polyplexes, tailored to each nucleic acid type.

1.6 PEI carriers using thioether linkages for gene delivery

The introduction of this paper emphasizes the critical need to achieve a delicate balance between stability, essential for facilitating cell internalization via endocytosis, and instability, necessary for disassembling after internalization to release nucleic acids [2,9]. This delicate balance is paramount for the effective performance of polyplexes. Within this context, the lipid component plays a central role in fine-tuning this balance. While extensive research has investigated the impact of the lipid nature on the efficacy of PEI derivatives, less attention has been given to the types of linkers used for lipid grafting onto the PEI backbone [26]. Chemical grafting is the primary method for introducing hydrophobic groups onto LMW PEIs, involving reactions such as quaternization, amidation, alkylation, acylation, or Schiff-base reduction[72]. Biologically degradable linkages, such as esters, amides, acetals, carbamates, or ureas, are preferred in these modifications to ensure biocompatibility and reduce cytotoxic effects.

Another well-studied aspect due to its vast application are sulfur-containing compounds [72]. Introducing sulfur moieties into molecules can significantly alter their biological activity by modifying atomic and binding parameters. These compounds allow for selective design and stepwise synthesis of substituent groups around the sulfur center, achieving specific functional goals [72]. Several studies have investigated the incorporation of sulfur atoms or thioether functional groups.

Drescher et al. [64] examined the integration of thioether non-polar functional groups into the hydrophobic domain of bolaphospholipids, replacing methylene units with sulfur atoms. Molecular modeling revealed that this substitution introduces structural kinks in the lipid chains while maintaining a relatively dense packing arrangement. Another study investigated thioether functional groups in ionic liquids by substituting a methylene unit of a C18:0 alkyl chain with a thioether group. The extent of melting point reduction compared to the saturated counterpart was found to heavily depend on the sulfur's placement within the side chain. This alteration notably decreased the melting point, suggesting significant supramolecular changes [74]. Bouraoui et al. [9] studied cationic amphiphiles featuring a thioether function at various positions along two 18-atom lipid chains, comparing them with analogues containing oleyl (C18:1) or stearyl (C18:0) chains. They investigated the physicochemical properties using fluorescence anisotropy and compression

isotherms. The fluidity of amphiphiles with the thioether function positioned centrally between the lipid chains was found to be intermediate compared to those with oleyl- and stearyl-containing analogues. As carriers for plasmid DNA delivery, these thioether-containing cationic amphiphiles showed effective transfection capabilities in human-derived cell lines, particularly those with the thioether moiety positioned in the middle of the chain, which demonstrated the highest efficiency. This study suggests that integrating a thioether function could substitute for unsaturation in aliphatic lipid chains of cationic amphiphiles, thereby adjusting their physicochemical properties and improving biological activities such as gene delivery.

Hu et al. [55] demonstrated that enhancing PEI's transfection efficiency can be achieved through thioether conjugation with the cationic peptide Indolicidin (IL) at either its C-terminus (ILC) or N-terminus (CIL) using cysteine. They utilized the heterobifunctional crosslinker GMBS to link the peptides' thiol groups to PEI's primary amines. PEI-ILC showed exposed hydrophobic ends, while PEI-CIL displayed cationic surfaces mainly due to the outer arginine residues of Indolicidin. IL's hydrophobic tryptophan was expected to aid peptide insertion into the lipid bilayer's hydrophobic region, inducing membrane perturbation.Moreover, converting PEI's primary amines to amide groups to link with peptides via thioether linkages aimed to reduce cytotoxicity and enhance functionality. This modification not only improved cell viability but also functionalized membrane perturbation domains, thereby enhancing endocytosis and facilitating the escape of internalized pDNA from endosomes.

Our group has developed a variety of cationic polymeric carriers by conjugating LMW PEIs with hydrophobic lipid groups using different types of linkages [65]. **Figure 1.7** depicts the lipid substitutions and linkers employed in these modifications, showcasing selected lipopolymers that have been applied in a range of therapeutic applications. Notably, two of these linkages incorporate sulfur atoms: disulfide-cross-linked and thioester-linked. Of particular interest are the significant outcomes achieved by introducing thioester linkages (tLAs) into linoleic acid (LA) conjugates of PEI1.2k [36]. This study demonstrated that these polyplexes exhibit reduced toxicity and enhanced transgene expression in primary mesenchymal stem cells (MSCs) compared to conventional PEI-LA formulations. Their transfection efficiencies were found to match or exceed those of widely used commercial agents such as PEI25k and Lipo2000. Moreover, they achieved transfection efficiencies that were markedly higher or comparable to those of commercial transfection reagents such as PEI25k and Lipo2000. Additionally, incorporating the electronegative thioester into lipid-modified PEI1.2k resulted in reduced DNA binding capability (up to 6-fold) and increased dissociation upon exposure to polyanionic challenges. Therefore, this research aimed to explore thioether linkages as a novel functionality, an approach that has not been previously investigated in the literature. By elucidating the roles of hydrophobic substituents and new linkers in non-viral carriers, this study seeks to broaden the spectrum of functional materials available for gene delivery applications.

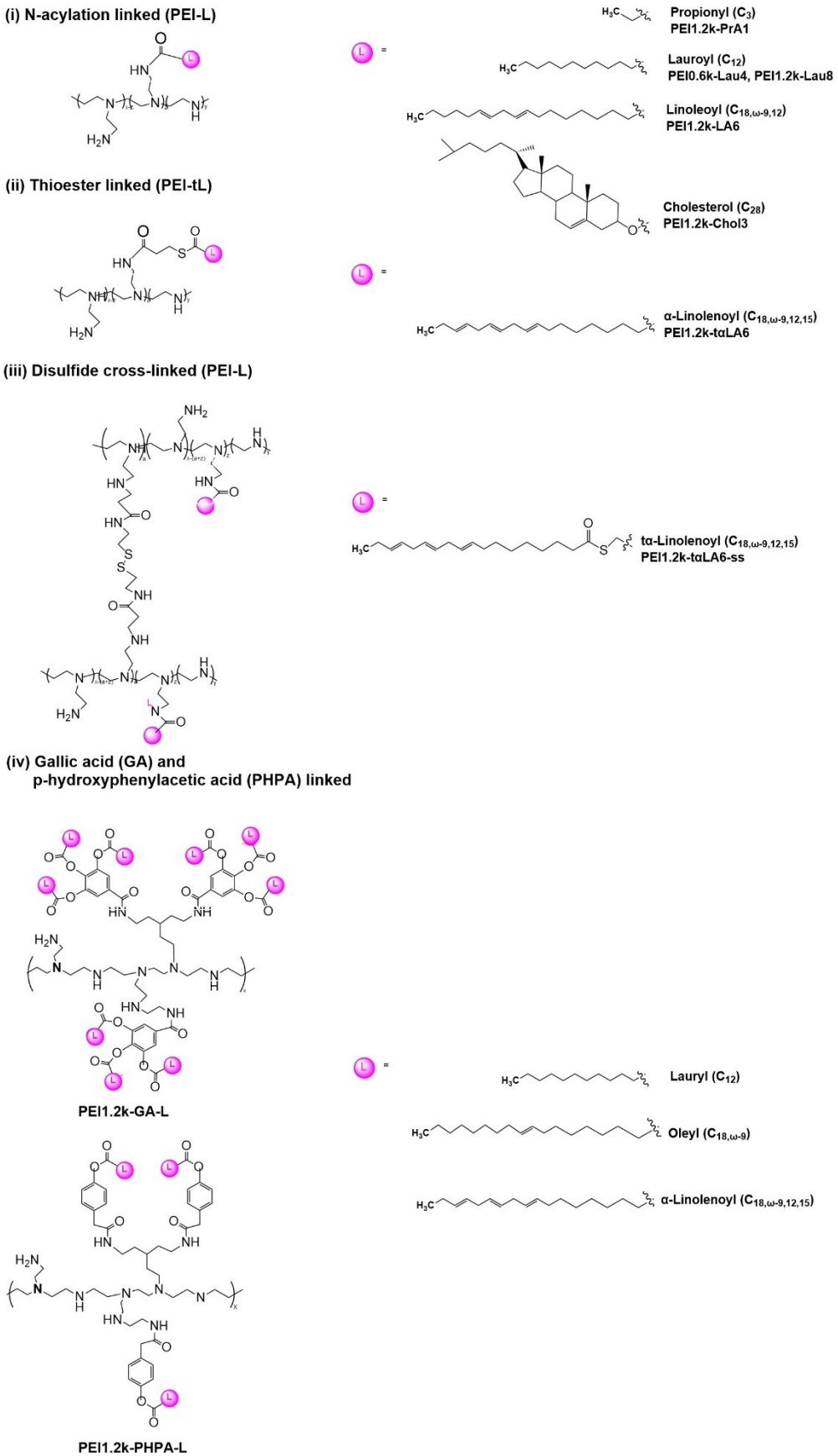

Figure 1.7 Summary of PEI conjugates with lipid substitutions and linkers synthesized by our group, utilized across various therapeutic applications (adapted from [26,65]).

Chapter 2: Synthesis of Thioether-Lipid Polyethyleneimine Conjugates for pDNA Delivery

2.1 Introduction

The success of gene therapy relies on developing efficient, non-toxic gene carriers that are stable enough to protect genetic material outside cells but can easily disassemble to release it intact upon cell internalization [2,9,10]. This incompatibility has posed significant challenges in the development of valuable and affordable treatments due to the sophistication of the process and the need for precise engineering of the delivery system.

To address these limitations, a competent intracellular delivery method employing nano-carriers as vectors has become increasingly attractive. These vectors are subdivided into two main categories, viral and non-viral. Viral vectors are characterized usually by their high transfection efficiency *in vivo*, but their inherent immunogenicity and high costs of manufacturing remain a hindrance to their widespread use [4,16]. As a result, non-viral vectors are increasingly explored as an alternative, since they have fewer limitations in condensing, protecting, and delivering genetic material [5], especially from the safety perspective. In particular, the cationic polymers demonstrate great potential owing to a more facile manufacturing, multivalent interactions with nucleic acids, ease of chemical functionalization to tailor their physicochemical properties, and non-immunogenicity [6,7].

Polyethyleneimine (PEI) has been initially used for effective non-viral delivery since it is capable of strong binding to nucleic acids and it can create an opportunity for endosomal escape due to the 'proton sponge effect' having an inherent high density of ionizable positive charges [6, 28]. These features provide a protective effect to prevent enzymatic degradation in the endosomal compartment and facilitate endosomal release of the nucleic acid cargo in intact form [1,7,8]. For a more proficient delivery, the high-molecular-weight (HMW) PEIs, in particular the 25 kDa MW branched PEI (PEI25k), have been studied considerably.

Even though HMW PEI has been extensively explored *in vivo* in animal models, its severe cellular toxicity caused by its strong cationic charge density, has been a deterrent to its clinical use due to the anticipated complex aggregation on the cell surface and induction of cell necrosis [4, 10]. Lower molecular weight (LMW) PEIs with MW in the 0.6 to 2.0 kDa range present acceptable toxicity profiles but, unfortunately, these small PEI polymers do not display efficacious nucleic acid delivery into cells [11,12], since they are inadequate to complex and transport the DNA across cell membranes [13]. A favorable design strategy to improving nucleic acid delivery involves incorporating hydrophobic segments into polymeric amines, since these substituents are expected to increase polymer interactions with lipophilic cell membranes and improve cellular uptake [12,16,26,28,32]. The incorporation of lipids generates relatively non-toxic PEI derivatives, which typically reduce binding efficiency and facilitate the unpacking of polyplexes in the cytoplasm [11,14, 40]. Previous research from our group achieved significant results by incorporating electronegative thioester

linkages into lipid conjugates of PEI1.2k, resulting in higher dissociation efficacy, lower toxicity, and greater transfection efficiency compared to commercial transfection agents [11]. This study aimed to investigate thioether linkages as an alternative functional group, which represents a novel approach not previously explored in the literature for constructing lipopolymers used in gene delivery.

Here, LMW PEI1.2k is modified with hydrophobic aliphatic lipid substituents (lauryl, palmityl, and stearyl) using thioether linkages at varying levels of substitution, and its *in vitro* transfection efficiency is assessed. Additionally, the impact of incorporating the polyanionic additive *Trans-Booster* into pDNA polyplexes was evaluated. This investigation of alternative non-viral vectors could provide insights into *in-situ* nucleic acid release, expected to produce therapeutic effects once internalized in cells [16].

2.2 Materials and Methods

2.2.1 Materials

The branched 25 kDa PEI (PEI25k), 1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide·HCl (EDC), N-hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) formaldehyde (38.7%), chloroform, hydrochloric acid and acetone were purchased from Sigma-Aldrich (St. Louis, MO). The branched 1.2 kDa PEI (PEI1.2k) which was used for modification with lipids in this study was purchased from Polysciences Inc. (Warrington, PA). All polymers and solvents were used in synthesis procedure as received without any purification. Lipofectamine™ 2000 (referred to as Lipo2000), 1-dodecanethiol (C12; 98%), 1-hexadecanethiol (C16; 98%), 1-octadecanethiol (C18; 98%), methyl acrylate (99%) and triphenylphosphine (99%) was obtained from ThermoFisher. Cell culture medium Roswell Park Memorial Institute (RPMI-1640), supplied with L-glutamine and 25 mM of N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES), and penicillin (10,000 U/mL)/streptomycin (10 mg/mL) mixture were obtained from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Avantor, Inc. (Radnor, PA). SYBR Green I was purchased from Cambrex BioScience (Rockland, MD). The pDNA used in this study (a sleeping beauty transposon vector for green fluorescent protein (GFP) expression by a CMV promoter, pT4-CMV-CAT) was obtained from Addgene (Watertown, MA) and expanded in-house to form a stock solution of 0.4 mg/mL in nuclease free water. The ALL-Fect transfection reagent as well as the anionic polymer additive utilized in polyplexes (*Trans-Booster*), hereafter referred to as PA, were obtained from RJH Biosciences Inc. (Edmonton, Alberta). Cy3-labeled pDNA was obtained by using a non-expressing pDNA gWIZ from Aldevron (Fargo, ND) and using Cy3 labeling kit from MIRUS (Madison, WI).

