Designing Nanomedicines for Breast Cancer Therapy: New Synthetic lipopolymers and Optimization of siRNA Formulations against Therapeutic Targets

by

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Abstract

In 2020, breast cancer became the most commonly diagnosed cancer worldwide. Conventional therapies like radiotherapy, chemotherapy and surgery have significant limitations and side effects that are mainly due to their inability to specifically target cancerous cells. Alternatively, gene therapy which can make use of both viral and non-viral carriers piqued the interest about 40 years ago. Viral carriers although available, are worrisome due to their potential insertional mutagenesis and high toxicity. Nonviral carriers, on the other hand, are preferred since they display lower immunogenicity and production costs. Short interfering RNAs (siRNA) carrying nanoparticles have a high potential to overcome the non-specificity of conventional therapies and reduce the expression of disease-associated proteins by mimicking the naturally occurring RNA interference mechanism.

Effective siRNA delivery relies heavily on the selected delivery method due to several challenges associated with siRNA, such as its immunogenicity, primarily through antiviral innate immunity mechanisms involving pattern recognition receptors. Additionally, off-target effects occur when the siRNA's antisense strand partially matches unintended mRNA transcripts. Another non-sequence-specific limitation is the saturation of the RNA interference machinery. These topics are discussed in detail in the first section of the introduction. The second section delves into a range of delivery platforms explored in research, including lipids (like liposomes, micelles, emulsions, and lipid nanoparticles), polymers, peptides (such as cell-penetrating and membrane-perturbing peptides), and combination therapies (like peptide-polymer, liposome-peptide, and lipid-polymers). These platforms are favoured for their unique benefits, such as the presence of lipids in cell membrane composition, versatile chemical properties of polymers, and precise targeting capabilities, alongside peptides' pH-responsive membrane disruption. A thorough

explanation of the induced toxicities of delivery systems, such as immune cell recognition by B-cells or activation of complement cascade, modulation of cellular gene expression or membrane impairment through protein kinase C inhibition, and generation of reactive oxygen species is provided in this section.

To improve cancer therapies, we are engineering novel non-viral lipid substituted PEI carriers specifically for the treatment of breast cancer. Our goal is to obtain high gene silencing through siRNA activity with the least amount of nonspecific toxicity. To address these, we hypothesize that lowering the lipopolymer ratio and adding negatively charged additives to our nanocomplexes will, respectively, reduce cytotoxicity and improve gene silencing ability by facilitating siRNA polyplexes uptake by the cells. To scrutinize our hypothesis, we investigated our nanocomplexes in Chapter 2 by experimenting with potential selected additives in various weight/weight ratios of polymer:additive:siRNA. The effect of different buffers for complex preparation was also explored to provide a better siRNA delivery environment. We first optimized our polyplexes in green fluorescent protein (GFP)+ MDA-MB-231 cells to effectively silence the GFP gene using GFP siRNA. After a series of screening experiments inclusion of phosphate pH 8.0 as complexation media and ratio 1 of lipopolymer proved to be the most effective formulation. Regarding the additives' functionality, addition of either *N*- Lauroylsarcosine Sodium Salt (LS) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in ratio 1 to siRNA was able to improve silencing by ~30% and achieve >60% silencing with the least amount of undesired cytotoxicity. Furthermore, these effects were shown to be persistent for at least 6 days in the time course study. Anti-apoptotic Survivin gene, which was shown to play an important role in breast cancer, was then selected to test our complexes for endogenous gene silencing in MDA-MB-231 cells since there is no strong drug (i.e., small organic molecule) for inhibition of its oncogenic

activity. qRT-PCR analysis and MTT assay revealed >90% silencing and ~70% cell death by the same formulations. These formulations were then examined by flow cytometry assay during which they demonstrated ~97% FAM-siRNA uptake after 24 hours which did not decrease more than 1%, on day 2 post-treatment. During these experiments, we were able to identify two effective additives for siRNA-polyplex formulation. The results show improved formulations for the development of modified PEI-lipopolymers to target selected endogenous genes in wild-type MDA-MB-231 cells.

Preface

The contents of this thesis include previously published material, as indicated below. Each chapter has been conceptualized, researched, and authored by myself, with guidance from my supervisors, Dr. Hasan Uludag and Dr. Carlos Velazquez Martinez. The distinct contributions of other collaborators in each chapter are duly recognized and delineated. Further acknowledgements can be found after each respective chapter.

In **Chapter 1**, we delve into a comprehensive literature review aimed at furnishing a thorough understanding of cancer, with a particular focus on breast cancer. This encompasses an exploration of its prevalence, associated risk factors, various subtypes, and the existing therapeutic interventions deployed in clinical settings. Subsequently, our attention shifts towards the realm of advanced personalized medicines, notably gene therapies, which have garnered significant attention in the context of breast cancer treatment. Within this discussion, we further scrutinize the nuances of siRNA therapies, highlighting the advantages of non-viral carriers and lipid modifications in enhancing their efficacy. Furthermore, we address some of the most prominent biological barriers impeding the efficacy of siRNA therapies, along with proposed solutions to overcome these obstacles.

Chapter 2 constitutes a published research paper authored by Abbasi Dezfouli, S., Rajendran, A. P., Claerhout, J., & Uludag, H. (2023). Designing Nanomedicines for Breast Cancer Therapy. Biomolecules, 13(10), 1559. This chapter encapsulates a pioneering investigation into a novel siRNA therapy intended for breast cancer treatment, along with its evaluation through in vitro studies. Serving as the lead author, I undertook responsibility for devising, executing, and analyzing the experiments, as well as drafting the manuscript. Rajendran, A. P. and Remant K.C. contributed to the synthesis of the polymers utilized in this research endeavour, while Jillian

Claerhout assisted with experimental procedures. The manuscript underwent meticulous editing by Remant K.C. and Hasan Uludag, with Dr. Uludag additionally furnishing invaluable guidance and oversight throughout the course of the study.

Chapter 3 encompasses overall conclusions and prospects for future research. Primarily drawn from the discussions and conclusions expounded in Chapter 2, as well as the insights garnered from the research presented within this thesis, this chapter offers a synthesis of comprehensive themes and implications, alongside outlining potential avenues for further investigation.

Acknowledgements

I extend my sincere appreciation to my supervisors, Dr. Hasan Uludag and Dr. Carlos Velazquez Martinez, for their invaluable guidance, patience, and expertise throughout the course of my master's program. Their insightful feedback, constructive criticism, and consistent support have played a pivotal role in shaping my research and academic development. I am deeply grateful for their mentorship and dedication to my academic growth.

I would like to express my deepest gratitude to my parents, Sepideh and Naser, and my siblings, Samin, Sobhan and Armin, for their unwavering support, encouragement, and sacrifice throughout my academic journey. Their endless love, guidance, and belief in me have been the driving force behind my pursuit of higher education. Their boundless encouragement, even from afar, has been a source of strength and motivation during challenging times. I am forever indebted to them for their immeasurable contributions to my success.

I am also profoundly thankful to my husband, Vahid, for his unwavering support, understanding, and encouragement throughout this journey. His love, encouragement, and belief in me have been a constant source of inspiration. His patience and understanding have been instrumental in balancing the demands of academia and personal life.

Furthermore, I would like to express my gratitude to my lab mates, Amarnath, Luis, Aysha, Remant, Cezy, and Daniel for their assistance, guidance, and support during my master's program. Their contributions have enriched my academic experience and shaped the outcome of this thesis.

To all those who have supported me in countless ways, I am truly grateful. This achievement would not have been possible without your unwavering support and encouragement. Thank you for believing in me and for being part of this remarkable journey.

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List of Abbreviations

ABC	Accelerated blood clearance
AchR	Acetylcholine receptors
ADAMTS13	a disintegrin and metalloproteinase with a thrombospondin type 1 motif, 13
ALC-0315	4-hydroxybutyl) azanediyl)bis (hexane-6,1-diyl)bis(2-hexyldecanoate
ALCL	Anaplastic large cell lymphoma
AML	Acute myeloid leukemia
APC	Antigen-presenting cells
ApoB	Apolipoprotein B
ASGPR	Asialoglycoprotein receptor
bPEI1.2	Branched 1.2 kDa Polyethylenimine
CD44	Cluster of differentiation-44
CDC20	Cell division cycle protein 20
CML	Chronic myeloid leukemia
CNS	Central nervous system
CPP	Cell penetrating peptide
CRT	Calreticulin
CSC	Cancer stem cell
CyPA	Cyclophilin A
DCA	Docosanoic acid
DCIS	Ductal carcinoma in situ
DLin-MC3-DMA	heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPC	Dynamic polyconjugates
DS	Dextran sulfate
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine
dsRNA	double-stranded RNA
DSTAP	1,2-distearoyl-3-trimethylammonium propane

ED_{50}	Effective dose for 50% silencing
EL	Endothelial lipase
EPC	Egg phosphatidylcholine
ER	Estrogen receptor
ES	E-Selectin
FBS	Fetal bovine serum
GA	Gallic acid
Gal8-GFP	Galectin 8-GFP
GalNAC	*N*-acetylgalactosamine
GDS	β-Glycerophosphate disodium salt hydrate
GFP	Green fluorescent protein
GO	Graphene oxide
gRNA	Guide RNA
HA	Hyaluronic acid
HBSS	Hank's balanced salt solution
HDL	High-density lipoprotein
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid
HER	Human epidermal growth factor receptor
HL	Hepatic lipase
HSC	Hepatic stellate cells
I.V.	Intravenous injection
IFN	Interferon
LDL	Low-density lipoprotein
LDV	Lue-Asp-Val tripeptide
LNP	Lipid nanoparticle
LPL	Lipoprotein lipase
LS	*N*- Lauroylsarcosine sodium salt
LSECs	Liver sinusoidal endothelial cells
MDDC	Monocyte-derived dendritic cells
miRNA	Micro RNA
MM	Multiple myeloma

MPP	Membrane perturbing peptide
MPS	Mononuclear phagocytic system
EPR	Enhanced permeability and retention effect
MPS1	Monopolar spindle 1
mRNA	Messenger RNA
MTT	Methylthiazolyldiphenyl tetrazolium bromide
NHP	Nonhuman primates
NLR	NOD-like receptors
NP	Nanoparticle
PA	Polyacrylic acid
PAMAM	Polyamidoamine
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PEG-DMG	1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol
PEI	Polyethylenimine
PKC	Protein kinase C
PKR	Activated protein kinase R
PLA1	A1 phospholipase
PLGA	Poly(lactic-co-glycolic acid)
PMPs	Pathogen-associated molecular patterns
PPI	Polypropylenimine
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
PPM	Potassium phosphate monobasic
PR	Progesterone receptor
PrP	Prion protein
PrP^{C}	Cellular prion protein
PrP^{RES}	Protease resistance prion protein
PRRs	pattern recognition receptors
PS	Phosphorothioate
RISC	RNA induced silencing complex

RLR	RIG-I-like receptors
RNAi	RNA interference
ROS	Reactive oxygen species
SAR	Structure-activity relationship
siRNA	Small interfering RNA
SM-102	heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy) hexyl) amino) octanoate
SNALPs	Stable nucleic-acid lipid particles
SPP	Sodium pyrophosphate
STR-mPEG	Stearyl-polyethylene glycol
TLR	Toll-like receptor
TNBC	Triple negative breast cancer
TSO	tri- Sodium orthophosphate
UTR	Untranslated region
WHO	World Health Organization
ZFN	Zinc finger nuclease

1. Chapter 1. Background and Scope

Parts of this chapter were published in:

Kalita T, **Dezfouli SA**, Pandey LM, Uludag H. siRNA functionalized lipid nanoparticles (LNPs) in management of diseases. Pharmaceutics. 2022 Nov 19;14(11):2520.

1.1 Introduction

Cancer has been defined by WHO as the uncontrolled growth and division of abnormal cells. It is the second leading cause of death globally after cardiovascular diseases and accounted for approximately 9.6 million deaths, or 1 in 6 deaths, in 2018. In males, the most common cancers are stomach, liver, colorectal, lung, and prostate; while in women, the most common cancers are thyroid, breast, colorectal, lung, and cervical. The worldwide prevalence of cancer is increasing, placing significant physical, emotional, and financial pressure on individuals, families, communities, and healthcare systems. Particularly in low- and middle-income nations, healthcare systems are often ill-equipped to handle this growing burden, leading to insufficient access to timely and high-quality diagnosis and treatment for numerous cancer patients worldwide. Conversely, in countries with robust healthcare systems, advancements such as accessible early detection, quality treatment, and comprehensive survivorship care have contributed to improving survival rates for various cancer types (1).

Cancer arises from genetic alterations that affect the normal functioning of cells, particularly their growth and division processes. These genetic changes can occur due to errors during cell division, exposure to environmental carcinogens like those in tobacco smoke and sunlight, or inheritance from parents. The body typically eliminates cells with damaged DNA, but this ability decreases with age, contributing to a higher cancer risk later in life. Each individual's cancer exhibits a distinct combination of genetic mutations, with additional changes accumulating as the tumour progresses. Moreover, different cells within the same tumour may harbour varying genetic alterations.