2.2.2 Methods

2.2.2.1 Synthesis of Lipopolymers (Lipid-S-PEI1.2k)

Using commercially-available alkyl thiols, the formation of the ether derivative of the lipid was carried out via the thiol-Michael reaction as shown in **Scheme 1**. A 2.0 M of the lipid thiol in a 20 mL scintillation vial was mixed with 0.4 mmol % phosphine catalyst in (45 wt. %) chloroform, and 2.0 M of the acrylate was added to obtain the product with subsequent hydrolysis using HCl/acetone to obtain the carboxyl end-capped aliphatic moiety. The latter was conjugated onto branched PEI1.2k through the EDC/NHS activation and coupling. The structural composition and level of lipid substitution of the synthesized thioether (Lipid-S-PEI1.2k) were elucidated using ^{1}H-NMR spectroscopy (Bruker 300 MHz, Billerica, MA) using D_2O as solvent, with tetramethylsilane (TMS) as an internal reference. The chemical shifts of the characteristic resonance protons corresponding to the alkyl and PEI were integrated and normalized for the protons in each peak. The transmittance in the FT-IR measurements were recorded using a Thermo Nicolet iS50 FT-IR (Thermo Fisher Scientific Co., Waltham, MA) spectrometer equipped with diamond crystal/built-in all-reflective diamond via attenuated total reflection (ATR) technique.

2.2.2.2 Formation of Lipid-S-PEI1.2k Polyplexes and Characterization

Considering that the electrostatic interactions between the cationic PEIs and the anionic phosphate groups of pDNA is expected to efficiently condense the pDNA into spherical and nanosized particles, measurement of the size and ***ζ***-potential of the resulting nanoparticles derived from the polyplexes were performed. This characterization can provide information on the mechanism of particle binding to the anionic cell surfaces that can facilitate cellular uptake of the particles. Particle size and surface charge (***ζ***-potential) of the polyplexes were measured in an aqueous medium using dynamic light scattering (DLS) of PSA Litesizer 500 (Anton Paar, Austria) equipped with He–Ne laser and operated at 10 mW. The polymers (1 mg/mL) were prepared and 40 uL of pDNA (25 ug/mL) was added to the polymer solution to give polyplexes with mass ratios of 5.0, 7.5 and 10.0 (polymer/pDNA). After 30 minutes of incubation at room temperature, the polyplexes were diluted to 1 mL with water and the particle size and ***ζ***-potential were measured.

2.2.2.3 pDNA Binding and Dissociation Assay

The pDNA binding capacity of the polymers and dissociation of the resultant polyplexes were determined using fluorophore SYBR Green in Fluoreskan Ascent 2.5 (Thermo Labsystems). The affinity of the SYBR Green to free pDNA in polyplexes prepared with different polymer/pDNA weight ratios (0.0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 1.5) in PBS medium was measured. After 30 min of incubation of polymers with pDNA at room temperature, 200 µL of 1X SYBR Green I was added to the tubes, and 200 µL of each sample was read on a 96-well plate (λ_{EX}, 485 nm; λ_{EM}, 527 nm) to quantify the amount of free pDNA. Fluorescence measured at 100% corresponds to pDNA as reference for no polymer binding. The sigmoidal binding curves were generated by plotting the % bound pDNA vs. polymer/pDNA ratio. The BC_{50} values were calculated at 50% of the pDNA binding. For heparin-induced dissociation assay, heparin concentrations ranging from 0 to 100 U/mL were added to the polyplexes (polymer/pDNA =7.5, w/w) for 1 h incubation. Subsequently, 100 uL of 1X SYBR Green were added to the polyplexes and 200 µL of each sample was read on the 96-well plate (λ_{EX}, 485 nm; λ_{EM}, 527 nm).

2.2.2.4 Cell Culture

The suspension-growing K562 cells (wild-type) and Jurkat T-cells with stable Green Fluorescent Protein (GFP) expression were used in this study. Cells were propagated in RPMI medium containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Typical passage of cells was done once a week where spent medium was discarded by centrifugation (600 rpm, 5 min) with 1:10 dilutions for sub-culturing. The cells were harvested and 300 µL were seeded in 48-well plates at the density of 100,000 cells/mL for transfection.

2.2.2.5 Analysis of GFP Expression

The GFP expressions in cells after treatment with polyplexes were investigated in suspension-growing Jurkat T-cells and K562-WT cells. PEI25k and Lipo2000 were used as reference reagents while the

untreated cells were used as a negative control. The polyplexes of various polymer/pDNA ratios (5.0, 7.5, and 10.0) with/without additive were prepared in 20 µL of RPMI to each well (20 µL duplicate) of the 48-well plates, followed by the addition of suspension cells (100,000/mL) for reverse transfection (cells added after the addition of nanoparticles). After 48 h of incubation under a humidified atmosphere, the cells were washed two times with 500 µL of Hanks' Balanced Salt Solution (HBSS) buffer and fixed with 100 µL of 3.7% formaldehyde solution. The induced GFP fluorescence was determined by a microplate Fluoreskan Ascent 2.5 reader (Thermo Labsystems).

2.2.2.6 Preparation of Additive Treatment in Polyplexes

The PA additive was incorporated to pDNA solution with varying ratios (PA/pDNA ratios of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0) and the desired transfection reagents were then added to the additive solutions for complex formation. The mixture was incubated for 30 min at room temperature to allow final complexation. **Table 2-1** indicates a typical study design for preparation of additive polyplexes using 1Palmityl (1Pa) where the ratio column indicates the ratio of transfection reagent to pDNA. This study design was also applied to 4Pa-S-PEI1.2k, 6Lau-S-PEI1.2k and 4St- S-PEI1.2k conjugates for the investigation of additive treatment.

Table 2-1. Representative study design in polyplexes

Carrier	Ratio (w/w)	Media (mL)	pDNA (mL)	Additive (mL)	Transfection Reagent (mL)	Total Vol. (mL)
NT	0.0	100.0	0.0	0.00	0.00	100
Lipo2000/pDNA-2.5.0	2.5	94.75	3.5	0.00	1.75	100
PEI25k/pDNA-7.5.0	7.5	91.25	3.5	0.00	5.25	100
1Pa/pDNA/Additive0	7.5	91.25	3.5	0.00	5.25	100
1Pa/pDNA/Additive0.5	7.5	86.87	3.5	1.75	7.87	100
1Pa/pDNA/Additive1.0	7.5	82.50	3.5	3.50	10.50	100
1Pa/pDNA/Additive1.5	7.5	78.12	3.5	5.25	13.12	100
1Pa/pDNA/Additive2.0	7.5	73.75	3.5	7.00	15.75	100
1Pa/pDNA/Additive2.5	7.5	69.37	3.5	8.75	18.37	100
1Pa/pDNA/Additive3.0	7.5	65.00	3.5	10.50	21.00	100
1Pa/pDNA/Additive4.0	7.5	56.25	3.5	14.00	26.25	100

2.2.2.7 Cellular Uptake of polyplexes

The intracellular delivery efficiency of the polyplexes was assessed by flow cytometry. K562-WT cells were seeded in 48 well plates with RPMI to quantitate pDNA uptake by using Cy3-labeled pDNA. After 48 h incubation, the medium was removed and the cells were collected by centrifugation at 1600 rpm for 5 min, washed and suspended in HBSS with 3.7% formaldehyde. The pDNA uptake was quantified using BD LSRFortessa-SORP (Flow Cytometry Core Facility, University of Alberta) flow cytometry instrument (10,000

events/sample). The instrument was calibrated so that the negative control sample (i.e., Cy3-labeled pDNA without any polymeric carrier) gives 1-2% positive cells as the background. The percentage of cells exhibiting Cy3-fluorescence and the mean fluorescence in the total cell population were determined.

2.2.2.8 Cytotoxicity Evaluation by MTT Assay

In vitro cytotoxicity of the polyplexes was studied by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay in 96-well plates. The Jurkat T-cells and K562-WT cells were added to the wells at 30,000 cells/well in RPMI medium and allowed to incubate for 30 min with 20 µL polyplexes. After 72 h of incubation at 37 °C in a humidified 95/5% air/CO_2 atmosphere, 100 µL of MTT solution (5 mg/mL in HBSS) was added to each well. After an additional 30-60 min incubation until formazan crystals were formed, the medium was removed, and 200 µL of DMSO was added to dissolve the formed MTT crystals. The absorbance was measured in a spectrophotometer (Spectramax 250) at a wavelength of 570 nm. The absorbance of the polymer-treated samples was normalized with the absorbance of untreated cells, which were used as a reference control (i.e., 100% cell viability), to calculate the % relative cell viability.

2.2.2.9 Statistical Analysis

All results were presented as mean $\pm$ standard deviation (SD) and the data was analyzed using the GraphPad Prism (Version 8.4.3) software. The two-tailed Student's *t-test* was used to calculate the statistical differences between the group means. The p-values *($p^* < 0.05$ and $p^{**} < 0.01$)* were considered statistically significant and the number of independent experiments used to generate the data (n) is indicated.

2.3 Results and Discussion

2.3.1 Synthesis of Lipid-Substituted PEI1.2k with Thioether Linkages

Three aliphatic lipids, Lauryl (12-C), Palmityl (16-C), and Stearyl (18-C) bearing thioether linkages were covalently conjugated to PEI1.2k via amide linkages using the EDC/NHS synthesis (**Figure 2.1A**). These lipids were employed in this study considering their successful use in gene delivery vectors in the past [14, 17]. The results of the synthesis are summarized in **Table 2-2**. The structure of Lau-S-PEI1.2k and degree of lipid substitution of the synthesized polymers were elucidated using ^{1}H-NMR analysis (**Figure 2.1B**). The chemical shifts corresponding to the lipid (~0.8−2.0 ppm), thioether (~2.6 and ~3.1 ppm), and methylene groups of PEI1.2k (~2.4−3.5 ppm), were observed in the spectrum confirming the proposed synthesis route. Likewise, characteristic FT-IR absorption peaks of the Lau-S-PEI1.2k were shown in **Figure 2.1C**. The presence of the strong band at 1650 cm^{-1} (C=0 stretch) can be ascribed to the formed secondary amide bond between the lipid and PEI1.2k while absorptions at 720 cm^{-1} and 1080 cm^{-1} (C-S-stretch) can be assigned to the thioether linkage [66]. The peak at 3260 cm^{-1} (N-H stretch) demonstrated that PEI was successfully grafted with the lipid. The degree of lipid substitution is beneficial to assess the actual conjugation level of lipids in the polymer, and whether good control exists during the synthesis for grafting. An increasing lipid substitution with increasing lipid:PEI feed ratio for Lauryl substituent was evident for 2, 5 and 7 feed ratios with approximately 1.8, 4.7 and 5.8 lipid substitutions per PEI, respectively, indicating a controlled and reproducible synthesis for modification of PEI1.2k (**Table 2-2**).

2.3.2 Characterization of polyplexes

The binding efficiency and stability of Lipid-S-PEI1.2k polymers with pDNA were evaluated. Binding capacity was measured using BC_{50}, the polymer/pDNA ratio required for 50% DNA binding. The lipid-substituted PEIs exhibited higher BC_{50} values compared to native PEI1.2k and PEI25k, which had values in the range of 0.03 to 0.04. This was expected due to the reduced cationic charge density of the amine groups when substituted with lipids. The binding curves (**Figure 2.2A**), generated through the SYBR Green binding assay, indicate a higher BC_{50} with highly-substituted stearyl polymers. This increased BC_{50} could potentially be ascribed to steric hindrance during the binding of the fluorophore, possibly caused by the longer carbon aliphatic chain of stearyl compared to its shorter counterparts (lauric and palmitic acid), when condensing pDNA. The relationship between the extent of lipid substitution and BC_{50} was further evident in **Figure 2.2B**, where stearyl substituents showed a proportional increase in BC_{50}. In contrast, lauryl and palmityl-substituted PEIs did not exhibit a clear dependence of BC_{50} on the extent of lipid substitution. The higher BC_{50} values of stearyl-substituted polymers resulted in a much weaker binding with pDNA, up to a 10-fold change over the native PEIs. This reduction in pDNA binding capacity, resulting from aliphatic lipid substitution in PEIs, is attributed to primary amine consumption and steric hindrance which was also reported in several studies [14,16-20].

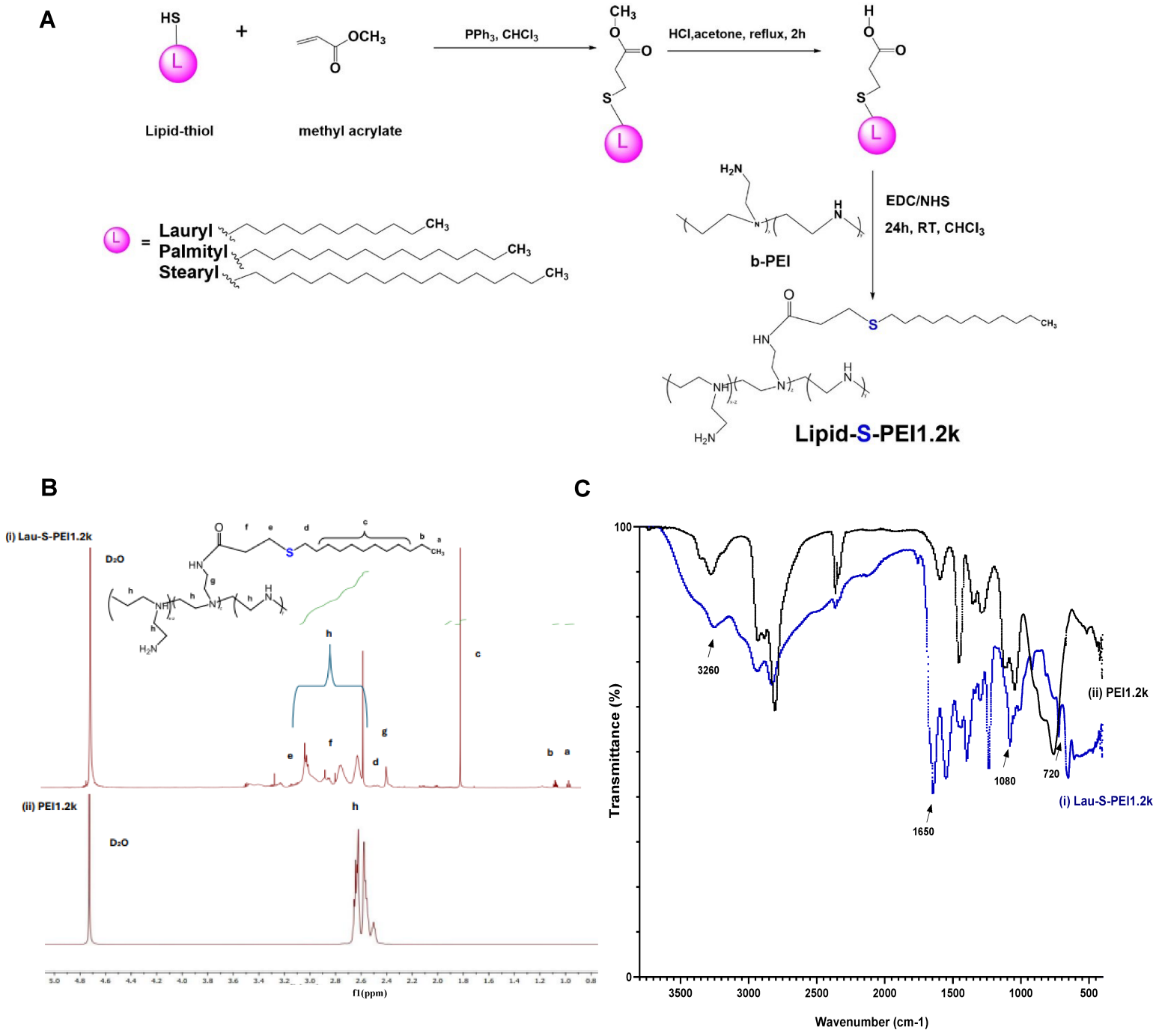

Figure 2.1. Synthesis of Lipid-S-PEI1.2k conjugates via thioether linkage using aliphatic lipids

(A) Reaction scheme. (B) Typical ^{1}H-NMR and (C) FT-IR spectra of (i) Lau-S-PEI1.2k conjugates (Table 2-2, entry 1) and (ii) native PEI1.2k

Table 2-2. Characteristics of Lipid-S-PEI1.2k conjugates

No.	Designation	Lipid	Lipid:PEI Feed Ratio	Substitution (Lipid/PEI)	Notation in Lipid-S-PEI1.2k
1	2Lau	Lauryl	2	1.8	2Lau
2	5Lau		5	4.7	5Lau
3	7Lau		7	5.8	6Lau
4	2Pa	Palmityl	2	0.8	1Pa
5	5Pa		5	4.3	4Pa
6	7Pa		7	5.9	6Pa
7	2St	Stearyl	2	0.75	1St
8	5St		5	2.9	3St
9	7St		7	4.4	4St

To explore the binding stability, the heparin-induced dissociation of polyplexes was studied (**Figure 2.2C)**. The heparin concentration for 50% dissociation (DC_{50}) of the pDNA from the polyplexes was calculated. These values denote the propensity for complex dissociation in the presence of negatively charged macromolecules in a physiological medium [14]. The DC_{50} values of the polyplexes varied between 16 and 3 U/mL, contingent upon the type and extent of lipid substitution. These polyplexes exhibited lower DC_{50} values compared to those prepared from native PEIs. Notably, the DC_{50} values for the native PEIs were similar (~20 U/mL), indicating comparable stability among these polyplexes.

The highly substituted conjugates of stearic acid exhibited reduced effectiveness in binding to pDNA, releasing it from polyplexes at lower heparin concentrations compared to native PEIs. This observation established a clear correlation between DC_{50} values and lipid substitutions, indicating an increased tendency for complex dissociation with greater stearyl substitution (**Figure 2.2D**). Conversely, no distinct dependence on DC_{50} and the extent of substitution was observed with lauryl and palmityl substituents. Accordingly, a significant relationship between binding efficiency (BC_{50}) and complex dissociation ability (DC_{50}) was observed for the modified PEI1.2k taken all together (**Figure 2.3**). Similar trends were observed in studies involving PEI750k acylated with varying carbon chain lengths [20], as well as in previous work of the author's group where linoleoyl acid (LA) was grafted onto PEI1.2k via thio-ester linkages [36]. In both cases, weaker pDNA binding capability and enhanced complex dissociation were demonstrated owing to lipid substitution.

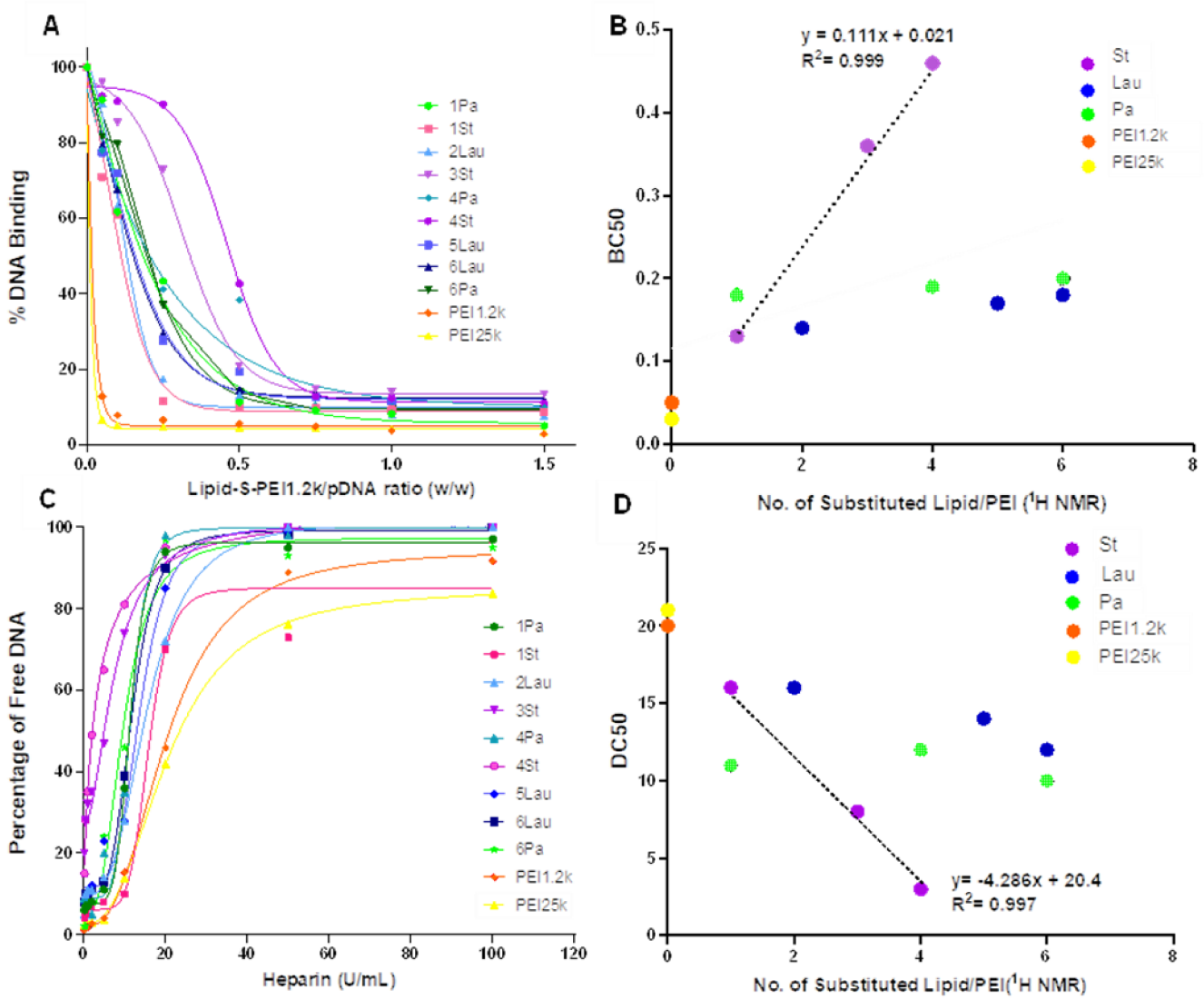

Figure 2.2 (A) SYBR Green binding assay between the lipid conjugates and pDNA. The legend indicated the individual lipids used from Table 2-2 (B) Correlation between BC_{50} and lipid substitution on PEI1.2k for polymers substituted with stearic acid (St), lauric acid (Lau), palmitic acid (Pa) and the unmodified PEIs of 1.2 and 25 kDa (C) Heparin dissociation assay for polyplexes between lipid-substituted polymers and pDNA. The legend indicated the individual lipids used from Table 2-2 (D) Correlation between DC_{50} and lipid substitution in lipid-polymer conjugates. Note that the dashed lines in B and D represent the simplest fit for stearic acid conjugates, showing a clear correlation between BC_{50}/DC_{50} and the number of substituted lipids. In contrast, no such correlation was observed for other lipid-substituted PEI1.2k.

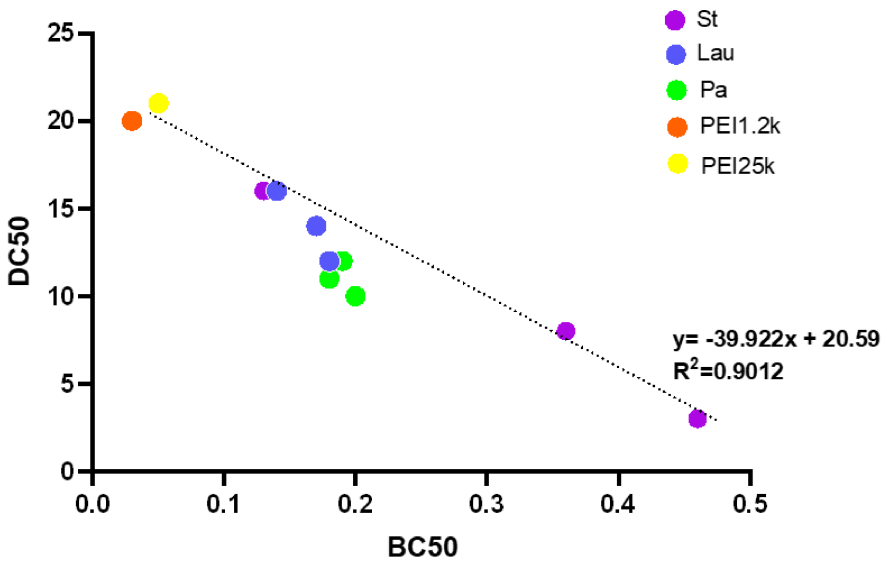

Figure 2.3. Correlation between the BC_{50} and DC_{50} values for Lipid-S-PEI1.2k polyplexes *(*p<0.05)*.

The particle size and ζ-potential of the polyplexes are shown in **Figure 2.4**. Particle size ranges from 140±1 nm to 330±10 nm for the longer aliphatic stearyl-substituents of PEI1.2k polyplexes. The size increases when additional lipids are substituted in PEI1.2k which can be explained by the lesser PEI amine content required for pDNA condensation [14]. Moreover, varying the polymer/pDNA mass ratio (5.0, 7.5 and 10.0) did not have a significant impact on the particle size. Previous studies have proposed the diameter of not more than 200 nm to be the optimal particle size for cell internalization since they have been shown to enter and accumulate in cells via endocytosis, which is better and more rapidly than larger-sized nanoparticles [4,22,31,41,53]. Both the hydrophobic and electrostatic interaction between pDNA and lipopolymer facilitate pDNA condensation, resulting in the formation of compact and stable polyplexes. In several studies, the exposed hydrophobic surfaces of lipid substituents in water were shown to cause aggregation of particles which increases the size drastically [36,17, 23,50].