Genetic alterations contributing to cancer primarily affect three types of genes: proto-oncogenes, tumour suppressor genes, and DNA repair genes, often referred to as "drivers" of

cancer. Proto-oncogenes regulate normal cell growth and division, but when mutated or overactive, they can become oncogenes, promoting abnormal cell growth and survival. Tumor suppressor genes normally control (suppress) cell growth, and mutations in these genes can lead to uncontrolled cell division. DNA repair genes are responsible for repairing damaged DNA, and mutations in these genes can result in additional uncontrolled mutations and chromosomal changes, ultimately leading to cancer development. Understanding these molecular changes has led to the development of targeted cancer treatments aimed at specific gene mutations, which can be effective across various cancer types harbouring the targeted mutation, irrespective of the origin of the cancer cell.

Cancer cells exhibit distinct characteristics compared to normal cells, including uncontrolled growth, resistance to signals that regulate cell division and death, invasion into surrounding tissues, stimulation of blood vessel growth to support tumour growth, evasion of the immune system, accumulation of chromosomal abnormalities, and altered nutrient metabolism. Therapies targeting these abnormal behaviours have been developed to inhibit tumour growth and survival, such as preventing blood vessel formation to starve tumours of essential nutrients (2).

Early detection of cancer leads to better treatment outcomes, reduced morbidity, and lower treatment costs. Two primary strategies for early detection are early diagnosis, which identifies symptomatic cases at the earliest stage possible, and screening, which detects abnormalities in asymptomatic individuals for prompt diagnosis and treatment referral. Treatment options include surgery, medications, and/or radiotherapy, tailored by a multidisciplinary team based on tumour type, stage, and patient preferences. Palliative care enhances patient and family quality of life, while survivorship care addresses long-term effects and recurrence monitoring (1).

Cancers are characterized in two ways: by the type of tissue from which they originate (histological type) and by the primary site, or the region in the body where the cancer first appeared. From a histological approach, there are hundreds of distinct tumours that are classified into six main categories: carcinoma (originating from epithelial tissue, constitutes 80-90% of cancers with two main subtypes adenocarcinoma and squamous cell carcinoma depending on its origin), sarcoma (arises from supportive/connective tissues like bone, tendons, or muscle and is common in young adults), myeloma (develops in the bone marrow's plasma cells), leukemia (cancers of bone marrow, entail overproduction of immature white blood cells, leading to weakened immunity and red blood cells, resulting in anemia, blood clotting issues, and fatigue), lymphoma (arises in lymph nodes or glands, categorized as Hodgkin or Non-Hodgkin lymphoma identified by the presence of Reed-Sternberg cells in the former), and mixed type (components of which can be from the same category or distinct categories) (3).

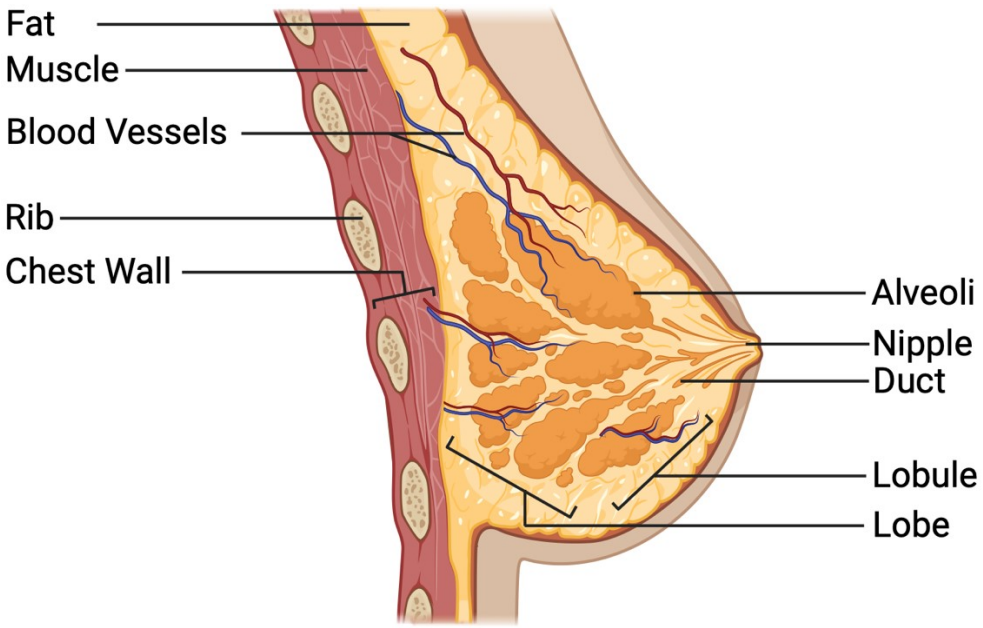

Figure 1.1. Breast anatomy. [created on Biorender]

The breast organ comprises three primary components: lobules, ducts, and connective tissue. Lobules function as milk-producing glands, ducts transport milk to the nipple, and the connective tissue, composed of fibrous and fatty elements, encases and supports these structures (4). Breast cancer involves the uncontrolled growth of abnormal breast cells forming tumours (5). The cancer stem cell (CSC) theory and stochastic theory propose two different mechanisms for breast cancer initiation and progression. The CSC theory proposes that all tumour subtypes stem from the same pool of stem or transit-amplifying cells, with various tumour phenotypes arising from acquired genetic and epigenetic mutations in these cells. Conversely, the stochastic theory suggests that any breast cell can accumulate random mutations over time, eventually transforming into a tumour cell. While both theories are supported by substantial evidence, neither provides a comprehensive explanation for the initial development of human breast cancer (6). In recent years, female breast cancer incidence has risen steadily at 0.6% annually since the mid-2000s, primarily

mainly due to localized-stage and hormone receptor-positive diagnoses. Notably, younger women (<50 years) saw a steeper increase (1.1% vs. 0.5% per year) than their older counterparts. Contributing factors include declining fertility and increasing obesity, though obesity isn't linked to premenopausal breast cancer. Mammography rates remained stable, suggesting that screening rates are not a significant factor (7). In 2024, an estimated 310,720 new cases and 42,250 deaths from breast cancer among women will occur in the United States (8). There are several types of breast cancer determined by which cells in the breast become cancerous. Most breast cancer originates in breast milk ducts or lobules. Early stages, like in situ cancer, are non-life-threatening and detectable. Invasion leads to tumor formation causing lumps or thickening. Invasive cancers spread to nearby tissues or lymph nodes, potentially metastasizing to other organs, posing life-threatening risks, and could result in fatality if untreated (5). Breast cancer stands as the most prevalent noncutaneous cancer in U.S. Women, with 56,500 cases of ductal carcinoma in situ (DCIS) and 310,720 cases of invasive disease reported. Men represent 1% of breast cancer cases and fatalities (8).

Personal history of breast cancer, family history of any kind of cancer, mutations in the BRCA gene, large breasts, late menopause, late or no pregnancy, hormonal replacement therapy, obesity and estrogen are among the main risk factors of breast cancer. Early detection is crucial for effective management and improved outcomes. Mammography, ultrasound, and biopsy are crucial diagnostic tools in breast cancer detection. Mammography utilizes low-dose X-rays for imaging, while ultrasound employs high-frequency sound waves to differentiate between solid tumours and cysts. Biopsy, the gold standard, involves tissue removal for laboratory examination. Various biopsy methods exist, including core and lymph node biopsies, as well as fine needle aspiration, each serving specific diagnostic purposes in breast cancer assessment (6).

Triple-negative breast cancer (TNBC), characterized by a lack of expression of estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptors (HER) (9), accounts for 20% of breast cancers and is characterized by aggressive behaviour, high metastatic potential, and poorer survival compared to other subtypes, irrespective of stage. TNBC exhibits a higher rate of distant relapse within the first five years of diagnosis. Race (black and Latinx vs. white), age (below 40 years old) and genetic mutations (mutations in the BRCA1 gene) are some of the risk factors that contribute to higher chances of TNBC. The lack of receptors poses multiple challenges in treatment advancements compared to targeted therapies for other subtypes (10). The treatment for TNBC differs depending on the stage of the cancer, size of the tumour and responsiveness to previous treatments. For stages I-III TNBC, treatment options vary based on tumour size and lymph node involvement. Surgery, often followed by adjuvant chemotherapy or radiotherapy, is a common approach. Non-adjuvant chemotherapy may precede surgery, with additional treatments like oral chemomedicine capecitabine, targeted drugs like olaparib for those with BRCA mutations, or pembrolizumab if needed post-surgery. In stage IV TNBC, initial treatment involves chemotherapy, often in combination with targeted drugs like PARP inhibitors or immunotherapy if specific biomarkers or high genome instability are present. For recurrent TNBC, options include pembrolizumab with chemotherapy (for local recurrence) or antibody-drug conjugates like sacituzimab govitecan with chemotherapy if recurrence happens in other parts of the body (11).

The fundamental barriers to chemotherapeutic cancer treatments are the development of anticancer drug resistance and off-target damage. For instance, anthracyclines such as doxorubicin and epirubicin can lead to bone marrow suppression, and cardiotoxicity, and are limited by cumulative dose limitation and resistant development. Taxanes like paclitaxel, docetaxel and

cabazitaxel might cause peripheral neuropathy, gastrointestinal issues and hypersensitivity reactions. Platinum drugs like cisplatin and carboplatin oxaliplatin may result in kidney damage and allergic reactions along with bone marrow suppression, peripheral neuropathy and gastrointestinal issues. In terms of other treatments, surgery for example has the risk of disease residual that recurs and has limited effectiveness against metastasis in advanced stages. Additionally, radiation causes myelosuppression, skin reaction and lymphedema. Moreover, PARP inhibitors like olaparib and talazoparib might cause myelodysplastic syndrome, acute myeloid leukemia and immunosuppressive-induced sepsis as well as fatigue and drug resistance. Finally, P13/AKT/mTOR Inhibitors such as ipatasertib and everolimus suffer from problems like pulmonary toxicity, elevated liver enzyme mucositis and hyperglycemia (9).

To overcome such limitations, scientists are looking for alternative treatments. Gene therapy, which is defined as the modification of one's genetic material to treat a disease or relieve its symptoms, sparked the interest of researchers about 50 years ago due to its simple principles. Both DNA and RNA can be used for gene therapy. In comparison to DNA, RNA molecules are safer since they do not have the risk of integration into the genome. They are also biologically less stable, and their application is relatively simple, rapid, and cost-effective (12). Gene therapy can be used to either induce or inhibit the expression of targeted genes. The latter makes use of a naturally occurring mechanism known as "RNA interference" (RNAi) (see Figure 1.2.). Large-scale production of RNA molecules as well as the discovery of stabilizing building blocks have enabled the emergence of RNA molecules as a new therapeutic option on blood diseases. Five primary kinds of therapeutic RNAs have found applications in therapy, which include (i) short (small) interfering RNA (siRNA) and antisense RNAs, (ii) messenger RNA (mRNA) that generates recombinant proteins, (iii) RNA aptamers that target proteins, (iv) guide RNAs (gRNAs)

that guide DNA-modifying CRISPR enzymes, and (v) RNAs that modify genetic sequences, such as trans-splicing ribozyme.