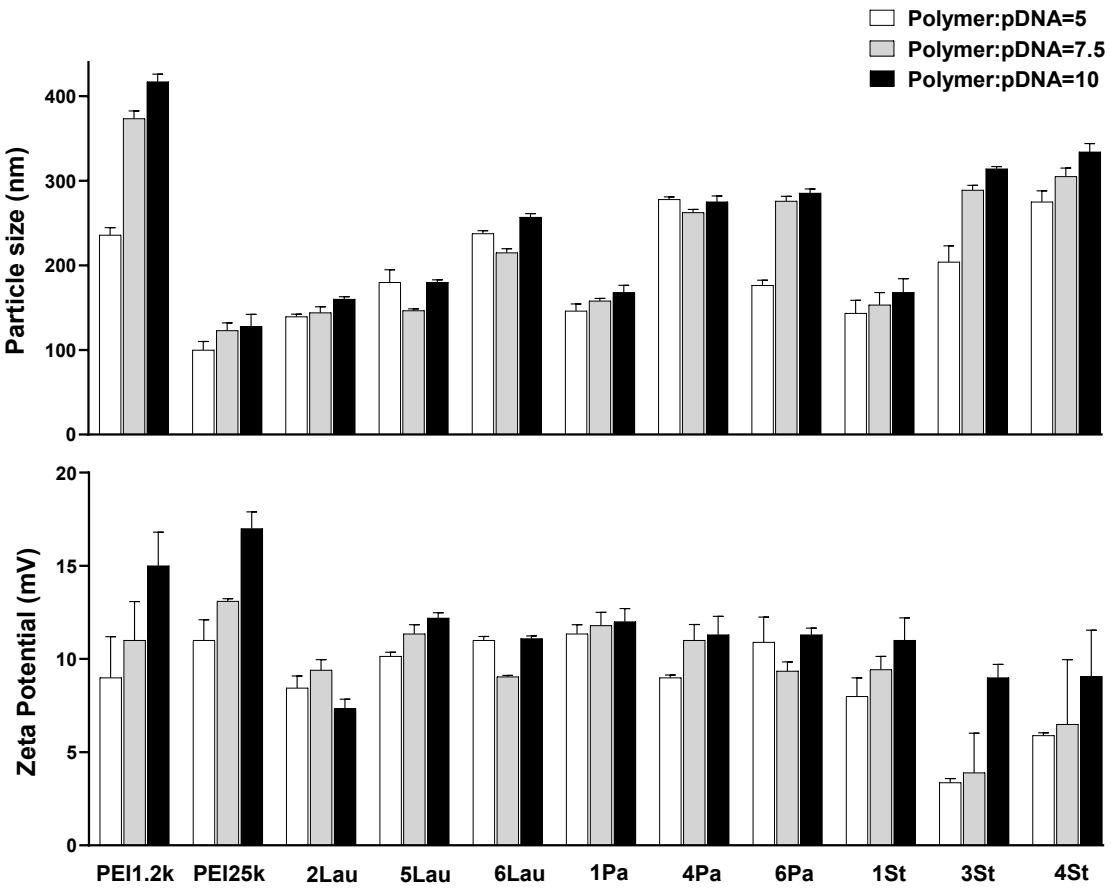

Figure 2.4. Particle size in nm (top) and zeta-potential in mV (bottom) at different ratios of polymer/pDNA polyplexes (5, 7.5 and 10). The horizontal axis indicates the specific polymer conjugate used from Table 2-2.

All polymers exhibited positive ζ-potentials in the range of +4 to +12 mV, indicative of their cationic nature. The incorporation of lipids into PEI1.2k led to a slight reduction in surface charge for most of the formed polyplexes compared with native PEIs. Particularly noteworthy was the apparent decrease in ζ-potentials observed in polyplexes incorporating highly-substituted PEIs with stearyl lipids. This similar result was observed with the N-terminal stearylation of octaarginine, where the surface charge of resulting pDNA polyplexes was relatively reduced, possibly due to partial shielding of the cationic charge by the stearyl substituent. It was assumed that upon complexation with pDNA, a combination of ionic and hydrophobic interactions would occur. However, the presence of exposed stearyl moieties on the surface of the polyplexes partially shield the cationic charge of arginine residues, leading to a decreased ζ-potentials compared to polyplexes of arginine without stearyl substituents [23,25]. The study further revealed that while the cationic charges of polyplexes alone were insufficient to induce high cellular association, the combination of cationic charges and hydrophobic interactions facilitated high levels of adsorption of stearylated polyplexes to the cell surface, thereby enhancing internalization via endocytosis. This pattern

was consistent with previous studies involving partial acetylation [73] and acylation of PEI [20], where despite the reduced surface charge of the polyplexes due to hydrophobic modification, they still exhibited significant non-specific association with the cell membrane, leading to improved transfection efficiency.

2.3.3 Transfection Efficiency and Ternary Complexes in Suspension Cells

The *in-vitro* transfection activity of the synthesized Lipid-S-PEI1.2k conjugates was assessed in "hard-to-transfect" anchorage-independent K562 WT and Jurkat T-cells. The cells display minimal surface area and have low levels of endocytosis, compared to spread, anchorage-dependent cells [40]. The GFP-expressing pDNA as a reporter gene was used at different polymer/pDNA weight ratios ranging from 5.0, 7.5 and 10.0. Building upon the previous experience of improving the transfection efficiency of lipid-modified PEI polyplexes through the incorporation of an additive, as evidenced by previous studies [36,29,30,42,44,45], this approach was employed in these experiments. The additives, typically polyanionic molecules, such as polyaspartic acid, polyglutamic acid, poly(acrylic acid), poly(ethylene glycol) derivatives, dextran sulfate (DS), hyaluronic acid, are believed to enhance transfection through various mechanisms. They stabilize the polyplexes by coating or shielding them from external molecules [44,45], reducing the associated cytotoxicity on target cells, and promoting effective self-assembly [30] and sometimes improving the dissociation of the polyplexes [42]. Additionally, they could enhance the cellular uptake of nucleic acids[29].

Figures 2.5A and **2.5B** illustrate the transfection efficiency of Lipid-S-PEI1.2k/pDNA polyplexes without and with additives, respectively, while **Figure 2.5C** shows the relative efficiency between the two conditions in K562 WT cells. Polyplexes without additives displayed lower transfection efficacy compared to the commercial transfection agent Lipo2000 *(**p<0.01*). However, there was no apparent correlation between the extent/nature of the lipid substituent and the obtained GFP expression since even the lowest lipid substitution (~1 lipid/PEI) was adequate in allowing transgene expression *(*p<0.05*). All three aliphatic lipids employed in this study improved the ineffective LMW PEI1.2k carrier into a more effective pDNA delivery agent. When PA additives were incorporated in the polyplexes, a boost in mean GFP fluorescence was notable with All-Fect, 6Lau-S-PEI1.2k, 4Pa-S-PEI1.2k and 4St-S-PEI1.2k. These polyplexes outperformed the reference standard PEI25k, while 4St-S-PEI1.2k and 4Pa-S-PEI1.2k exhibited transfection efficacy comparable to Lipo2000 at ratios of 7.5 and 10 (w/w), respectively *(*p <0.05).* While some polyplexes did not benefit from the additive formulation, the enhanced fluorescence intensity for certain polyplexes (e.g., as high as 18-fold for 6Lau), as illustrated in **Figure 2.5C**, highlights the impact of additive treatment in these polyplexes.

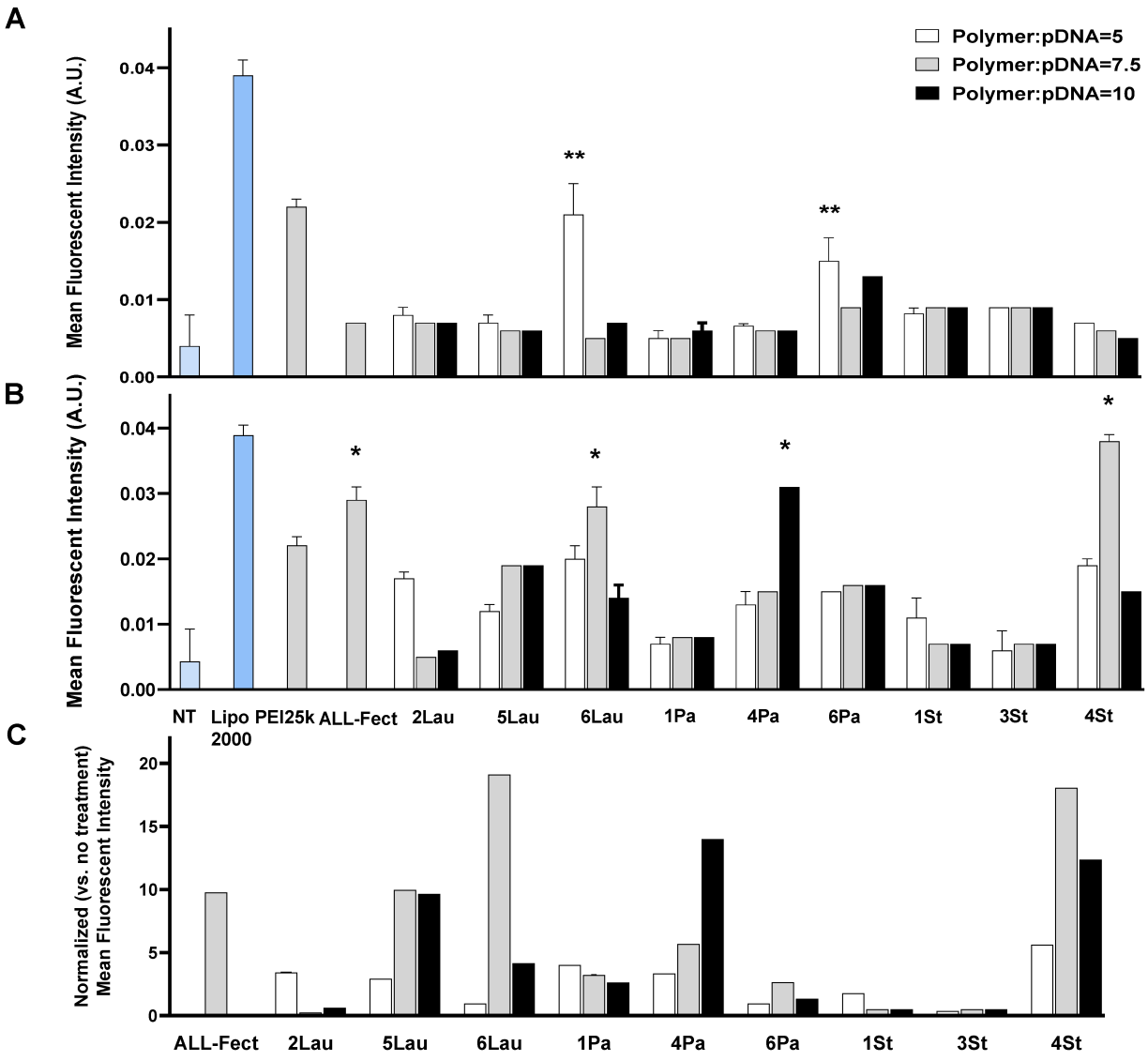

Figure 2.5. Transfection efficiency of Lipid-S-PEI1.2k/pDNA polyplexes in K562 WT cells after 48 hours of transfection. Mean GFP fluorescence of polyplexes without the additive (A) and with the additive (B). The polyplexes were prepared at polymer/pDNA ratios of 5, 7.5 and 10 (w/w), with pDNA/additive ratio of 1:1. (C) Relative GFP expression of polyplexes with and without additives. The ratio of GFP expression with vs. without additive is shown after removing GFP of non-treated cells from each group (***p<0.01* versus Lipo2000, **p<0.05* versus PEI25k; *n*=3).

To further investigate the cellular interaction and internalization of the lipid-modified polymers, the uptake efficiency was evaluated with K562 WT cells **(Figure 2.6).**

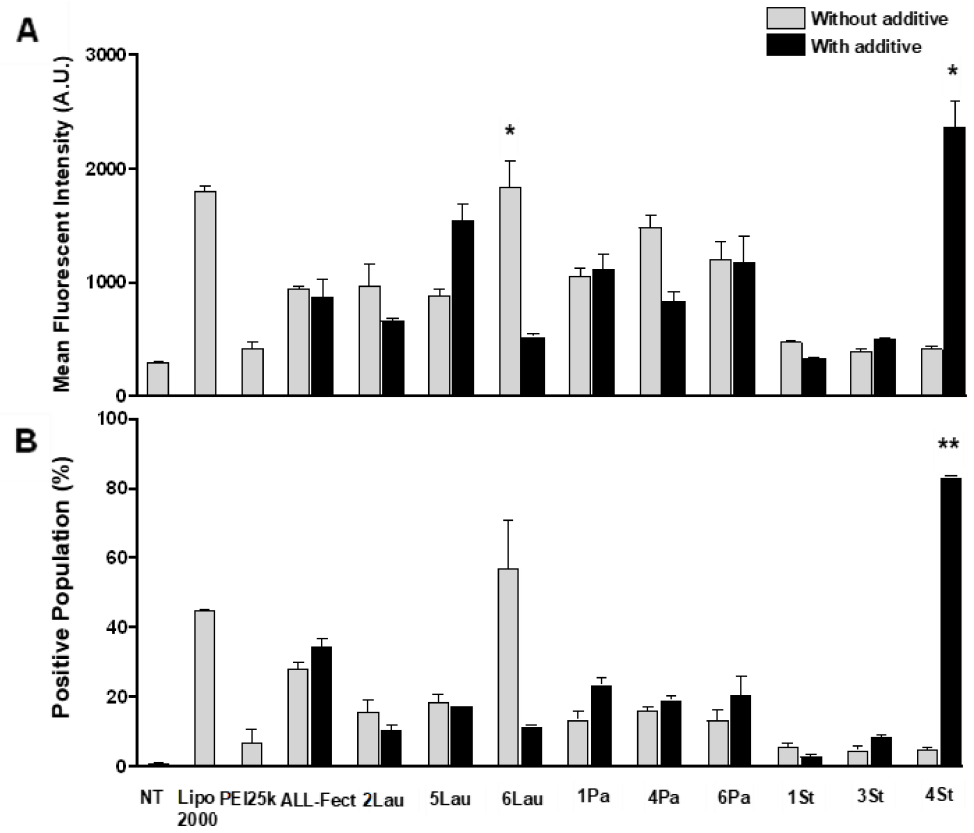

Figure 2.6. Cellular uptake of lipid-substituted PEI1.2k/pDNA polyplexes in K562 WT cells by flow cytometry analysis. (A) Mean fluorescence Intensity (MFI) and (B) Percentage of positive cell population for polyplexes formed with Cy3-pDNA after 72 hr treatment at ratio 7.5 (w/w) ratio *(*p<0.05* versus PEI25k; ***p<0.01* versus Lipo2000, n=2).

Notably, significant enhancements in uptake were observed with the highest stearyl-substituted PEI particularly when additives were included, compared to its less-substituted counterparts *(*p<0.05).* This finding by flow cytometry analysis showed strong agreement with transfection efficiency. The polymers 4St-S-PEI1.2k and 6Lau-S-PEI1.2k demonstrated a beneficial impact of increased lipid substitution on pDNA delivery, with *in-vitro* transfection competence better than PEI25k and comparable cell uptake to Lipo2000, as indicated by mean Cy3-pDNA levels in cells *(*p<0.05)*.

Furthermore, the superiority of 4St-S-PEI1.2k among the synthesized polymers was evident by achieving a notable one-fold higher positive cell population (~80%) compared to Lipo2000 (~40%) *(*p<0.01).* Lipid grafting can modify the physicochemical properties of polyplexes, affecting their cellular uptake pathways [32,39,41]. The improved lipophilicity of the stearyl-substituted polyplexes can be suggested as a possible reason for the higher uptake, which induced compatibility with cell lipid membranes resulting in subsequent uptake and cell internalization, *in vitro* [16,17,46]. This modification results in reduced polymer/pDNA binding strength, which increases the dissociation of Lipid-S-PEI1.2k polyplexes within the cytoplasm [36,40,73], as shown by the binding profile data. Similar improvements in cellular uptake were noted, owing to the lipophilicity post-modification of LMW PEIs by using dodecyl/hexadecyl [27], linolenic acid, lauric acid, and

oleic acid (linked via gallic acid, GA, and p-hydroxybenzoic acid, PHPA) [26], as well as lipoic acid [28]. Moreover, the incorporation of additives into stearyl polyplexes also played a role in enhancing uptake. These anionic polymers facilitated the preferential localization of the hydrophobic groups on the surfaces of the polyplexes. This was achieved by increasing the polyanionic charge within the core of the polyplexes, thereby suppressing the penetration of hydrophobic groups into the core and ultimately boosting surface lipophilicity [17,36].

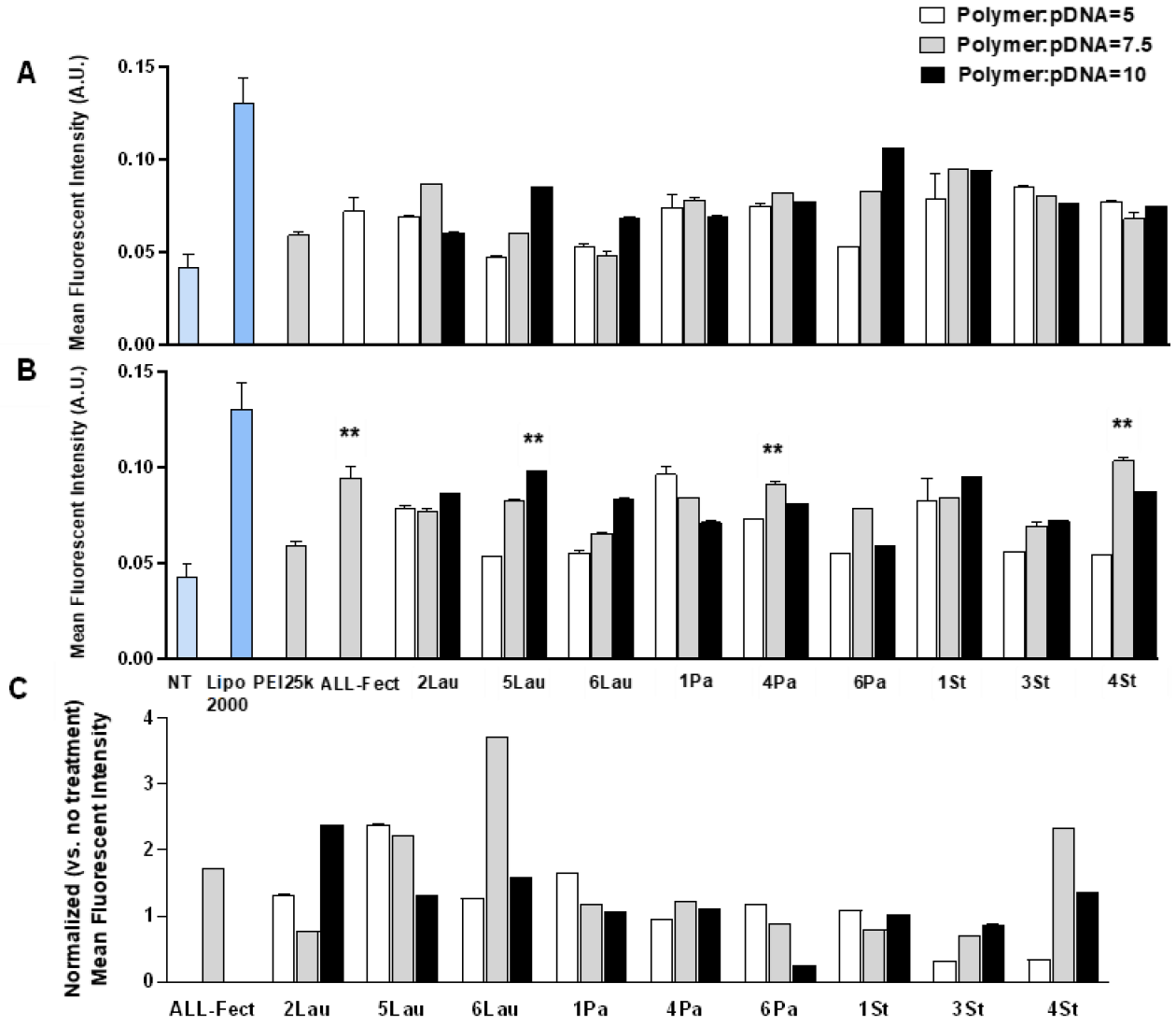

Figure 2.7. Transfection efficiency of Lipid-S-PEI1.2k/pDNA polyplexes in Jurkat T cells after 48 hours. (A) Mean GFP fluorescence of polyplexes without additives and (B) with additives. (C) Relative GFP expression of polyplexes with and without additives. The ratio of GFP expression with vs. without additive is shown after removing GFP of non-treated cells from each group (** $p<0.01$ versus PEI25k; n=3).

The transfection activity of the synthesized Lipid-S-PEI1.2k conjugates was further assessed in Jurkat T-cells (**Figure 2.7).** The polyplexes without additives (**Figure 2.7A**) exhibited lower transfection efficacy compared to Lipo2000 (*p*<0.05*). However, when most polymers (e.g., All-Fect, 5Lau-S-PEI1.2k, 4Pa-S-PEI1.2k and 4St-S-PEI1.2k) were treated with additives (**Figure 2.7B**), they exhibited significantly higher mean GFP fluorescence intensity than the positive control PEI25k *(**p<0.01).* Notably, high lipid-substituted polymers 4St-S-PEI1.2k and 5Lau-S-PEI1.2k showed transfection efficacy comparable to Lipo2000 at ratios of 7.5 and 10.0 (w/w), respectively (*p*<0.05*). Nonetheless, polymers 4St-S-PEI1.2k and 6Lau-S-PEI1.2k still demonstrated the most pronounced effect of additive treatment in the polyplexes (**Figure 2.7C**), consistent with their performance in K562 WT cells.

To investigate further on the impact of integrating additives on the transfection efficiency of Lipid-S-PEI1.2k polyplexes, various additive/pDNA combinations were examined (**Figure 2.8**). Selected lipid-substituted PEI1.2k polymers that had shown improved transfection with additive treatment in Jurkat T-cells were prepared. Results indicate that the addition of PA was found to improve the performance of all polyplexes compared to non-treated *(*p<0.01*). In particular, an additive ratio as low as 1.0, with the exception of 4Pa-S-PEI1.2k, has showed a profound effect on transfection efficiency of the polyplexes. However, further increasing the additive ratio led to a decrease in mean GFP levels, indicating a limitation in its positive effect. The effect peaked again at a ratio of 4.0, but there was no significant difference between additive formulations at ratios of 1.0 and 4.0 (**p<0.01).

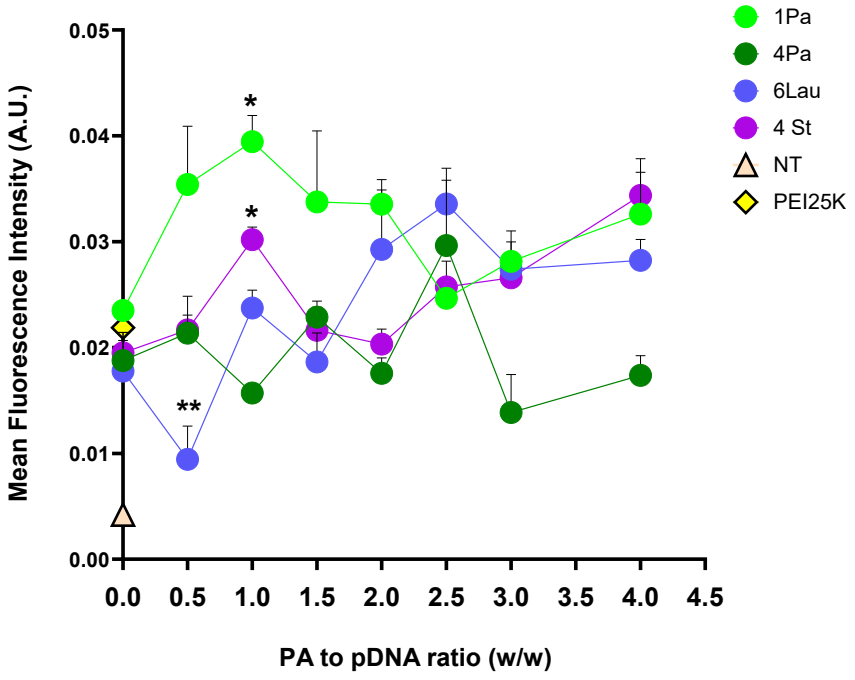

Figure 2.8. Transfection efficiency of Lipid-S-PEI1.2k/pDNA polyplexes at a ratio 7.5 (w/w) with varying PA additive concentrations (0.5 to 4.0) in Jurkat T cells (*p<0.05,* versus PEI25k; ***p<0.01,* versus non-treated; *n*=3).

Additionally, the inclusion of additives in polyplexes was more effective with lower palmityl substitution (1Pa-S-PEI1.2k) when compared to its higher lipid grafting counterpart (4Pa-S-PEI1.2k). Interestingly, the higher stearyl-substituted (4St-S-PEI1.2k) polyplexes obtained comparable improvement in transfection efficiency with the lower palmityl-grafted polyplexes at the optimal additive ratio of 1.0 (*$p<0.05$). Therefore, the impact of the additive treatment was dependent on the type of lipid substituents of the PEI1.2k polyplexes but not on its degree of substitution *(*$p<0.05$)*. In general, incorporating the additive to the Lipid-S-PEI1.2k polyplexes at a ratio of 1.0 enhanced transfection in Jurkat T-cells compared to untreated polyplexes and performed better than PEI25k polyplexes *(*$p<0.05$*). This notable surge in transfection efficiency, observed even with an additive inclusion as low as 1.0 for the nucleic acid polyplexes, aligns with findings from previous studies [36,29,42,45]. Additionally, Rajendran's study [29] confirmed that polymer/pDNA formulations beyond a ratio of 7.5 and the presence of higher PA additive/pDNA ratios exceeding 2.5 resulted in a diminished capacity to transfect Jurkat T-cells.

2.3.4 Evaluation of Cell Toxicity

The primary challenge associated with HMW PEI polymers is usually attributed to their cationic surface charge since the electrostatic interactions that draw polycations to anionic membranes also trigger cytotoxic effects in cells [24,66]. The modification of LMW PEI with lipid substituents improves its ability to deliver nucleic acids to cells, but it may also simultaneously decrease cell tolerance due to enhanced cellular interaction, as observed in previous studies [10,14,16,24,28,32].

The cytotoxicity of the polyplexes was explored by employing an MTT assay in suspension cells and the results were expressed as percent viability loss relative to untreated control cells. Remarkably, the lipid conjugates were essentially non-toxic to Jurkat T-cells (**Figure 2.9A**), with cell viability around 97%, across various polymer/pDNA ratios (w/w: 5.0, 7.5, 10.0), in contrast to PEI25k (cell viability~80%). Also, even the highly substituted polymers showed a comparable safety profile to commercial Lipo2000 at a ratio of 2.5 (w/w). Furthermore, the incorporation of the PA additive (**Figure 2.9Aii**) did not induce toxicity in the cells *(*$p<0.05$*),* consistent with previous findings from the author's group [26], which showed negligible toxicity with the additive treatment in Jurkat T-cells. Likewise, when these polyplexes were introduced to K562 WT cells (**Figure 2.9B**), even the highly-substituted polymers were well tolerated by cells in the ratio of 5.0 and 7.5. However, at the highest polymer/pDNA mass ratio of 10, highly substituted Lauryl polymers resulted in decreased cell viability (~56%). Hence, a correlation between the extent of lipid substitution and cytotoxicity for the lipid-substituted PEI1.2k was obtained *(*$p<0.05$*),* showing the impact of additive treatment (**Figure 2.10**). Notably, it demonstrates the enhanced tolerance of cell toxicity observed with the additive polyplexes compared to those lacking additives. This imminent decline in cell viability for highly lipid-substituted PEI1.2k in K562 WT cells is a predictable consequence of designing polymers with enhanced cellular affinity [14,32]. Achieving an optimal concentration of polyplexes becomes imperative to reduce the undesired effect of cytotoxicity.