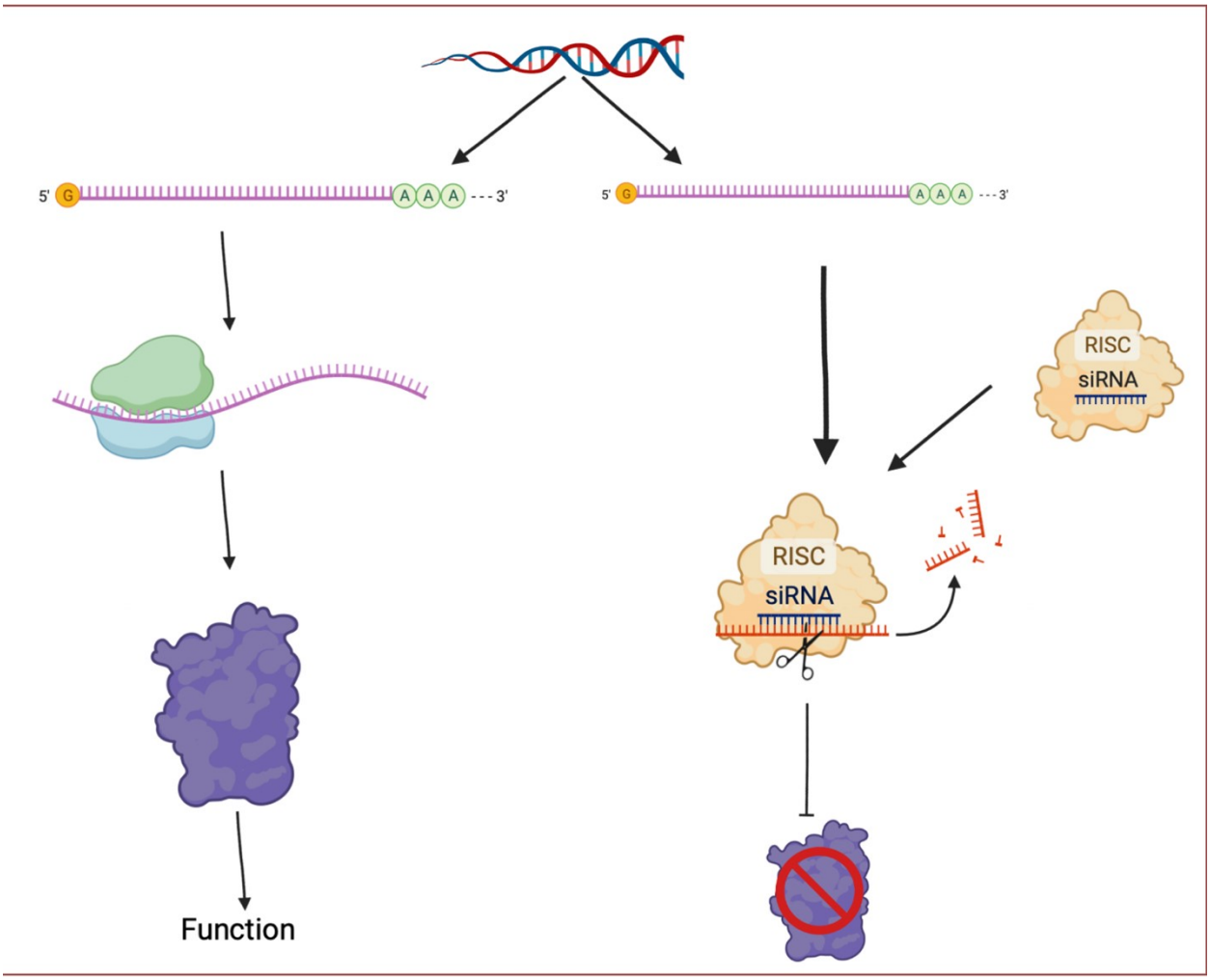

Figure 1.2. RNAi mechanism.

Nanomaterials with distinct physicochemical properties have gained popularity as the future of drug delivery means. Nanomaterials' small size enables enhanced drug loading capacity, extended blood circulation time, and higher cellular internalization and tissue penetration. Nanostructures can be finetuned for adjustable drug loading and release, tailored distribution to disease sites and enhanced biological efficacy. Lipid nanoparticles (LNP) have the potential to overcome the limitations of viral deliveries by simplifying procedures and enabling redosing. The

biodistribution and therapeutic effects of LNPs are significantly affected by their physiochemical features like size, zeta potential, and dispersity. However, early attempts to manufacture LNPs were plagued by problems such as high particle sizes, large batch-to-batch variance, and difficulty in mass production, rendering LNPs unsuitable for clinical application (13). Table 1.1. summarized the siRNA drugs that have been approved by FDA for clinical use.

Table 1.1. FDA-approved siRNA therapeutics

Drug (Brand Name)	Target	Total Dose	siRNA Formulation
Patisiran (Onpattro)	hereditary transthyretin-mediated amyloidosis (hATTR)	0.3 mg/kg IV infusion	lipid nanoparticle (LNP)
Givosiran (Givlaari)	acute hepatic porphyria (AHP)	2.5 mg/kg SC	ESC-GalNAc-siRNA conjugate
Lumasiran (Oxlumo)	primary hyperoxaluria type 1 (PH1)	3 mg/kg SC followed by 1.5 mg/kg	ESC-GalNAc-siRNA conjugate
Inclisiran (Leqvio)	lowering LDL cholesterol	300 mg SC	ESC-GalNAc-siRNA conjugate

1.1.1 siRNA Delivery Limitations

1.1.1.1 Inherent Limitations of Naked siRNA as a Therapeutic

The naked siRNA has poor pharmacokinetic properties including limited cellular uptake, the possibility of eliciting an immune response(s), nuclease-susceptibility, and fast clearance. Some of these shortcomings have been overcome with the development of chemical siRNA analogs while others require an effective delivery vehicle that can protect the siRNA and deliver it intact to its destination to implement the RNAi. The delivery systems explored will be articulated in Section 1.1.2. Here we will delve into the intrinsic limitations of siRNA molecules, which are categorized into 3 main groups (14) and are important to understand and fully implement siRNA-based RNAi.

1.1.1.1.1 Immunogenicity

The exogenous RNA is naturally recognized by innate immunity through pathogen-associated molecular patterns (PAMPs) as part of the antiviral mechanisms. RNA recognition

happens by pattern recognition receptors (PRRs), which involve immune responses such as secretion of Type I Interferon (IFN), chemokines and proinflammatory cytokines which, if excessive, can lead to organ damage, failure and death. Cytoplasmic PRRs involve RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and double-stranded (ds)RNA-activated protein kinase R (PKRs) whereas endosomal receptors include toll-like receptor 3 (TLR3), TLR7 and TLR8. The siRNA structure, sequence, length and delivery mode are among the factors that affect RNA recognition (15). Regarding the sequence, it has been reported that TLR3 recognizes dsRNA while TLR7/8 recognizes both siRNA and dsRNA with a higher affinity (16–18). TLR7/8 has been shown to use a sequence-dependent recognition where, for instance, U-rich sequences activate TLR7/8 to secrete proinflammatory cytokines in human peripheral blood mononuclear cells (PBMCs) (19) and GU-rich motifs activate NFκB and proinflammatory cytokines (IL-6 and TNF) in dendritic cells and macrophages (20). A minimum length of 19 base pairs (bp) is required for immune recognition even in the presence of immunostimulatory motifs (21,22). The capacity of hazardous siRNA to cause cell death was examined when the duplex size was lowered from 19 bp to 17 bp. Previous research has found that duplexes shorter than 19 bp target mRNA sequences are inefficient, implying that RISC fails to induce RNAi when duplex length falls below a certain threshold (23). When the length of 19-bp toxic siRNA was lowered by 2 bp (resulting in 17 bp length), toxicity decreased considerably, demonstrating that RISC entrance and/or processing were required for toxicity induction (24). The delivery mode also contributes to RNA immunogenicity; delivery through the endosomal pathway exposes the siRNA to TLR3 and TLR7/8 while delivery vesicles facilitating endosomal escape expose the siRNA to cytoplasmic PRRs and activate the RIG1/MDA-5 pathway involved in Type I IFN secretion. Moreover, the alternative pathway of complements could be activated by high phosphorothioate content (PS) in oligonucleotides. Ocular

administration for instance is advantageous since lower therapeutic doses are required for local action and the possibility of systemic toxicity is likewise limited within the local area. Similarly, the mucus on the surface of the gastrointestinal tract promotes direct delivery of RNAi formulations to its site of activity, reducing any unwanted effects and systemic toxicity to surrounding tissues and organs (25). Chemical modifications to reduce the immunogenicity may be needed and this approach has been compatible and necessary with clinically tested siRNAs (18,26). These alterations can involve nucleotide modifications and phosphate backbone modifications (27).

1.1.1.1.2 Hybridization-Dependent Toxicity

Unwanted effects may result from the hybridization of siRNA with intended (on-target) or unintended (off-target) transcripts downstream of RNA-induced silencing complex (RISC) due to partial complementarity of the antisense strand and mRNA transcripts (15). It has been reported that the complementarity of the siRNA 5'-end (seed region) with the 3′-untranslated region (3′-UTR) of the transcripts, also referred to as microRNA (miRNA)-like off-target effect, is primarily responsible for off-target transcript silencing by siRNAs (28,29). The thermodynamic stability and Watson-Crick pairing of the seed duplex created between the seed and target are required for sequence-induced off-target toxicity as well (30). Base pairing in the seed region was also reported to be the main cause of siRNA off-target effects; however, non-seed regions and target sequences were also found to be important, since the target sequence's GC-content and a subsection of the non-seed region's melting temperature could have an impact on the severity of siRNA off-target effects (31). Although siRNA design algorithms utilize seed area sequences with low complementarity to 3′-UTRs to reduce the potential miRNA-like off-target effects, some degree of similarity with some 3′-UTRs will inevitably occur. Nonetheless, several chemical alterations

have been shown to attenuate these unintended consequences (15). 2′-OMe substitution of the guide strand at position 2 (32), modification at unlocked nucleobase analogues in the passenger and guide strands (33), or into position 7 of the siRNA (34), along with the incorporation of locked nucleic acid (35,36), are highly effective to this end. The hepatotoxicity of the *N*-acetylgalactosamine (GalNAc) conjugated siRNAs in rats can be mitigated by modifying the seed area sequence or adding a destabilizing glycol nucleic acid nucleotide into it (37). The sense strand-mediated off-target gene silencing is the result of the passenger strand's assembly into the RISC complex, which is another mechanism of off-target effects. Reducing the passenger strand RISC assembly has been achieved through the successful application of several chemical modifications, such as 5′-biotinylation of the sense strand (38,39), 5-nitroindole modification at position 15 of the sense strand (40), and design of small internally segmented interfering RNAs (41), Dicer-substrate siRNAs (42), and asymmetric shorter-duplex siRNAs (43).

On-target toxicity, on the other hand, can occur by either excessive pharmacological effects in target cells (e.g., at supratherapeutic doses of siRNAs) or on-target effects on undesirable cells (15). Fitusiran, the GalNAc-conjugated siRNA that targets antithrombin to enhance hemostasis in hemophilia, was observed to cause thrombosis and disseminated intravascular coagulation in non-diseased animals at excess doses (44). Compared to on-target effects, off-target effects are considerably more susceptible to siRNA doses and may be the primary source of toxicity at large dosages (15), which is sometimes necessary due to limitations of delivery. By comparing various siRNA concentrations (1 to 25 nM), Caffrey et al. reported that by administering siRNA at relatively low concentrations (1 nM), off-target effects can be notably mitigated while still maintaining effective silencing of the desired target. This reduction in off-target effects was demonstrated for both modified and unmodified siRNAs directed towards STAT3 or hexokinase

II. At lower concentrations, the silencing of transcripts exhibiting complementarity to the siRNA seed region was diminished. Likewise, non-complementary off-targets, including genes associated with immune response up-regulation, were also diminished at lower doses. Notably, they observed a concentration-dependent off-target effect in the form of unintended induction of caspase activity upon treatment with a siRNA targeting hexokinase II (45).

1.1.1.1.3 Saturation of RNAi Machinery

The siRNA therapeutics possess the ability to impact gene expression in a manner that is not specific to a particular sequence. This arises from the saturation of siRNA machinery, influencing the processing of endogenous miRNA (15). An initial example of this effect was reported in a mouse model that displayed liver toxicity and fatalities due to excessive expression of shRNAs in hepatocytes (46) and saturation of exportin 5, which is crucial for transporting pre-miRNA from the nucleus to cytoplasm. This saturation resulted in a global decrease in levels of miRNA in hepatocytes. However, in scenarios involving siRNA and miRNA therapeutics entering the RNAi pathway at later stages, downstream processes beyond exportin 5 activity may contribute to the undesired activities. For example, externally introduced siRNAs and miRNAs may compete with endogenous miRNAs for RISC, binding sites on mRNA (specific or non-specific), or other downstream elements, thereby influencing widespread gene regulation (47). Consequently, while the intended transcripts are being suppressed, the expression of undesired genes might be simultaneously altered. Based on a mathematical model to explain how alterations in concentrations of miRNA could elucidate the unexpected positive effects of miRNA on their targets, the net positive impact of miRNAs on transcripts, presumed to be downregulated, stemmed from miRNA competition in an environment with multiple miRNA targets (48). These studies

emphasize the significance of fine-tuning the dosage of siRNA treatments to avoid overwhelming the cellular RNAi machinery.

1.1.2. Critical Aspects of siRNA Delivery Systems

The effective non-viral delivery of siRNA, which is currently the pharmacologically acceptable model of delivery in clinics, is reliant upon the formation of supramolecular complexes in delivery vehicles (49). The scalability, ease of synthesis and relatively lower immune recognition of non-viral delivery vehicles, including peptides, polymers, and lipids, have been considered advantageous (50–53). Lipids, by virtue of their presence in cell membranes, and unique solubility features capable of forming dynamic assemblies under aqueous conditions, have found utility to entrap and transport the siRNA at cellular and organ levels. Polymer-based delivery systems present a viable alternative due to their varied chemistries, structures, and adaptable properties suitable for tailoring (54–57). Peptides, including cell-penetrating peptides (CPPs) and membrane perturbing peptides (MPPs), have demonstrated efficacy in siRNA delivery owing to their ability to specifically target cells, their pH-responsive membrane disruption, their efficient encapsulation and their excellent membrane transport properties (58–61).