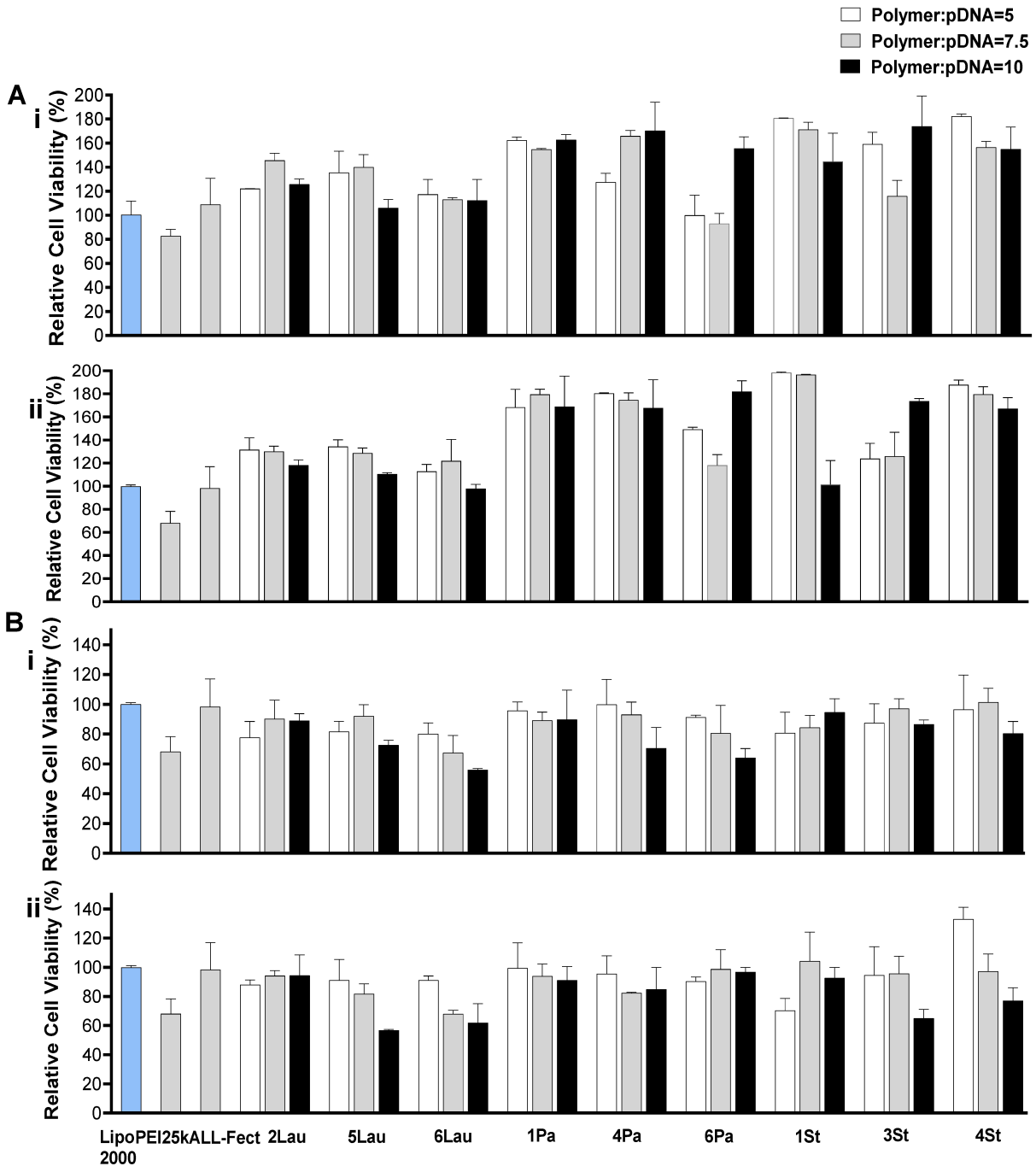

Figure 2.9. Cell toxicity profiles of Lipid-S-PEI1.2k polyplexes in (A) Jurkat T-cells and (B) K562 WT cells. The cells were treated with polyplexes at polymer/:pDNA weight ratios of 5, 7.5 and 10 (w/w) (i) without the additive and (ii) with the additive. All results are normalized against no treatment (NT), which was taken as 100% viability.

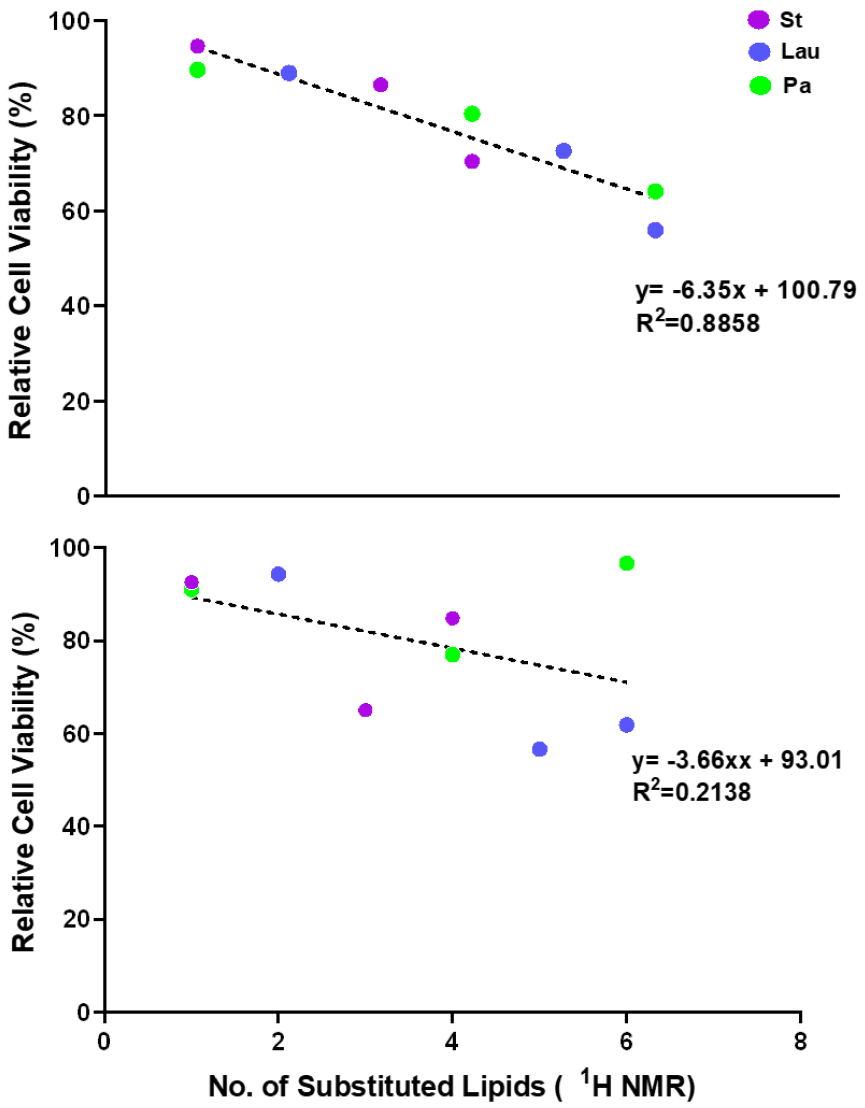

Figure 2.10. **Correlation between the obtained cell viability in K562 WT cells and the extent of lipid substitution on PEI1.2k without (top) and with PA additive (bottom), at polymer/DNA ratio of 10 (w/w).** The cell viability was investigated by the MTT assay, and it was expressed relative to untreated control cells, which was taken as 100% viability. There was an apparent correlation of the extent of lipid substitution and loss of cell viability of polyplexes without the use of additives (*$p<0.05$).

Chapter 3. Investigating Thioether-Lipid Polyethyleneimine Conjugates as siRNA Carriers

3. RNA Interference (RNAi) for the treatment of Chronic Myeloid Leukemia (CML)

Recent advances in understanding disease pathogenesis underscore the role of genetic mutations in various disorders, including cancer [5,12,78,79]. Genetic alterations such as gene deletions or mutations disrupt normal protein production, leading to cellular abnormalities characteristic of cancer [76,78]. Specifically, cancers arise from mutations in genes that regulate cell growth—protooncogenes when activated excessively or tumor suppressor genes when inactivated. This knowledge forms the foundation for gene therapies aimed at manipulating gene expression. These therapies involve introducing foreign genetic material, like DNA or RNA, to modify the activity of specific genes implicated in cancer development, offering tailored approaches for precision medicine [76,77,80].

Chronic myeloid leukemia (CML) exemplifies the success of targeted molecular therapies in leukemia treatment. CML is a myeloproliferative neoplasm resulting from a chromosomal translocation between chromosomes 9 and 22. This translocation fuses the Abelson murine leukemia viral oncogene homolog 1 (ABL) from chromosome 9 with the breakpoint cluster region (BCR) from chromosome 22, forming the BCR-ABL1 fusion gene [32,43,46]. The BCR-ABL fusion gene encodes a constitutively active tyrosine kinase (TK) protein known as p210BCR-ABL, which initiates aberrant downstream signaling pathways [71]. These pathways lead to abnormal cell proliferation, reduced adherence of cells to bone marrow stroma, and decreased apoptosis, contributing to the uncontrolled expansion of malignant myeloid cells in the hematopoietic system [8,43].

Over the past decades, a range of highly specific small molecule tyrosine kinase inhibitors (TKIs) has been introduced for clinical use, leading to significant improvements in patient surviva [32,46]. However, these drugs often lose their effectiveness within 6–12 months in a considerable number of patients due to acquired resistance mechanisms [76]. Even breakthrough TKIs like imatinib, which initially revolutionized the treatment of chronic phase CML, have demonstrated limitations [32,46].

Leukemic cells exhibit inherent plasticity and employ diverse resistance mechanisms that enable them to adapt to drug therapies, resulting in high rates of relapse. Resistance is compounded by a rare subset of leukemic stem cells (LSCs) capable of evading treatment [76]. Current therapeutic strategies primarily focus on TKIs targeting the ABL enzymatic function to disrupt oncogenic tyrosine kinase activity [32,46,76]. While these treatments have boosted overall survival rates, acquired TKI resistance and early clinical relapse persist as significant challenges requiring attention [32,71]. Moreover, some CML patients do not respond to TKIs, highlighting the urgent need for new treatment approaches. Stem cell transplantation serves as an

alternative when TKI therapy fails; however, it carries risks such as chronic graft-versus-host disease and mortality [32, 43, 71].

The potential of RNA interference (RNAi) for gene silencing presents a promising alternative in CML therapy. RNAi, facilitated by small-interfering RNA (siRNA), achieves sequence-specific gene silencing by targeting mRNA, leading to the degradation of oncogenic transcripts and down-regulation of specific proteins involved in cellular overgrowth and inhibition of apoptosis [5,32,43,71,76,79]. Unlike tyrosine kinase inhibitors (TKIs) that target BCR-ABL enzymatic activity, siRNA acts upstream at the mRNA level, offering a complementary therapeutic approach.

However, translating gene therapies like siRNA into effective clinical treatments for diseases like CML requires overcoming significant delivery challenges. Exogenous siRNA is prone to degradation by nucleases in serum, can activate an innate immune response, and lacks inherent cell membrane penetration capability [32,34,81]. Physical methods such as electroporation, while effective in delivering siRNA, often cause high levels of cell death [8,14,65], limiting their practical use. To address these challenges, advanced non-viral delivery systems like polyethyleneimine (PEI) have been developed. PEIs encapsulate siRNA, protecting it from degradation and facilitating its delivery into cells, thereby enhancing therapeutic gene silencing efficiency [5,8,34,79]. Thus, PEIs represent a crucial advancement toward realizing the full potential of gene therapy in precision medicine [8].

3.1 Materials

The branched 25 kDa PEI (PEI25k), 1-ethyl-3-(3-dimethylaminopropyl)- carbodiimide·HCl (EDC), N-hydroxysuccinimide (NHS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) formaldehyde (38.7%), chloroform, hydrochloric acid and acetone were purchased from Sigma-Aldrich (St. Louis, MO). The branched 1.2 kDa PEI (PEI1.2k) which was used for modification with lipids in this study was purchased from Polysciences Inc. (Warrington, PA). All polymers and solvents were used in synthesis procedure as received without any purification. Lipofectamine™ 2000 (referred to as Lipo2000), 1-dodecanethiol (C12; 98%), 1-hexadecanethiol (C16; 98%), 1-octadecanethiol (C18; 98%), methyl acrylate (99%) and triphenylphosphine (99%) was obtained from ThermoFisher. Cell culture medium Roswell Park Memorial Institute (RPMI-1640), supplied with L-glutamine and 25 mM of N-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES), and penicillin (10,000 U/mL)/streptomycin (10 mg/mL) mixture were obtained from Invitrogen (Grand Island, NY). The Leu-Fect C transfection reagent was obtained from RJH Biosciences Inc. (Edmonton, Alberta). The negative control siRNA (siNC) and a custom-synthesized BCR-ABL siRNA (5'-GCA GAGUUCAAAAGCCCTT-3' and 3'-TTCGUCUCAAGUUUUCGGG-5') were obtained from IDT (Coralville, IA).

3.2 Methods

3.2.1 Synthesis of Lipopolymers (Lipid-S-PEI1.2k)

Using commercially-available alkyl thiols, the formation of the ether derivative of the lipid was carried out via the thiol-Michael reaction. A 2.0 M of the lipid thiol in a 20 mL scintillation vial was mixed with 0.4 mmol % phosphine catalyst in (45 wt. %) chloroform, and 2.0 M of the acrylate was added to obtain the product with subsequent hydrolysis using HCl/acetone to obtain the carboxyl end-capped aliphatic moiety. The latter was conjugated onto branched PEI1.2k through the EDC/NHS activation and coupling. The structural composition and level of lipid substitution of the synthesized thioether (Lipid-S-PEI1.2k) were elucidated using ^{1}H-NMR spectroscopy (Bruker 300 MHz, Billerica, MA) using D_2O as solvent, with tetramethylsilane (TMS) as an internal reference. The chemical shifts of the characteristic resonance protons corresponding to the alkyl and PEI were integrated and normalized for the protons in each peak. The transmittance in the FT-IR measurements were recorded using a Thermo Nicolet iS50 FT-IR (Thermo Fisher Scientific Co., Waltham, MA) spectrometer equipped with diamond crystal/built-in all-reflective diamond via attenuated total reflection (ATR) technique.