1.1.2.1 Lipidic Delivery Systems

In vivo delivery of siRNA can be implemented using various lipid assemblies, including liposomes, micelles, emulsions, and LNPs (60-64). Lipofection, i.e., lipid-mediated transfer of nucleic acids into animal and human cells, was first used in 1987 (65,66) and resultant liposomes have been adopted for carrying a range of pharmaceuticals, including siRNA. Figure 1.3. illustrates the key components of LNPs and their role in successful siRNA delivery. The LNPs, a highly

versatile delivery system approved by the FDA and EMA, consist of four lipids; (i) a neutral helper lipid that protects the cargo in a hydrophobic medium by creating a lipid core, (ii) cholesterol that provides a balance between fluidization and condensation of the lipid bilayer, (iii) a polyethylene-glycol (PEG)-lipid that helps to increase stability by preventing aggregation and prolonging the circulation time via preventing the interactions with serum proteins and (iv) an ionizable or cationic lipid to reversibly bind the nucleic acid. Ionizable lipids such as Dlin-MC3-DMA or C12-200 are neutral at physiological pH, so they limit interactions with anionic membranes of blood cells and improve biocompatibility. The lipids become protonated after internalization due to lower endosomal pH and facilitate endosomal escape. The cationic lipids, on the other hand, are permanently charged and readily interact with anionic nucleic acids under physiological conditions (12,14).

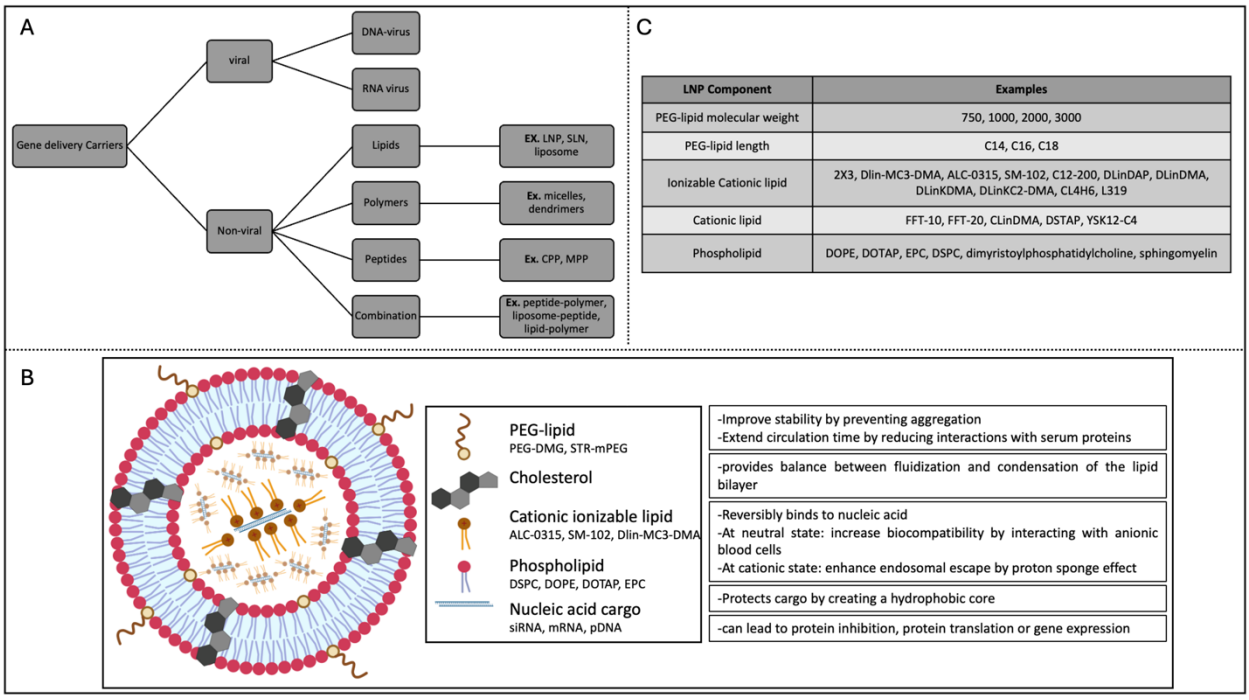

Figure 1.3. Gene delivery carriers with a focus on LNP A. Different gene delivery carriers. B. LNP components. C. Examples of variation in LNP components and intended actions.

Besha et al. investigated the differences among four ionizable cationic lipids, namely DLinDAP, DLinDMA, DLinK-DMA, and DLinKC2-DMA, with respect to their uptake, intracellular trafficking and gene silencing in primary antigen-presenting cells (APCs) macrophages and dendritic cells. They reported DLinKC2-DMA as the most potent formulation evident through its superior silencing and intracellular delivery. This formulation was effective for silencing the target gene in APCs in the spleen and peritoneal cavity in vivo. Moreover, to achieve more specific silencing in APCs, they compared the biodistribution of different sizes of LNPs (80, 240 and 360 nm) and observed redirection of the LNPs from hepatocytes to APCs with increasing size. The LNPs in this study were administered by intra venous (I.V.) bolus injection (62). In another attempt to use novel cationic lipids, Nozaki et al. utilized a combination of experimental trials and the Bayesian Optimization method. This machine learning technique can predict optimum values based on a small number of experiments and is particularly useful for multifactorial optimization. They developed new LNP formulations incorporating either of the two newly synthesized cationic lipids: FFT-10 and FFT-20. These formulations proved to be more efficient than both Lipofectamine 3000 and the original composition without the cationic polymers, which consisted of mole ratios of 28% DOPE, 28% DOTAP, 41% cholesterol, and 3% DMG-PEG for LNP delivery to Human PBMCs. Furthermore, by testing LNPs formulated with both polymers in different ratios for their luciferase activity, they also showed that slight variations in molecular ratios of the lipid compositions can affect the gene delivery efficiency. Finally, their results show that the Bayesian Optimization method can be beneficial in designing the lipid composition of LNPs for target cells, thereby expanding their clinical applications (63).

Liver tropism of LNPs is determined by the protein corona spontaneously formed around the LNPs in the bloodstream. Lipid conjugates of siRNA have been shown to support extrahepatic delivery but with lower tissue accumulation and silencing compared to those in the liver (64). In a systematic evaluation to investigate the impact of siRNA scaffold on docosanoic acid (DCA)-conjugated siRNA, structurally asymmetric (5nt-overhang), conventional (2nt-overhang) or blunt-end siRNAs carrying different numbers of phosphorothioate modifications and varying linker chemistries were compared with respect to their delivery and efficacy. It was reported that structural asymmetry showed no impact on tissue accumulation although essential for improving extrahepatic activity. With similar tissue accumulation, asymmetric and conventional siRNA showed better efficacy in the lung, kidneys, heart, spleen, muscle and adrenal glands. Moreover, linker chemistry (cleavable vs. stable) altered the activity but not the tissue accumulation. A cleavable linker showed enhanced silencing across tissues. Additionally, higher PS content, despite being crucial for siRNA stability and tissue accumulation, led to an elevated accumulation of asymmetric compounds but lowered the efficacy. Finally, it was concluded that tissue accumulation does not define efficacy but rather endosomal escape as well as intracellular re-distribution originating from the details of chemical structures influence the obtained activity (as articulated in Section 1.1.1.1.2 as well) (65).

New lipid-like molecules continued to be synthesized and tested for their efficiency in carrying siRNA. Researchers from MIT and Alnylam Inc. synthesized a library of over 1,200 structurally different lipid-like molecules, called lipidoids, and tested their efficiency in carrying siRNA or single-stranded antisense 2′-O-methyl (2′-OMe) oligoribonucleotides targeting microRNA (miRNA) delivery and tested their safety and efficacy in mice, rats and nonhuman primates. To be able to create a large library their synthesis methodology relies on the conjugate

addition of alkyl-acrylates or alkyl-acrylamides to primary or secondary amines. Unlike many conventional lipid synthesis approaches, this specific chemistry enables reactions to occur without the requirement of solvent or catalysts. It yields solely lipidoid products, thus obviating the necessity for protection and deprotection steps, as well as purification or concentration processes. Based on their in vitro results, 17 formulations were tested in mice models to target factor Vll in the liver. A range of silencing activity was observed in the liver with >90% being the highest which was achieved by a formulation called 98N12. After testing five variants of this formulation with different tail numbers, 98N12-5 (5 tail) was selected for in vivo delivery to rats. This formulation then showed significant dose-dependent activity (40% by 1.25 mg/kg, 80% by 2.5 mg/kg and 90% by 5 mg/kg) after I.V. administration. The lack of silencing activity with formulated control siRNA implies the specificity of the observed activity (66). To optimize the delivery, factors such as particle size, lipid composition, drug-to-lipid ratio, and manufacturing process must be addressed. While the composition of lipidic carriers facilitates interaction with cell membranes, enabling efficient cellular uptake of siRNA (67), they can be also engineered with ligands that confer specific affinity towards receptors expressed on the surface of the target cells. Such ligand-receptor interactions allow targeted delivery of siRNA, enhancing the therapeutic index while minimizing off-target effects and the associated toxicity. Swart et al. developed a functionalized LNP using a chemically modified Leu-Asp-Val (LDV) tripeptide, which is a specific ligand for VLA-4 receptor expressed on all leukocytes and plays a key role in homing to and retention of hematopoietic stem and progenitor cells (HSPC) in the bone marrow. Adding a benzyloxycarbamidophenylurea group to the terminal amino acid of the tripeptide increased the binding potency, offered protection against enzymatic hydrolysis, and inhibited T-cell adhesion. Biodistribution studies using female c57/B16J mice revealed improved bone marrow accumulation and retention time with LDV-LNP

formulations with both mature and immature hematopoietic stem cells (14). Kusumoto et al. designed multifunctional liposomes with targeting capabilities by using egg phosphatidylcholine (EPC)/cholesterol/stearyl-polyethylene glycol 2000 (STR-mPEG) and incorporating lung endothelium-targeting GALA peptide to deliver siRNA and eradicate lung metastasis. The GALA peptide (WEAALAEALAEALAEHLAEALAEALEALAA) was intended to target sialic acid-terminated sugar chains on the pulmonary endothelium and to subsequently deliver the encapsulated cargoes to the cytosol via endosomal membrane fusion (68).

LNPs, particularly those formulated with ionizable lipids, are known to exhibit low immunogenicity, reducing the likelihood of eliciting an undesired immune response. This property is significant for siRNA-based therapeutics where repeated administration may be necessary (69). If immune-stimulatory features are desired, incorporating class B CpG oligodeoxynucleotides1826, a murine toll-like receptor-9 agonist, into LNP with ionizable lipids could be attempted to robust innate immune response activation in lymph nodes, contrary to the unformulated, soluble CpG with systematic distribution (70). The manufacturing process of LNPs is scalable and adaptable, but having multiple components may lead to complications in scale-up. This flexibility to alter lipid components underscores the potential of LNPs as a platform technology for siRNA delivery (71). Further understanding the interactions of LNPs will pave the way for improved therapies for different diseases.

1.1.2.2 Polymeric Delivery Systems

Polymers with their ability to form multivalent interactions with nucleic acids have emerged as a viable alternative to lipid-based carriers, offering unique properties that could benefit the deployment of siRNA therapeutics. Similar to LNPs, polymers offer enhanced stability to siRNA molecules by protecting them from degradation by endogenous nucleases present in the

bloodstream. Polymers can be engineered to release their cargo over an extended period, thereby maintaining therapeutic concentrations of siRNA in target cells and potentially improving treatment outcomes (72). Through functionalization, polymers can be designed to specifically target diseased cells, thereby minimizing off-target effects, for instance, by conjugation with ligands or antibodies that bind to receptors overexpressed on the surface of target cells (72). Integrin receptors, the heterodimeric adhesion receptors involved in extracellular matrix interactions such as the VLA-4 receptor, Lymphocyte Peyer patch adhesion molecule, and the lymphocyte function-associated antigen-1, have been explored for targeting hematological malignancies (14). Biocompatible polymers can be selected or engineered to have minimal toxicity and immunogenicity, thereby enhancing patient safety. Several polymers used in drug delivery applications, such as polyethyleneimine (PEI) and chitosan, have shown relatively low immunogenicity, making them suitable for long-term siRNA therapies (73,74). Polymers are inherently versatile, allowing for a variety of functional modifications to improve their delivery efficiency and compatibility with siRNA. They can be tailored to carry multiple therapeutic agents, opening up possibilities for combination therapies that could enhance treatment efficacy for complex disorders (75). In an attempt to design an effective therapeutic agent for Anaplastic large cell lymphoma (ALCL), Zeng et al. prepared a protamine-derived nanoparticle containing the cytotoxic drug doxorubicin and ALK siRNA and decorated it with CD30 aptamers to specifically target lymphoma cells. The nanoparticles bound and killed lymphoma cells with little off-target toxicity (76). Ongoing research continues to refine these properties to maximize their therapeutic potential in other applications.