3.2.2 Cell Culture

The suspension-growing K562 cells (drug-resistant, IMR) and Jurkat T-cells with stable Green Fluorescent Protein (GFP) expression were used in this study. Cells were propagated in RPMI medium containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37 °C in a humidified atmosphere of 95% air/5% CO2. Typical passage of cells was done once a week where spent medium was

discarded by centrifugation (600 rpm, 5 min) with 1:10 dilutions for sub-culturing. The cells were harvested and 300 µL were seeded in 48-well plates at the density of 100,000 cells/mL for transfection.

3.2.3 Cell Growth Inhibition by BCR-ABL Silencing

For the treatment of CML K562-GFP cells, various polymer ratios (w/w) of 2.5, 5.0, and 7.5 were employed, each at a final siBCR-Abl concentration of 50 nM. Similarly, K562-IMR cells were subjected to BCR-Abl gene silencing using polymer-siRNA polyplexes at three different siBCR-Abl concentrations (40, 60, and 80 nM) with a fixed ratio of 7.5 (w/w). In each experiment, polymer-siRNA polyplexes were added in duplicate (100 µL/well) to a 48-well plate for reverse transfection, followed by the addition of 300 µL (100,000 cells/mL) of K562 cells per well, and incubated at 37°C. After incubation, cell viability was assessed using the MTT assay. MTT reagent (5 mg/mL in HBSS) was added to each well and incubated for 30 minutes to 1 hour at 37°C. Subsequently, cells were harvested in microcentrifuge tubes, formazan crystals were sedimented by centrifugation, and dissolved in DMSO. Absorbance readings were obtained using a spectrophotometer (Spectramax 250) at a wavelength of 570 nm. The absorbance values of polymer-treated samples were normalized against untreated cells, which served as the reference control (defined as 100% cell viability), to determine the percentage relative cell viability.

3.2.4 Statistical Analysis

All results were presented as mean + standard deviation (SD) and the data was analyzed using the GraphPad Prism (Version 8.4.3) software. The two-tailed Student's t-test was used to calculate the statistical differences between the group means. The p-values ($p^* < 0.05$ *and* $p^{**} < 0.01$) were considered statistically significant and the number of independent experiments used to generate the data (n) is indicated.

3.3 Gene-Silencing by the Lipid-Conjugated Polymers

Considering the synthesized Lipid-S-PEI1.2k polymers' demonstrated efficiency in delivering anionic pDNA to suspension cells, the potential to extend this ability to deliver other anionic nucleic acids, such as small interfering RNA (siRNA) for gene silencing, was hypothesized. Previous studies from our group have already investigated an expanded library of polymers with hydrophobic substitution (aliphatic or cholesterol) for siRNA delivery, particularly targeting the BCR-ABL oncogene in chronic myeloid leukemia (CML) cells [32,34,46,71,75, 77].

To explore this approach, the functional delivery of BCR-ABL siRNA (siBCR-ABL) using the synthesized Lipid-S-PEI1.2k polymers was initially assessed in CML K562-GFP cells, along with commercial reagents Leu-Fect C and Lipo2000 serving as positive controls. Cell viability using the MTT assay was measured following transfection, at the 3rd and 6th day post-treatment with polyplexes at different polymer/siBCR-ABL ratios (w/w) of 2.5,5.0 and 7.5, at a final siBCR-ABL concentration of 50 nM (**Figure 3.1).**

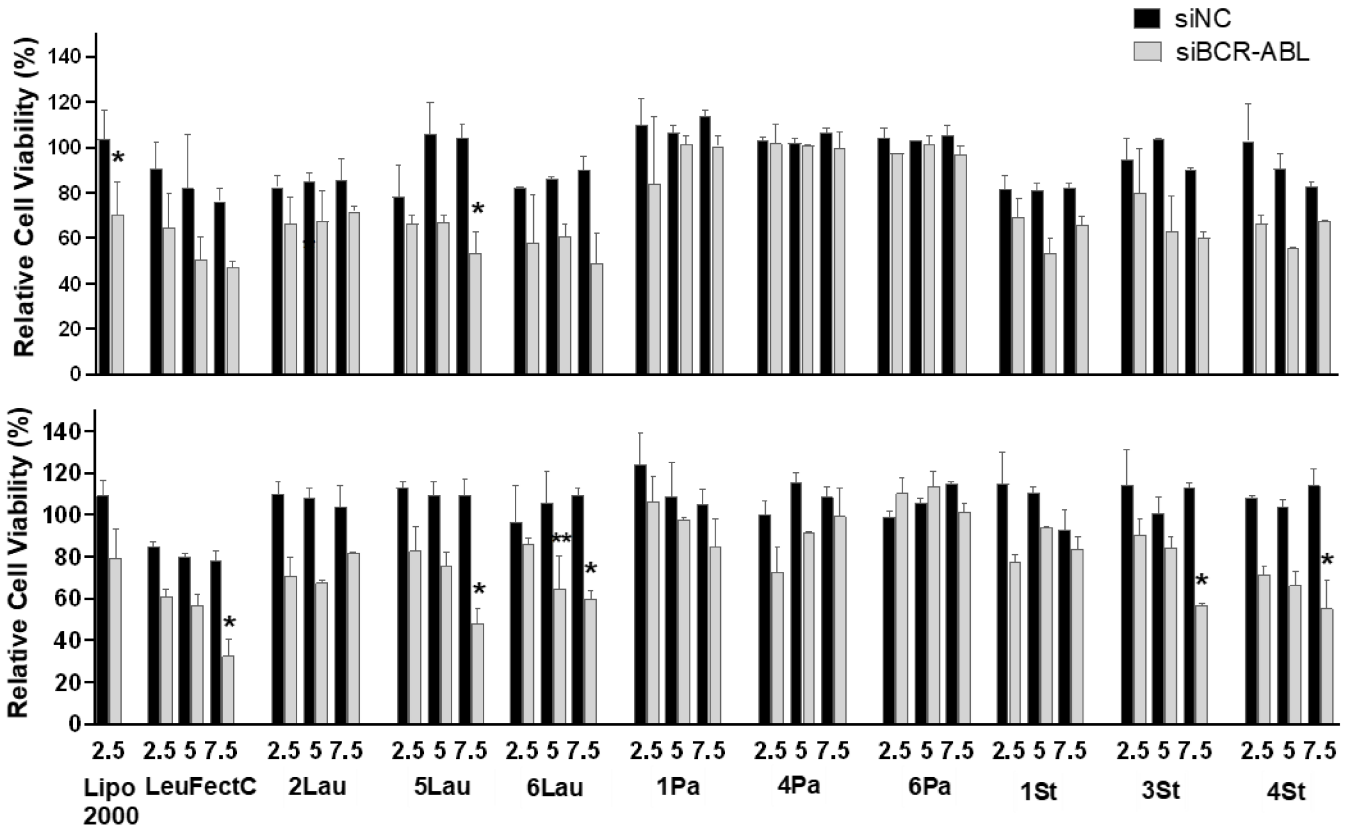

Figure 3.1. Growth inhibition in K562-GFP cells treated with siBCR-ABL polyplexes at 3rd (top) and 6th (bottom) day post-transfection (*$p < 0.05$; **$p < 0.01$). The polyplexes were formed with a negative control siRNA (siNC) and a BCR-ABL specific siRNA at polymer/siRNA ratios of 2.5, 5, and 7.5.

On day 3, the siBCR-ABL complexes formed with Lipo2000 (33%) and 5Lau-S-PEI1.2k (51%) demonstrated significant inhibitory effects on cell proliferation. However, on day 6, complexes comprising Leu-Fect C (45%), 5Lau-S-PEI1.2k (61%), 6Lau-S-PEI1.2k (49%), 3St-S-PEI1.2k (56%), and 4St-S-PEI1.2k (59%) notably diminished cell viability. This suppression of cell growth, in comparison to the negative control (NC) siRNA group, indicates successful delivery of siBCR-ABL via Lipid-S-PEI1.2k complexes in K562 cells, thus demonstrating the potential of the polymers for siRNA therapy. Among all weight ratios, complexes at a 7.5 ratio (w/w) exhibited the most substantial inhibition of growth (~60%), doubling the effectiveness of the commercial transfection agent, Lipo2000.

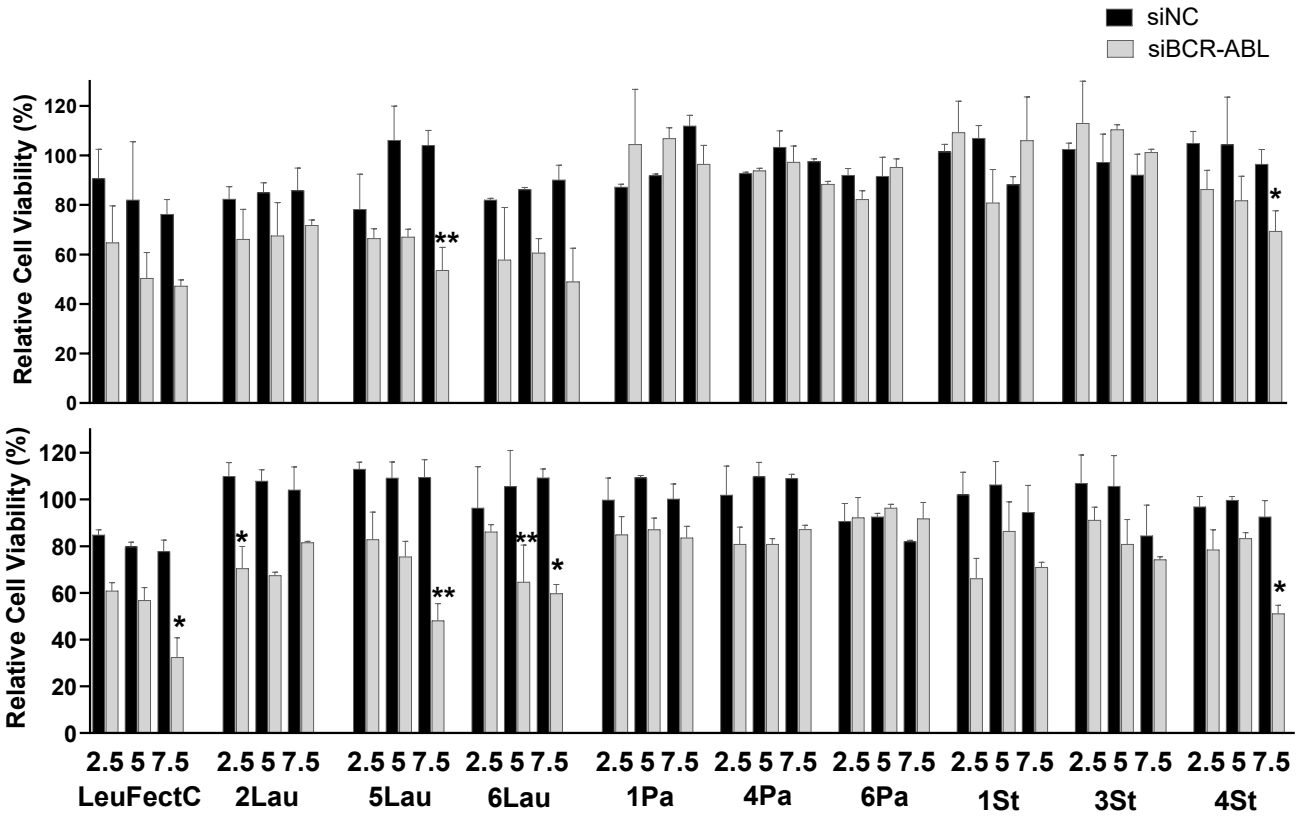

Figure 3.2. Growth inhibition in drug-resistant K562-IMR cells treated with siBCR-ABL polyplexes at 3rd (top) and 6th (bottom) day post-transfection *(*p < 0.05; **p < 0.01*). The polyplexes were formed with a negative control siRNA (siNC) and a BCR-ABL specific siRNA at polymer/siRNA ratios of 2.5, 5, and 7.5. MTT assay was used to determine the viability of the cells and results are normalized against the non-treated cells, which is taken as 100%.

Furthermore, the therapeutic potential of Lipid-S-PEI1.2k polymers in silencing the endogenous oncogene BCR-ABL in drug-resistant CML cells (K562-IMR) was investigated **(Figure 3.2).** The BCR-ABL gene is known to generate a fusion oncoprotein with aberrant tyrosine kinase activity, which plays a crucial role in promoting the uncontrolled proliferation of CML cells [32,76]. To assess the impact of suppressing BCR-

ABL on abnormal cell proliferation, cell viability using the MTT assay was measured following transfection. Polymer/siBCR-ABL polyplexes were formulated at different ratios (2.5,5.0 and 7.5 w/w) with a final siBCR-ABL concentration of 50 nM

As before, the cell viability was evaluated 3 and 6 days post-treatment. Polyplexes containing Leu-Fect C (48%), 2Lau-S-PEI1.2k (31%), 5Lau-S-PEI1.2k (48%), 6Lau-S-PEI1.2k (39%) and 4St-S-PEI1.2k (41%) demonstrated maximum inhibition of cell growth, with an approximate ~48% inhibition *(*p<0.05)* observed on day 6. Conversely, only 5Lau-S-PEI1.2k (33%) and 4St-S-PEI1.2k polyplexes (27%) displayed significant inhibitory effects on day 3. The pharmacological impact of siBCR-ABL was also investigated in the BCR-ABL negative Jurkat T-cells (**Figure 3.3**), where the effect was insignificant compared to CML model K562-IMR cells. This absence of growth inhibition in Jurkat T-cells confirms the specificity of siBCR-ABL polyplexes' therapeutic effect on BCR-ABL-positive K562 cells with the fusion oncogene [76].