1.1.2.3 Combinational Delivery Systems

The amalgamation of multiple modes of transport and delivery vehicles of contrasting features, including peptide-polymer, liposome-peptide, and lipid-polymer combinations, has provided improved siRNA delivery systems (77–79). Bioconjugation systems based on polymers and liposomes have gained significant traction for the delivery of DNAzymes, antisense oligonucleotides, and plasmid DNA (67,80,81). Dynamic PolyConjugates (DPCs) derived from polymer-siRNA conjugates facilitated targeted siRNA delivery to hepatocytes (82–84), analogous to conventional LNPs. Liposome-siRNA-peptide complexes showed in vitro efficacy in delivering prion protein (PrP) siRNA to acetylcholine receptors (AchR)-expressing cells, inhibiting cellular prion protein (PrP^{C}) expression and eradicating the protease-resistant isoform (PrP^{RES}) formation (53,85–87).

The low molecular weight PEI (<2 kDa), with its low toxicity albeit ineffective siRNA delivery capability, was substituted with lipids linoleic acid, α-linoleic acid or cholesterol, which were tested concerning their delivery and silencing efficacy in primary Chronic myeloid leukemia (CML) cells. An effective internalization, silencing of the BCR-Abl oncogene and inhibition of colony formation by bone-marrow aspirates were achieved. However, discrepancies between different cell samples/polymers underscored the patient-to-patient variabilities that need to be addressed in going forward. Silencing activity and colony formation inhibition of 17%-45% and 24%-41% were achieved, respectively. This study showed that low doses of siRNA (60 nM) can be used to achieve effective silencing in malignancies (88). In a preclinical animal model, Ansari et al. used aliphatic lipid grafted 1.2 kDa PEI polymers, in complex with FLT3 and BCR-Abl siRNA for the treatment of acute myeloid leukemia (AML) and CML xenografts in vivo and were able to achieve 50% and 65% reduction in tumour volumes in AML and CML models,

respectively. Combination therapy with the tyrosine kinase inhibitor Gilteritinib led to earlier remission than Gilteritinib monotherapy (9 vs. 12 days) with higher median survival (66 vs. 45 days) (89). Highly hydrophobic cholesterol-grafted PEI also showed therapeutic efficacy in a CML model (K562 cells in culture) observed through reduced colony formation, increased caspase activity and improved apoptosis. Cholesterol substitution led to smaller polyplexes as well as enhanced zeta potential of the nanoparticles. These polymers were reported to be able to fully protect the siRNA in fresh serum. Interestingly, the delivery efficacy was proportional to the substitution degree and inversely related to the molecular size of the polymer backbone (90). In another study, Morales et al. compared the biodistribution of commercially available lipid-substituted PEIs, Leu-Fects, with LNP-formulated siRNAs in a subcutaneous CML-bearing mice model. A markedly higher delivery of siRNA was observed with LeuFect carriers to extrahepatic organs like the spleen, bone marrow, and lungs after 1 and 7 days (91). Recently, Guimaraes et al. developed a series of lipid-polymer/siRNA nanoparticles which target Cyclophilin A (CyPA) and tested them for the treatment of MM. CyPA is an abundant inflammatory protein that promotes colonization, proliferation, and resistance in blood vessels of bone marrow in MM patients. In this study, they created a library of 15 nanoparticles using low molecular weight PEI polymer, C15 epoxide-terminated lipids and PEG-lipid conjugate with varying (i) tail length of lipid-PEG (C14, C16, C18), (ii) molecular weight of PEG (750, 1000, 2000, 3000 kDa) and (iii) PEG mole percentage (20% or 30% by weight). Unique DNA barcode strands were incorporated into each formulation to be recognized in each tissue by deep sequencing. Their siCyPA-NP was able to inhibit the target gene in bone marrow endothelial cells, prevent MM extravasation, and sensitize the MM cells to chemotherapy in vitro. This approach also reduced the tumour burden and increased the survival of animal models with or without FDA-approved antineoplastic medicine

bortezomib (92). In a follow-up study by the same group, their optimized formulation ([PEI_{600} + epoxide-terminated lipid= lipid-polymer hybrid 7C1] + $C_{18}PEG_{1000}$ at mole ratio 70:30 of 7C1 to PEG-lipid) when complexed with siRNAs for adhesion receptor E-selectin (ES) showed efficient gene silencing in vitro, which decreased MM cell adhesion and migration through endothelial monolayers. The same formulation was then complexed with both siES and siCyPA for simultaneous inhibition, which extended the survival of animals with or without bortezomib (93). Table 1.2. restates some of the efforts in manufacturing combinational therapeutics for siRNA delivery.

Table 1.2. Examples of combinational therapies for more efficient siRNA delivery systems. L319: 9-[4-(dimethylamino)-1-oxobutoxy]-heptadecanedioic acid, 1,17-di-(2Z)-2-nonen-1-yl ester

Initial component	Modification Type	study	Reference
PEI [0.6, 1.2, and 2.0 kDa]	Substitution with linoleic acid, α-linoleic acid, or cholesterol	-low toxicity and delivery -effective silencing -reduced colony formation	Valencia-Serna et al. 2019
PEI [1.2 kDa]	linoleic acid and lauric acid	-effective silencing -if combined with Gilteritinib; earlier remission and increased median survival	Ansari et al. 2024
PEI [0.6, 1.2, and 2.0 kDa]	Cholesterol	-reduced colony formation, caspase activity -increased apoptosis -reduced size and enhanced zeta potential -full protection for siRNA in serum -delivery efficacy was proportional to substitutional degree and invertedly related to MW of polymer backbone	K C et al. 2020
PEI (LeuFects)	Lipid	-Increased siRNA delivery to extrahepatic organs: spleen, BM, lungs after 1 and 7 days	Morales et al. 2024
PEI_{600}	C15 epoxide terminated lipids	-effective silencing in BMEC -prevented MM extravasation -reduced tumour burden -increased survival and chemotherapy sensitization	Guimarães et al. 2023
PEI_{600}	Epoxide terminated lipids	-effective silencing led to increased MM migration - complexed with siCyPA led to extended survival of animals with or without bortezomib	Figueroa-Espada et al. 2023
L319 lipid	Replacement of 9,10-cis double bond with ester of linoleyl chain (L319) + including bio-cleavable groups in the tail	- facilitated excretion -considerable tolerability -increased potency in rodents and NHPs -the closer the ester bond is to the head group, the lower the silencing efficacy and the more the effect on pKa is. -the further the ester bond is from the head group, the higher the liver accumulation is.	Maier et al. 2013

In an attempt to compare CPPs and cationic liposomes for their silencing efficiency and siRNA delivery capabilities, Vysochinskaya et al. used the CPP EB1 and 1,26-bis(cholest-5-en-3-yloxycarbonylamino)-7,11,16,20-tetraazahexacosane tetrahydrochloride (2X3) (2X3)-DOPE-PEG liposomes in K562 CML cell lines. Their results showed robust delivery and endosomal release as well as a more pronounced therapeutic effect (inhibition of cell proliferation) by the formulated liposomes (94). Finally, in a study to compare the efficacy of 0-/1-/2-dimensional nanocarriers for siRNA delivery, CD47-specific siRNA was delivered to AML, lung cancer and liver cancer cells using LNPs with ionizable lipids, modified boron nitride nanotubes [BNNT-polymer] and graphene oxide (GO) modified with PEG dendrimers [GO-PEG-PAMAM] in co-culture with human macrophages. All delivery vehicles had high transfection efficiency in all cell lines; however, it seemed that small GO-PEG-PAMAM used a different pathway for the elimination of cancer cells than the LNP and BNP carriers. Given that downregulating CD47 on cancer cells exposes calreticulin (CRT) to macrophages and restores their phagocytic activity, when small GO-PEG-PAMAM was used, the presence of CRT on cancer cells was required for macrophage phagocytosis, whereas this was not the case when LNP and BNP were used, and phagocytosis occurred with or without CRT blockers, indicating the involvement of other pathways in the cancer cell elimination process. (95).

1.1.2.4 Conjugates for siRNA Delivery

Conjugate-mediated delivery is a clinically feasible siRNA delivery strategy due to advantages such as reduced immunogenicity and increased stability against nuclease degradation, in addition to the critical benefit of eliminating particulates (LNP) during delivery. This avenue of delivery necessitates complete chemical stabilization of siRNAs, as unmodified siRNAs are

rapidly destroyed (by nucleases) and removed from circulation by renal filtration, resulting in little bioavailability in tissues. Chemical scaffolds that substitute every 2' hydroxyl, modify terminal nucleotide links (11,12), and stabilize the 5' phosphate enhance the in vivo activity of siRNA (64). Specialized siRNA conjugates have been engineered to improve targeting and cellular uptake of siRNAs.

1.1.2.4.1 *N*-Acetylgalactosamine (GalNAc) Conjugates

The success of GalNAc conjugates of siRNA, specifically in targeting hepatocyte, stands out with four FDA-approved products. Tris-GalNAc conjugates target the asialoglycoprotein receptor (ASGPR) present on the surface of hepatocytes, facilitating their rapid endocytosis. Using this conjugate technology, Givosiran (GIVLAARI®), Lumasiran (OXLUMO®), Inlcisiran (Leqvio®) and Vutrisiran (AMVUTTRA®) were developed for subcutaneous administration, which received FDA approval in 2019, 2020, 2021 and 2022, respectively. A fundamental distinction between particulate siRNA and siRNA conjugates lies in their structure, with particulate siRNA enclosed within a particle while the siRNA conjugate directly encounters the physiological environment. Minimizing siRNA's interaction with serum proteins in conjugates becomes paramount to avert potential toxicities attributed to the siRNA structure. In siRNA conjugates, the PS content in the siRNA backbone poses a dual role: on one side, by binding to plasma proteins, it prolongs siRNA's half-life thus extending its renal elimination and bolstering intracellular accumulation (15,65). On the other hand, higher phosphorothioate content enables siRNA to bind to proteins in the alternative complement pathway, potentially triggering the innate immune system. Moreover, the phosphorothioate content in oligonucleotides correlates with

thrombocytopenia, possibly due to their binding to platelet proteins such as Receptor Glycoprotein VI and Platelet Factor-4 (96).

The potential toxicity of the GalNAc moiety should not be overlooked. A hypothesis regarding the Revusiran-triggered neuropathy highlights GalNAc-induced demyelination, drawing parallels to reports of Guillain–Barre syndrome in patients treated with exogenous gangliosides (97). In GalNAc-siRNAs, the likelihood of ASGPR saturation at high doses may impact the distribution of these compounds. For instance, a singular dose of 10 mg/kg of a novel GalNAc-siRNA revealed a twofold greater accumulation in the kidney than in the liver. Surprisingly, even higher doses of 30 mg/kg divided into multiple doses did not yield higher kidney accumulation (98). Consequently, dose adjustments are vital to avoid kidney accumulation of GalNAc conjugates. The GalNAc-conjugated siRNAs exhibit the ability of long-term silencing in humans due to the buildup of degradation-resistant siRNA in acidic intracellular compartments, forming a reservoir for these molecules (99). While superior durability and minimum dosing frequency are desirable, the potential cytotoxic effects of siRNAs on disrupting endogenous endocytic processes via effects on endocytic vesicles and proteins merit attention (15).

1.1.2.4.2 Lipophilic Conjugates for siRNAs

Lipid-conjugated siRNAs, such as cholesterol-conjugated siRNAs, primarily accumulate in the liver, but they also show accumulation and effective silencing in other organs such as the kidney (100), muscle (101), and placenta (102). Localized administration of cholesterol-modified siRNA triggers functional gene silencing in various tissues, including the brain (103,104), vagina (105), and skin (106). Cholesterol makes up 15-30% of the cell membrane and when co-incubated with cells, spontaneously intercalates into lipid membranes to fulfil its biological function of promoting membrane structure and fluidity. Cholesterol, when conjugated to siRNAs, can deliver

the siRNA into the cells via two mechanisms, endocytosis and interaction with lipoprotein receptors (107). Cholesterol is a good candidate for local in vivo delivery particularly to the skin, eye, and brain parenchyma due to rapid internalization of cholesterol-conjugated siRNAs in vitro which provides restricted biodistribution at the site of injection (107). Cholesterol was the first conjugate described for systemic siRNA delivery, which demonstrated a significant enhancement in the pharmacokinetic characteristics of siRNA through extending half-life and increased availability of siRNA in serum. The initial rationale behind the cholesterol-conjugated siRNAs was based on their potential to target the liver, owing to the fenestrated endothelium in the liver and hepatocytes' intrinsic ability to incorporate cholesterol in natural lipid metabolism pathways (55). However, subsequent studies revealed that cholesterol and other lipid-conjugated oligonucleotides interact with circulating lipoproteins, such as low-density lipoprotein (LDL), and their uptake by the liver relies on the expression of LDL receptors (108). Several cholesterol-conjugated siRNAs have progressed to clinical trials. RXI-109 by RXi Pharmaceuticals (currently known as Phio) was tested in Phase II trials to reduce hypertrophic scarring and in Phase I/II trials in the eyes of age-related macular degeneration patients at risk for subretinal fibrosis. Another example is Arrowhead Pharmaceuticals' cholesterol-conjugated oligonucleotides to target the liver in Hepatitis B. In this case, a polymeric carrier was also co-administered to increase the endosomal release and thus in vivo potency. These studies were later discontinued due to the toxicity of the cationic formulation in non-human primates (107).

Regarding the effect of lipid conjugates on siRNA biodistribution, a study by Osborn et al. undertaken with a panel of structurally diverse lipids (cholesterol, lithocholic acid, docosahexaenoic acid and docosanoic acid) attached to 3'-end of the sense strand of a siRNA via a C-based and an amide-based linker, revealed that lipid conjugation alters the hydrophobic

properties of modified siRNAs and the resultant pharmacokinetic behaviour by facilitating their selective integration into endogenous lipoprotein pathways. Intravenous injection led to a preferential association of more hydrophobic complexes with circulating low-density lipoproteins (LDLs) whereas less hydrophobic complexes bound to high-density lipoprotein (HDL). Lipid modification enhanced the efficacy of siRNA-mediated mRNA silencing in tissues enriched with lipoprotein receptors, such as the liver, adrenal gland, ovary, and kidney. Of note, siRNA internalization was not fully driven by endocytosis but phosphorothioate modifications seemed to play a role as well. These observations suggested that the extent of hydrophobicity plays a crucial role in determining the tissue distribution pattern of lipid-conjugates, beyond the initially intended effect of specific receptor and/or cell targeting (109). Biscans et al. have also studied a panel of 15 lipid conjugates on siRNA tissue biodistribution and silencing efficiency. The conjugates were covalently attached to the 3'-end of the sense strand. Although most of the conjugates accumulated in clearance organs, some accumulated in other organs such as heart, lung, fat, muscle, and adrenal glands with 3-10-fold higher accumulation than cholesterol-conjugated siRNAs. Altering the conjugate structure could improve the extrahepatic delivery and the chemical nature of conjugates affected tissue-dependent cellular and intracellular trafficking (64).

Conjugates with ionizable cationic lipids are another type of conjugate under study. Currently, there are 3 clinically approved ionizable lipids as part of a LNP formulation, DLin-MC3-DMA (heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino)butanoate), ALC-0315 (4-hydroxybutyl) azanediyl)bis (hexane-6,1-diyl)bis(2-hexyldecanoate), and SM-102 (heptadecan-9-yl 8-((2-hydroxyethyl) (6-oxo-6-(undecyloxy) hexyl) amino) octanoate). They are all used in similar molar ratios in formulations, of ionizable cationic lipid (~50%), cholesterol (~38.5%), DSPC (1,2-Distearoyl-sn-glycero-3-phosphocholine; ~10%), and PEG-DMG (1,2-dimyristoyl-

rac-glycero-3-methoxypolyethylene glycol-2000; ~1.5%). However, the latter two share the same branching and functional groups and have been developed for mRNA delivery in SARS-CoV-2 vaccines of Pfizer/BioNTech/Acuitas and Moderna, respectively. In a head-to-head comparison of LNPs with ALC-0315 and DLin-MC3-DMA for hepatotoxicity and siRNA delivery efficiency to silence Coagulation Factor FVII in hepatocytes and ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, 13) in hepatic stellate cells (HSCs) in mice, at lower doses of 1 mg/kg, LNPs with ALC-0315 showed superior silencing efficacy (2-fold for FVII and 10-fold for ADAMTS13). On the other hand, higher doses of 5 mg/kg led to elevated liver toxicity markers such as alanine transaminase and bile acids by LNPs formed with ALC-0315 but not with DLin-MC3-DMA (110). In another study, conjugation of DLin-MC3-DMA to siRNA enhanced the endosomal escape in cell culture without compromising siRNA efficacy. In mice, on the other hand, siRNAs exhibited a similar tissue distribution to cholesterol-conjugated siRNAs, due to similar hydrophobicity. However, DLin-MC3-DMA conjugated siRNAs showed significantly higher accumulation in vascular tissues, suggesting a structural effect on tissue distribution. Notably, tissues with high DLin-MC3-DMA siRNA accumulation displayed significant non-specific gene modulation, highlighting the need for fine-tuning conjugate properties to optimize endosomal escape while minimizing toxicity for therapeutic siRNA applications beyond the liver (111).

Another siRNA conjugate developed by Alnylam for central nervous system (CNS) delivery is 2'-O-hexadecyl (C16) lipid, which provides lipophilicity to attach to the cell membrane and its proteins in multiple cell types in CNS, lung and eye. The ALN-APP is an investigational RNAi therapeutic to treat cerebral amyloid angiopathy and Alzheimer's disease, which is injected intrathecally (into the spinal cord) (112).

Using docosahexaenoic acid, eicosapentaenoic acid, or myristic acid, a panel of mono-, di- and tri-meric lipid-conjugated siRNAs was created to evaluate the effect of lipid structure and valency on siRNA delivery. The results revealed significant changes in physiochemical properties like hydrophobicity and micelle formation which influenced the siRNA clearance and distribution profile. While trivalent siRNA conjugates were mostly retained at the injection site with minimal systemic exposure, monovalent siRNA conjugates were quickly released into circulation and accumulated mostly in the kidney. Divalent siRNA conjugates, however, showed an intermediate behaviour with preferential accumulation in the liver with functional distribution in organs like the lung, heart, and fat. Additionally, chemical nature/structure (i.e. carbon length, degrees of unsaturation and self-association properties) proved to be a better predictor of extrahepatic accumulation necessary for efficient gene silencing than hydrophobicity, with Myr-d siRNA showing ~3 times higher lung accumulation and thus better gene silencing than other conjugates with similar hydrophobicity, underscoring the importance of precise engineering to fine-tune the therapeutic siRNAs for enhancing extrahepatic delivery (113). Finally, it is noteworthy to highlight the work of Kubo et al., who condensed 21-nt single-strand RNAs (ssCATs) modified with an amine at the 5′-end with 16 saturated and unsaturated fatty acids. By targeting β-catenin in colorectal cancer HT-29 cells, excellent cellular uptake was observed with conjugates of 16–18C lipids and they revealed better β-catenin silencing with prolonged effect compared to unmodified siRNAs (formulated with a lipofection reagent). Notably, siRNA conjugates having 18C chains in trans isoform (elaidic and trans-vaccenic acids) had lower gene silencing efficiency than the conjugates with cis isoform (oleic and cis-vaccenic acid) (114).

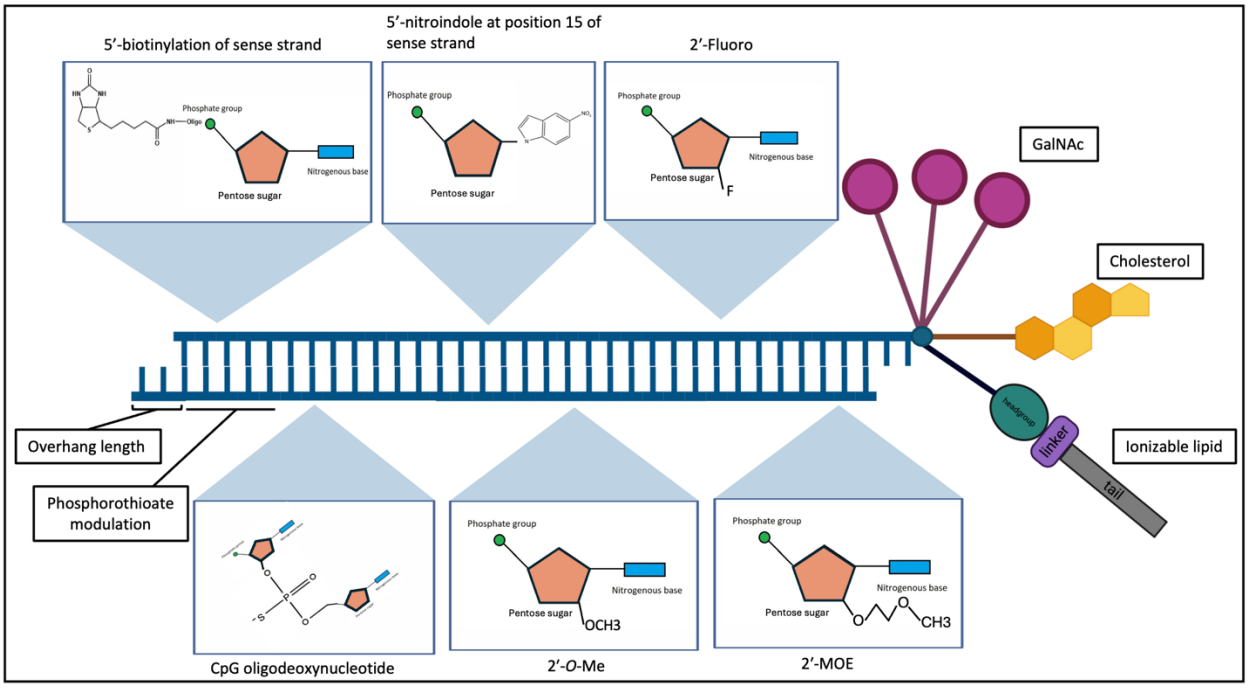

Figure 1.4. Different siRNA modification (point changes in structure) and conjugations (linking of another compound) strategies.

1.1.3. Adverse Effects Associated with Delivery Systems

The delivery systems used to carry the siRNA to its intended site may further contribute to the pharmacological activity independent of the delivery per se. Once downsized to the nanoscale, many normally ‘inert’ materials can become substantially more reactive owing to dramatic increases in total surface area and exposure of previously hidden ingredients. This results in more extensive interactions between the ‘non-materials’ and the biological milieu, causing damage to the human body at the organ, tissue and cell levels with the manifestation of ‘nanotoxicity’ (115). The use of delivery systems can be justified only when the naked siRNA (native or chemically modified) is not capable of implementing RNAi at practical doses. While sufficient delivery efficiency is expected to be attained, the delivery systems should not alter the normal cellular

physiology and remain ‘stealth’. Below we inspect the mechanistic aspects of toxicity related to deployment of siRNA for therapeutic purposes.

1.1.3.1 Recognition by Immune Cells

The presence of TLR ligands on nanoparticles can trigger B-cells. For instance, IgG and IgM antibodies were reported to be secreted against cholesterol components, phosphate and sulfate esters, and other epitopes like Apolipoprotein H on the surface of lipid-based nanomaterials (116–118). LNPs can activate the classical complement cascade when antibodies bind to them or when C-Reactive Protein attach to their surface (119). The complement pathway can be activated through lectin-mediated or alternative pathway via cationic liposomes or Fab-fraction of bound antibodies, or by phosphatidylinositol/mannose-containing liposomes (120,121). In monkeys, the parenteral administration of cationic LNPs was linked to activation of the complement system (122). Lipid-based nanoparticles can incite pro-inflammatory reactions; cationic LNPs, if administered systemically, can trigger Type I IFN responses and elevate Th1 cytokines such as IL-2, IFN-γ and TNF-α by activating TLR4 in immune cells (123). Moreover, LNP201 comprising of the cationic lipid CLinDMA can induce both pro- and anti-inflammatory cytokines such as IL-6, TNF-α, and IL-10 in vivo (124). Another study reported that empty LNPs spurred the maturation of monocyte-derived dendritic cells (MDDCs), increased CD40 expression and ultimately elicited a Type I IFN response (125). Additionally, by stimulating Type III IFN secretion, lipoplexes could generate an antiviral response. In an intriguing application, this response was employed to lessen the off-target accumulation of subsequently administered nanoparticles in the cancer treatment (126). The concept revolves around the hypothesis that the injection of lipoplexes triggers the production of IFN-λ, which fortifies healthy epithelium, preventing the deposition of subsequently administered nanoparticles. Yet, lacking the immune response fortification within the tumour

microenvironment, these nanoparticles accumulate in the tumour tissue (126). One common strategy used to prevent such an immunotoxicity is premedication or co-administration of dexamethasone (124), and use of Jak inhibitors (127) as a general immunosuppressant.

PEGylation has been investigated to make delivery vehicles more tolerant, although repeated administration of PEGylated nanocarriers can activate the immune system, triggering complement activation and hypersensitivity reactions (128). Incorporating cleavable PEG-lipids could potentially bypass the immune response induced by PEGylated liposomes (129,130). Even with ionizable cationic lipids, where the lipids gain cationic charges in low pH endosome compartment only, excessive accumulation of the ionizable LNPs in specific tissues, such as the liver, can lead to proinflammatory cascades (131,132) and fast neutrophil infiltration in the skin and muscle (133). In the latter study, inflammation occurred independent of the delivery route with or without mRNA while removal of the ionizable lipid from the formulation abolished leukocyte infiltration. The ionizable lipid in the first authorized siRNA drug, DLin-MC3-DMA, has an extended half-life in the liver (134,135). Pharmacokinetic analysis from Patisiran's Phase II study in hATTR amyloidosis revealed that siRNA-LNPs can reach the liver within 1 hour of intravenous administration, however clearance from the liver involved 3 days (136). Next-generation biodegradable lipids commonly integrate labile esters in their hydrophobic tails to facilitate clearance (137). The immune reaction seen with ionizable lipids, however, may be beneficial for some immunotherapy indications as long as it is tightly controlled, but this has been linked to LNP's PEG-lipid components (128,138). GalNAc conjugates are beneficial in reducing accumulation at other sites (131), or creating lipids with cleavable esters may reduce their in situ residence time (139).