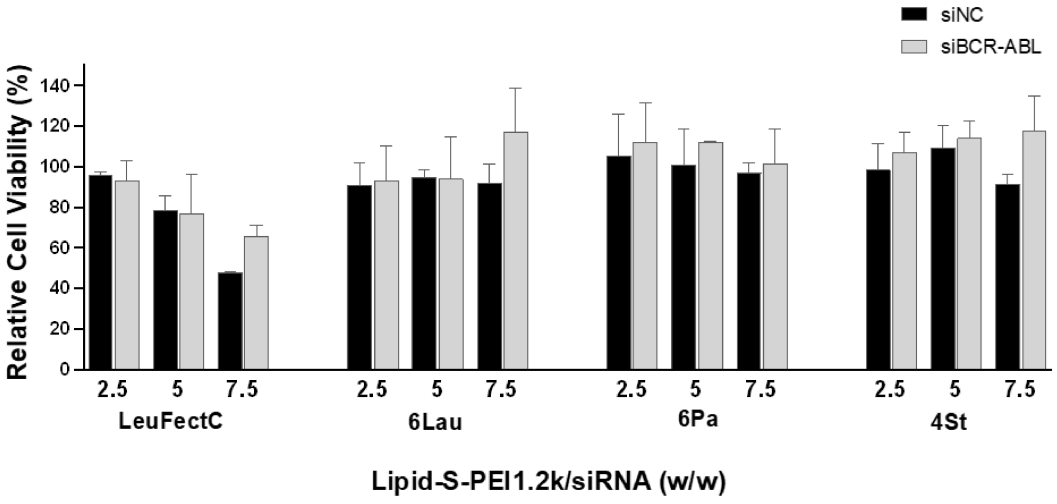

Figure 3.3. Growth inhibition in Jurkat Cells assessed by MTT Assay. The cells were transfected with the indicated polyplexes to give a final concentration of 50 nM siRNA for 3 days before proceeding for the MTT assay. Cell growth is expressed relative to the growth of nontreated cells (taken as 100%).

Finally, the efficacy of siBCR-ABL polyplexes in providing long-term silencing of the BCR-ABL gene was evaluated by assessing their impact on the growth of K562-IMR cells (**Figure 3.4**). Three final siRNA concentrations (40, 60 and 80 nM) were tested at a 7.5 ratio (w/w) using various Lau polymers (2Lau-S-PEI1.2k, 5Lau-S-PEI1.2k and 6Lau-S-PEI1.2k) and highly-substituted polymers (4St-S-PEI1.2k and 6Pa-S-PEI1.2k), all of which had demonstrated promising therapeutic performance in the previous assessment. The functional outcome of siBCR-ABL delivery to suppress the growth of CML K562-IMR cells *in vitro* have been achieved using commercial and non-commercial agents, with effective siRNA concentrations ranging from 70 nM to 2000 nM [60]. However, previous studies by the authors' group [71,75] indicated successful

silencing effects even at lower concentrations, ranging from 40 to 80 nM, which were thus incorporated into this study.

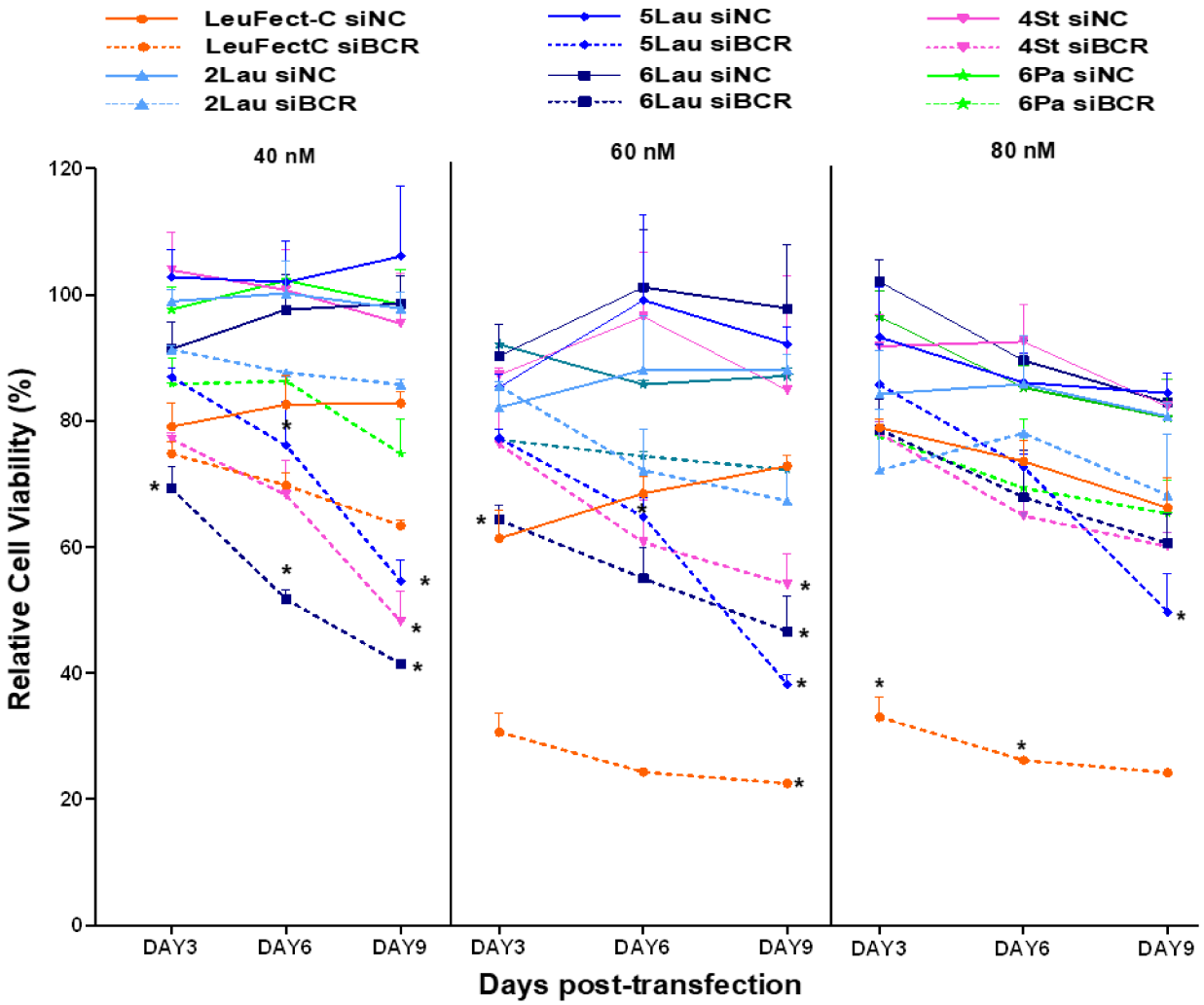

Figure 3.4. Growth inhibition in drug-resistant K562-IMR cells after treatment with different siRNA concentrations at polymer/siRNA ratio of 7.5 (w/w). The MTT assay was conducted after 3, 6 and 9 days post-transfection and cell viabilities were expressed as a percentage of non-treated cells *(*p < 0.05).*

The BCR-ABL complex treatments at both 40 and 60 nM demonstrated comparable significant results, while the highest siRNA concentration tested (80 nM) did not yield the greatest growth inhibition due to a reduction in cell viability, indicating a potential cytotoxic effect at this concentration *(*p<0.05)*. A steady inhibitory effect on cell growth was noted over the 9-day study period, particularly for polyplexes involving Leu-Fect C, 5Lau-S-PEI1.2k, 6Lau-S-PEI1.2k and 4St-S-PEI1.2k. These polyplexes consistently achieved their maximum reduction in cell growth, typically observed on day 9. Specifically, the 40 nM siRNA treatment showed a considerable decrease in % viable cells for polyplexes such as 5Lau-S-PEI1.2k (51%), 6Lau-S-PEI1.2k (57%), and 4St-S-PEI1.2k (47%). At 60 nM, Leu-Fect C (54%), 5Lau-S-PEI1.2k (58%), 6Lau-S-PEI1.2k (55%) and 4St-S-PEI1.2k (38%) displayed the highest inhibitory effects, while at 80 nM, LeuFect C and 5Lau-S-PEI1.2k obtained the highest reduction at 47% and 34%, respectively. The lower-substituted polymer 2Lau-S-PEI1.2k together with 6Pa-S-PEI1.2k obtained a marginal ~20% growth inhibition throughout the 9-day period, even across the three varying siRNA concentrations.

4. Conclusions, and Future Directions

4.1 Conclusions

This thesis has investigated non-viral gene therapy approaches for delivering nucleic acids (NAs), such as plasmid DNA (pDNA) and small interfering RNA (siRNA), through the incorporation of three different aliphatic lipid substituents into low molecular weight (LMW) PEI1.2k via thioether linkages using a thiol-Michael addition reaction. These designed carriers were evaluated for their ability to deliver genes *in vitro* using suspension-growing K562 leukemic cells and Jurkat T-cells.

Effective gene therapy relies on the ability to protect negatively charged therapeutic NAs and deliver them precisely to their intended sites within cells, such as the nucleus for DNA and the cytosol for siRNA. Similar challenges, including stability in systemic circulation, compacting into small nanoparticles for enhanced cellular uptake, and facilitating endosomal release, are critical in carrier design across various technologies. Carrier design strategies, such as condensation with cationic polymers and optimizing encapsulation efficiency, play crucial roles in protecting against degradation in serum and promoting effective cellular uptake. This thesis has addressed these obstacles comprehensively in Chapter 1, focusing on intracellular barriers to delivery and the mechanisms employed to overcome them. By enhancing the efficiency and safety profile of LMW PEIs through rigorous investigation in Chapter 2, this research contributes to advancing gene delivery technologies. Among the lipids explored for pDNA delivery, stearic acid proved to be most effective, achieving over 80% uptake of pDNA-positive cells, and outperforming common lipid-based Lipo2000 and the HMW PEI25k. The superiority of stearic acid may be attributed to its beneficial lipophilicity, which also reduced pDNA binding and increased dissociation ability of modified PEI1.2k. Additionally, the integration of anionic *Trans-Booster* additive to majority of Lipid-S-PEI1.2k polyplexes improved the transfection efficacy with as low as additive/pDNA ratio of 1 (w/w).

In Chapter 3, the study further investigated the Lipid-S-PEI1.2k polymers to deliver siRNA for gene silencing, using the well-established BCR-ABL(+) leukemic K562 cells. The siBCR-ABL polyplexes led to as much as ~58% reduction in growth of K562-IMR cells. This approach further confirmed potent growth inhibition in drug-resistant K562 cells with sustained effects lasting up to 9 days, especially with stearyl and lauryl-grafted PEI1.2k. Overall, the synergistic effects of lipid-grafting LMW PEI1.2k via thioether linkages demonstrated its versatility for improving transfection, minimizing toxicity, and promoting cellular internalization of both pDNA and siRNA in hard-to-transfect suspension cells.

4.2 Future directions

This thesis has demonstrated the versatility of lipid-modified PEI carriers in delivering both plasmid DNA (pDNA) and small interfering RNA (siRNA) to suspension cells. The study focused on using aliphatic lipids for the hydrophobic modification of PEI1.2k, chosen for their controlled grafting capabilities during the reaction [14]. However, an area yet to be explored involves incorporating lipid substituents featuring unsaturated bonds in the backbone chain, such as oleic acid (OA) and linoleic acid (LA). Future studies could explore the integration of thioether linkers into these lipids, known for their enhanced transfection efficiency compared to aliphatic analogues. This improvement is attributed to their polyunsaturated nature, which inherently enhances endosomal disruption activity [14,47]. Further exploration of the structure-activity relationship in these lipid-linker approaches is expected to provide valuable insights essential for optimizing the design of superior non-viral gene carriers. This investigation aims to enhance their overall performance in gene delivery applications by better understanding how these modifications influence crucial intracellular processes such as endosomal escape and nuclear delivery.

Considering the challenges associated with transfecting suspension-growing cells like K562 and Jurkat T-cells, alternative models such as 293T cells, which are easier to transfect, could offer valuable insights. Suspension-growing cells typically exhibit fewer cell surface proteoglycans, which often results in reduced nucleic acid internalization [26,40,43,46,48,75]. Comparing these cell types with adherent cells could provide significant insights into optimizing transfection outcomes.

While acknowledging the distinct physical properties and intracellular trafficking requirements of siRNA and pDNA, this study highlights the potential of synthesized lipopolymers for versatile delivery applications. Further optimization of lipid-modified thioether conjugates specifically for siRNA delivery is warranted. Additionally, exploring the incorporation of polyanionic additives like hyaluronic acid (HA), known for its biocompatibility and targeting capabilities via CD44 receptors overexpressed in certain cell types including mesenchymal stem cells and cancer cells [60,68], could enhance stability and cellular uptake efficiency of both pDNA and siRNA polyplexes, akin to polyethylene glycol (PEG)-based nanocarriers.

Moreover, the versatility of Lipid-S-PEI1.2k carriers warrants exploration in delivering a broader range of genetic materials, such as CRISPR/Cas9 gene-editing tools and mRNA. Expanding the application scope beyond the current study offers promising avenues for advancing genetic medicine and biotechnology. Finally, further validation of these non-cytotoxic, modified LMW PEI polymers in preclinical animal models will be crucial to assessing the efficiency and safety of formulated polyplexes *in vivo*.

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