The amine-rich polymers can also trigger an immune response by PRR recognition of specific nanoparticle components or protein corona. Repetitive surface epitopes on polymeric nanoparticles can activate complement pathways, while cationic coatings enhance the attraction of opsonins, resulting in enhanced phagocytosis. The protein corona can be influenced by physicochemical factors, such as size, shape, aggregation state, and surface chemistry. The size of Poly(lactic-co-glycolic acid) (PLGA) nanoparticles has been linked to their ability to incite a pro-inflammatory response, where larger PLGA particles activated the NF-κB pathways (140). In a recent study, Debnath et al. revealed the inhibitory effect of absorbed corona proteins on NLRP3 activation as a key component of the intracellular inflammasome. In serum-free settings, ionizable lipid of LNPs was found to play a crucial role in controlling the activation of the NLRP3 inflammasome. On the other hand, the corona layer on LNPs significantly decreased the activation of the inflammasome mainly through reduction of lysosomal rupture when serum proteins were present. Furthermore, the endocytic pathways for LNPs in macrophages were significantly influenced by the lipid formulation when the cellular uptake in macrophages was observed to be markedly lowered upon protein corona formation (141). Additionally, cationic particles can induce inflammatory responses via oxidative stress; for instance, exposure to PEI nanoparticles in the airways has been associated with immune responses through oxidative stress-induced Th2 cytokine secretion (142). Not surprisingly, complement activation-related pseudoallergy was noted after IV administration of a high molecular weight form of PEI in a swine model (143). Therefore, understanding the immunogenicity arising from the lipidic and polymeric components of the nanoparticles warrants a thorough investigation during the preclinical toxicity assessment.

1.1.3.2 Direct Toxicities

Cationic lipids and polymers can cause cytotoxicity directly. The PEI, as one of the leading polymers for delivering nucleic acids with good cell membrane interaction and endosomal escape features can cause significant in vitro and in vivo toxicity. Uncomplexed PEI can interact with anionic proteins in the serum or red blood cells and cause aggregation and hemolysis. In polyplex form, it can also be released when internalized by the cells and lead to delayed toxicity by interacting adversely with cellular components. It is recognized that higher molecular weight and branched PEI isoforms are more toxic, and its limited biodegradability contributes to this. While smaller PEI molecules can be removed by simply renal filtration, hydrolysable analogous of PEI or other polymers with degradation linkages with half-lives in the order of days, such as poly(amino esters), can help to lessen their footprint. Chitosan as a natural polymer has been shown to display minimal toxicity in siRNA delivery, no proinflammatory cytokines in vitro and low allergenic potential in vivo. Polypropylenimine (PPI) was developed as a cationic dendrimer with hopes for low cytotoxicity, but later showed alterations in gene expression related to dendrimer synthesis (115).

Cationic lipids in nanoparticles are recognized for triggering cytotoxicity directly. Triangular cationic head, hydrophobic hydrocarbon backbone, and linker connecting head and tail are the three main components of cationic lipids, which are essentially ‘surfactants’ facilitating pore formation and destabilization of cellular membranes (115). The prototypical cationic lipid Oligofectamine® upregulated expression of apoptotic genes such as HSPD, BCL2A1, HSP70, and caspase 8 isoform c in vitro. The gene-modulating effects of Lipofectin® were relatively lower, but it still upregulated the RPA1 gene significantly and suppressed the expression of genes like EDNRB1/2 and ribosomal protein S16 that could potentially increase the tendency for apoptosis

induction (144,145). Soenen et al. showed that transferring cationic lipids from liposomes to cell membrane constitutes a primary mechanism for membrane destabilization. The high curvature of nano-sized liposomes enhances this transfer (146), presumably by lowering the thermodynamics barrier to transfer. With cationic polymers used to create polyionic complexes, Vaidyanathan et al. suggested that the unbound fraction of polycations, not directly linked to siRNA, can also act as surfactants as well and create pores in cellular membranes by intercalating into lipid layers (147). This is the case when the N:P (amine to phosphate) ratio in polymeric nanoparticles exceeds 1 considerably so that the polyplex possesses a net positive charge and unbound carriers. Multivalent polymers can interact with cell membranes' phospholipids, causing destabilization in both blood cells and intracellular organelles like mitochondria (15). The acute toxicity with PEI-PEG siRNA nanoparticles was linked to the promotion of erythrocyte aggregation affected by the N:P ratio, particle size, and extent/pattern of the PEGylation (148).

Another mechanism of toxicity involves polycations catalyzing the hydrolysis of phospholipids by facilitating proton transfer, which induces the formation of inverted hexagonal phases within the membrane (149). Monnery et al. reported this to be the primary mechanism behind membrane disruption, citing observations of acid-catalyzed lipidic phosphodiester bond hydrolysis induced by PEI in 1,2-dioleoyl-sn-*glycero*-3-phosphocholine (DOPC) liposomes in vitro (142). Several studies have suggested a direct link between the toxicity of polycations and their molecular weight (150,151). Additionally, factors such as charge density and structural traits—like linear versus branched configurations—significantly influence polycation toxicity (151). Modifications such as PEGylation and structural alterations, such as introducing anionic groups into polymers, have demonstrated reduced toxicity of these carriers (148,152,153). Moghimi et al. reported that while the initial impact of disrupting cellular membranes results in

necrotic cell death, a subsequent phase of toxicity associated with linear or branched PEI takes place after pore formation in the outer mitochondrial membrane. This process leads to the secretion of proapoptotic cytochrome c, prompting mitochondrial depolarization and eventual apoptotic cellular demise. This depolarization can further induce the generation of ROS (154). Finally, it must be noted that the beneficial effect of cationic lipids and polymers, such as polyamidoamine (PAMAM) dendrimers and PEIs, to destabilize the lysosomal membrane by the proton sponge effect can lead to another toxicity mechanism if it becomes excessive (15).

Table 1.3. Example of modifications in NP and/or biomaterial features to improve siRNA delivery. NP: nanoparticle, LNP: lipid nanoparticle, EPR: enhanced permeability and retention, Ab: antibody, LSEC: liver sinusoidal endothelial cells

Modifications	effect
Incorporation of tertiary amines and disulfide bonds in tail of polymer	-improves silencing in the presence of vitamin E in scaffold[235]
Incorporation of ester motifs in polymer	-increases biodegradability[168] -have controlled biodegradation[135, 169, 170]
N:P ratio >> 1 in NP composition	-unbound fraction of polycations can act as surfactants as well and create pores in cellular membranes by intercalating into lipid layers[15]
Introducing anionic groups to polymers	-reduces toxicity of polycations[148, 152, 153]
PEGylation	-reduces toxicity of polycations[148,152,153] -improves transfection of anionic LNPs[229] -reduces LNP recognition by MPS and renal filtration clearance[162] -reduces opsonization by serum proteins[164] -reduces reticuloendothelial clearance[164] -increases EPR[165]
Cleavable PEGylation	-prevents PEG-specific Ab induction[166]
dePEGylation	-favours intracellular transposition of NPs[162]
Anionic and neutral liposomes	-prevents oxidative-stress-related pulmonary toxicity[157]
Ester motifs	-increases biodegradability[168]
Composition of ionizable lipid	-affects protein corona[189]
Size of NPs	-size was not heavily involved in liver accumulation but affects silencing activity -lower activity and stability with smaller particles[195]
Apparent pKa (in the range of 5.5-7.5)	-small changes in pKa results in significant changes in intrahepatic distribution and silencing activity -low pKa, were unable to convert cationic moieties in endosome thus poor endosomal escape -as pKa rises, the silencing activity improves in LSECs -pKa is more important than size and siRNA entrapment in overcoming biological barriers -LNPs with pKa < 5.5 were not successful in vitro nor in vivo[194]

1.1.3.3 Biochemical Activation

Another membrane damage mechanism is attributed to the activation of biochemical pathways. Protein Kinase C (PKC) inhibition was observed with certain derivatives of cationic cholesterol (155). The creation of Reactive oxygen species (ROS) was seen in the presence of cationic materials. High cation density on liposomes promotes ROS generation, impacting cellular functions (156). While anionic and neutral liposomes demonstrate no such effect, cationic liposomes might prompt oxidative stress-related pulmonary toxicity, underscoring the influence of cations in ROS production (157). Increased levels of intracellular ROS may react with various cellular macromolecules non-specifically (15). To address the toxicity of cationic lipids, Soenen et al. used the cationic magnetoliposomes (MLs; FeO cores enwrapped by a phospholipid bilayer of dimyristoylphosphatidylcholine or sphingomyelin, doped with cationic lipids 1,2-distearoyl-3-trimethylammonium propane (DSTAP), as a model and showed that the mechanism of cytotoxic effects can be either dependent or independent of particle internalization. While the former is caused by ROS generation leading to Ca^{2+} influx, the latter is due to cell membrane destabilization. They further reported that cationic lipid toxicity depended on the nature of the lipid, size and physicochemical characteristics of liposome and can be reduced by ROS scavengers like N-t-butyl-α-phenylnitrone or Ca^{2+} channel blockers like nicardipine. The use of DSTAP instead of DOTAP or sphingomyelin instead of dimyristoylphosphatidyl choline as the neutral matrix lipids resulted in better membrane packing and decreased cytotoxicity (146). Wei et al. proposed another cationic liposome-induced toxicity mechanism based on impairment of Na^{+}/K^{+}-ATPase, giving rise to exposure of mitochondrial DNA and subsequently, triggering inflammatory responses and contributing to necrotic cell death (158).

Beyond such specific actions, general alterations in cellular gene expression represent another mechanism of cytotoxicity for siRNA delivery vehicles; cationic lipids used in commercial lipofection reagents can modify the expression of >20 genes involved in various cellular pathways, steering human epithelial cells toward early apoptosis (144). Similarly, cationic dendrimers derived from Polypropylenimine (PPI) can influence the expression of endogenous genes associated with cytokine signalling and apoptosis (159). PEI and to a lesser extent PEG-PEI were also reported to activate inflammatory and apoptotic genes depending on the concentration and cell line. Interestingly, the increase in target gene expression was not correlated to siRNA silencing activity, but rather a response to signals triggered by PEG-PEI. A full understanding of the genomic impact of delivery vehicles is vital for accurate interpretation of therapeutic outcomes and engineering platforms with minimum toxicogenomic effects (160).

1.1.4 Biological Barriers to Lipid Nanoparticle Delivery

To accomplish their mission, LNP formulations of nucleic acids must overcome several biological obstacles. Nucleic acids need to be first protected against digestion in physiological fluids by nucleases, which occurs through full encapsulation of nucleic acids by LNPs creating a physical barrier against nucleases. Secondly, they have to be able to evade mononuclear phagocytic system (MPS) interception and renal glomerular filtration following systematic administration. Due to its intrinsic role in regulating (monitoring) the body, MPS in the spleen and liver is a frequent destination for LNPs (161). By lowering the clearance of NPs by MPS we can prolong their circulation lifetime (162). This can be accomplished by utilizing biodegradable functional groups to facilitate clearance (163). One strategy is known as surface PEGylation which lessens the opsonization by serum proteins and reticuloendothelial clearance (164). Additionally, due to the greater permeability and retention (EPR) effect, pegylated NPs are more likely to

extravasate from tumor vasculature to solid tumors (165). PEG surface coating is exploited to govern the kinetics of cellular uptake and prevent PEG-specific antibody induction by dissociating eventually. Dissociation of PEG is necessary in that not dissociated PEG can lead to rapid systematic clearance of subsequent doses via accelerated blood clearance (ABC) (166). Adjusting the PEG structure can subside the ABC by attuning the kinetics of shedding and chain recognition (166,167). The intracellular transposition of NPs, which is a crucial step in the transport of biomacromolecules like siRNA to tumour cells, is favoured by dePEGylation, on the other hand (162). Ester motifs are one more strategy for enhancing biodegradability (168). In addition to being chemically stable, easily incorporated, and bio-cleavable, ester moieties also have controlled biodegradation (135,169,170). If surviving the filtration systems, the LNPs should be able to reach their target cells subsequently and escape endosomal maturation upon internalization, which is believed to be facilitated by LNP's hexagonal phase structure and pH-ionizable moieties (171). LNPs fuse electrostatically to the cell membrane and use an inverted non-bilayer lipid phase to enter the cells by endocytosis (172). Physiochemical properties of nanocarriers such as shape, size, surface charge, and surface composition (makeup) are also believed to affect the mechanism of cellular entry (173,174). Interestingly, LNPs can also be exocytosed (e.g. ~70% of LNP-siRNA formulations) which gives rise to another challenge to LNP delivery (175). Once inside the cell, nanocarriers will be routed into early endosomes and then to lysosomes, where the majority of nanocarriers will be degraded by digestive enzymes, through maturation to multi-vesicular late endosomes. Otherwise, the internalized nanocarriers can be degraded during endo-lysosomal trafficking via recycling pathways (175,176). The proton sponge hypothesis states that endosomal escape occurs as a result of gradual ATP-driven acidification from 6.5 to 5-6, promoting (encouraging) protonation of amine residuals in LNPs formulations, which allows cargo release

following disruption of endosomal membrane (177). The actual endosomal escape mechanism, according to later studies, can be more complex and is influenced by a number of different variables, including endosome size, late endosome formation, membrane leakiness, Rab7a localization on the surface of endosomes, and activation of mTORC1 for downstream signalling for protein synthesis (75,178). The nucleic acids, finally, should be released (freed from the carrier) either into the cytoplasm (in the case of mRNA and siRNA) where the endogenous machinery for RNA interference and protein translation is located, resulting in down-regulation (siRNA) or upregulation (mRNA) of target proteins, or released into the nucleus (in the case of pDNA) where transcription takes place (179–181).

The initial investigations using LNP-plasmid delivery showed limited success since this approach was impeded due to the inevitable requirement for nucleus entry. LNP technology, however, flourished in the case of siRNA delivery since this nucleic acid can function adequately if only be recognized by the RISC, which is present in the cytosol. This quicker approach led to the first robust gene silencing in nonhuman primates (NHPs) using stable nucleic-acid lipid particles (SNALPs) with siRNA payload, which were tailored against apolipoprotein B (ApoB) in 2006 (182). Twelve years later, the first LNP-siRNA drug (Patisiran) was authorized by the FDA to treat hereditary transthyretin-mediated amyloidosis (183).

Multiple intracellular and extracellular barriers have been explained in the following section concisely, along with some related studies highlighting attempts to overcome the respective issues.

1.1.4.1 Liver Accumulation

One major feature and, limitation at times, with LNP delivery is their propensity for accumulation in the liver, where they will be taken up by the reticuloendothelial system (184). Upon systematic administration (185), various serum electrolytes, proteins, and lipids will adsorb onto the LNP's surface and form the so-called "biomolecular corona" (186). This crown can significantly influence the journey of systematically administered nanoparticles from biodistribution and cellular uptake (187) to systematic circulations and nano-bio interactions (188). According to Miao et al., the composition of ionizable lipid particles can have a considerable effect on the formed corona (189). ApoE is one of the most implicated serum proteins that plays a significant role in the clearance and endogenous targeting of LNPs to hepatic cells (190). The delivery of nucleic acids to the liver is partly attributed to the organ's well-perfused nature and its fenestrations as well (181). It has been recognized that LNPs accumulate in different cells within the liver (191–193). Particle size, lipase sensitivity and apparent pKa are some characteristics that govern the intrahepatic distribution of LNPs (194). Chen et al. investigated the effect of particle size on influencing the tissue penetration and *potency of lipid nanoparticle formulations* of siRNA. They injected the LNPs intravenously into mice and found that regardless of the size, the majority of LNPs were found in the liver 24 hours after injection. Less than 1% of the LNP formulations were found in the pancreas, kidney, lung, femur, and heart. However, ~10% of the 80 nm size LNPs were found in the spleen. Since the particle size did not seem to be heavily involved in liver accumulation, they further investigated other parameters that can reduce the potency of small LNPs for silencing. In the end, they concluded that there was a clear hierarchy of LNP-formulated siRNAs' capability for gene silencing (78 nm > 42 nm > 38 nm >> 27 nm > 117 nm). The LNPs with ~80 nm size demonstrated the maximum silencing activity, which was considered to be the result of two factors; lower activity and stability of smaller-sized particles along with the inability

of particles larger than 100 nm to access the hepatocytes (195). In another study investigating the relationship between LNPs' physiochemical properties and the efficiency of siRNA delivery to liver cells, Sato et al. once again showed a size-dependent reduction in gene silencing for 172 and 433 nm particles. In contrast, 76.5- and 117-nm particles consistently showed high gene silencing activity in hepatocytes. Interestingly enough, for targeting liver sinusoidal endothelial cells (LSECs), they concluded that adjusting the LNP size to around 200 nm, which is larger than the fenestrae size in mice, will lead to an increased specificity (194).

Regarding the pKa of ionizable moieties of LNPs, the same study demonstrated that the intrahepatic distribution of siRNA will be significantly changed due to small changes in pKa value, which will subsequently affect gene-silencing activity in both hepatocytes and LSECs. The authors further showed that the ED_{50} for gene silencing (i.e., effective dose for 50% silencing) vs. pKa curve in hepatocytes was bell-shaped, with maximum activity at a pKa of 6.45. At the same time, the same pKa value was not optimal in terms of specificity for hepatocyte gene silencing. They then concluded that since formulation with low pKa results in poor endosomal escape because of the inability to convert to cationic moieties in endosomes, a new mechanism should be introduced to determine the balance between specificity and activity in hepatocytes. In LSECs, on the other hand, a sigmoid curve was observed for gene expression versus pKa value, indicating improved gene-silencing efficiency in response to a rise in pKa value (194).

Intrahepatic localization and activity of LNPs can also be modulated by lipid sensitivity to phospholipase. Based on the knowledge of the existence of extracellular lipases on the surface of liver cells' membrane, including hepatic lipase (HL), lipoprotein lipase (LPL), and endothelial lipase (EL) (196), and the fact that HL is expressed only on the surface of hepatocytes, and LPL and EL on the surface of LSECs (197), Sato et al. hypothesized that EL-sensitive ionizable cationic

lipids in the hepatocyte-specific LNP-siRNA systems is degraded by either EL or LPL on the surface of LSECs but not on the surface of hepatocytes. To reveal this involvement, they used GSK264220A (an inhibitor of LPL and EL) and orlistat (an inhibitor of LPL) as cotreatments. Considering that EL is primarily an A1 phospholipase (PLA1) and GSK264220A inhibits the activity of the EL (177), and that the PLA1 activity of HL is much lower than that of EL (198), they estimated that the LNPs are inactivated by the PLA1 activity of the EL (176).

A potential strategy for non-hepatocyte delivery is to deviate from ApoE-dependent pathways of delivery/targeting by increasing the PEG-lipid content in LNPs, which was not successful in terms of prolonged circulation and redirection to extrahepatic targets (199). Conjugation of targeting ligands on the surface of the LNPs proved to be effective in facilitating the uptake by specific organs in small-scale settings. For instance, conjugated antibodies against vascular cell adhesion molecules PECAM-1 or VCAM-1 and CD-4 were employed to redistribute the LNPs from liver to lung, cerebral endothelium during brain edema and into all T cells (naïve, central, memory, and effector) in both spleen and lymph, respectively (200–202). Moreover, successful localization of LNP-siRNAs to the antigen-presenting cells' cytoplasm has been reported (62). Another approach examined by Saunders et al. involved the pre-treatment of mice with a liposome that occupies liver cells temporarily prior to LNP delivery; this approach decreased the uptake of tested LNP-RNA formulations by the reticuloendothelial system (RES) and ultimately led to enhanced bioavailability of the bioactive RNA, increasing the protein production in the case of mRNA and better silencing in the case of siRNA (184).

1.1.4.2 Spleen Accumulation

LNPs have also been observed to accumulate in the spleen upon systematic administration (191). This has been attributed to protein adsorption on the surface of LNPs followed by surface

opsonization and subsequent uptake by splenic macrophages of the mononuclear phagocytic system (203). Although targeting the spleen for LNP delivery can be considered a promising approach for vaccine development, and oncology purposes (204), lipid and nucleic acid accumulation can trigger undesired immunological responses like cytokine release syndrome by the massive production of IL-6 in the spleen (124).

1.1.4.3 Maintaining Prolonged Protein Expression

Gene editing is another application of nucleic acid delivery-LNP systems. Many studies on enzyme and protein replacement therapies, as well as siRNA-based interventions, have confirmed their potential for correcting genetic diseases. However, the problem with these approaches is their temporary nature (205). Viral vectors and mRNA encapsulating LNPs can be employed for long-lasting treatments to edit the genes by modifying the DNA itself through either loss-of-function or gain-of-function mutations (189,206). Although successful in terms of its mission, viral vectors have not gained much interest for several reasons, such as excess cytotoxicity and immune reaction, the potential of off-target genomic integration and the inability to administer a repeat dose because of the host's adaptive immunity towards the carrier. The LNP approach with mRNA as a nonviral vector, on the other hand, can produce permanent outcomes. Conway et al. managed to knock down the TTR or PCSK9 gene by over 90% by utilizing LNPs containing Zinc finger nuclease (ZFN) coding mRNA. Another approach was the codelivery of mRNA along with a guide RNA inside an LNP in CRISPR-based studies, which showed promising outcomes in vitro and in vivo (206,207). One example was recently demonstrated by Da Silva Sanchez et al. for cystic fibrosis treatment (208).

1.1.4.4 Immunological Responses

Nucleic acids can be recognized as invading pathogens via various cellular sensors (209). Synthetic siRNA can stimulate innate immune responses, especially in the presence of lipidic or polycationic carriers, which utilize endosomes to facilitate intracellular delivery (26). Synthetic siRNAs have been shown to be potent inducers of inflammatory cytokines and interferons through toll-like receptors (22) when used in nonviral delivery vehicles (21). The immunostimulatory potency of nucleic acids is sequence-dependent, suggesting that the motifs can be modified for minimal immunostimulatory activity (194). Chemical modifications of the nucleic acid can prevent the recognition of lipid-encapsulated siRNAs by PRRs (26). After the activation of innate immune responses, dsRNA-dependent protein kinase phosphorylates eIF2a, which downregulates the mRNA translation (210). Modifying mRNA by N1-methyl pseudouridine increased both translational capacity and overall mRNA biological stability when evaluated in mammalian cells in vitro and in vivo. It also decreased its immunogenicity. It has been hypothesized that protein synthesis can be prohibited by RNA-dependent protein kinase activated by structural motifs present in mRNA-containing uridine (211) but not in Ψ modified mRNA. They also highlighted that superior translation might be the reason behind enhanced stability via protecting the mRNA by high ribosome occupancy (212).

1.1.4.5 Endosomal Escape

Despite being recognized for a long time, endosomal escape remains one of the unresolved bottlenecks in the way of effective LNP design (213). Following the cellular entry, LNPs will be trapped in endosomes, from which only a small fraction may be able to successfully escape. It has been estimated that only 2% of designed RNA delivery systems can escape the endosomes effectively (214). Endosomal escape can occur via membrane fusion, rupture, or pore formation

(177). Numerous new formulations of LNPs are continuing to be reported that can more efficiently overcome the endosomal entrapment. To better understand the endosomal escape steps, Herrera et al. employed a screening method based on a Gal8-GFP reporter fusion (Gal8-GFP) (215) to create a robust galectin 8-GFP (Gal8-GFP) cell reporter platform to directly visualize the endosomal escape capabilities of LNP-encapsulated mRNA. This sensor system uses the rapid and sensitive distinction of endosomal membrane integrity as an indicator of cytosolic availability of mRNA(216). Modelling of the delivery process was recently tackled by Mihaila et al., who designed an ordinary differential equation-based model as a predictive tool for optimizing the LNP-mediated delivery of siRNAs. This mathematical model can be effectively used as a screening tool to compare the relative kinetics of different classes of LNPs towards choosing the most efficacious option prior to hands-on experiments. This model employs critical steps of the intracellular RNAi pathway involved in the delivery (i.e., cell entry through plasma membrane, endosomal escape/unpackaging, loading of siRNA onto RISC, and mRNA knockdown) to predict the knockdown efficiency induced by novel LNP formulations of siRNA in vitro (217).

The endosomal escape process has not been fully understood, but it is clear that cationic lipids may facilitate the fusion by increasing the electrostatic interactions with anionic endosomal membrane components leading to the cargo leak to the cytoplasm (171). Ionizable lipids are unique in that they have a neutral charge at physiological pH while being protonated at low pH and thus becoming cationic, which can promote endosomal membrane destabilization and facilitate endosomal escape (218). Numerous ionizable cationic lipids with various structures have been and are being developed, which share certain core characteristics:

(i) Headgroups containing tertiary amines which are uncharged (zwitterionic) under physiological pH and become protonated at acidic pH (171),

(ii) Lipid tails that promote self-assembly into a nanoparticle due to hydrophobic association (171). The tail properties can further affect the endosomal escape capability of LNPs. For instance, due to the stronger protonation at endosomal pH, branched-tail lipids demonstrate improved endosomal escape in comparison with their linear counterparts (168). Lipid type and ratio can also enhance endosomal escape (219–224).

(iii) Protonated lipids which contribute to an elevated propensity for membrane fusion in acidified endosomes in target cells (171). Optimizing the pKa values of the ionizable lipids can positively affect the endosomal escape. Alabi et al. showed that among the three key variables, LNP size, LNP pKa and siRNA entrapment, the strongest correlation with overcoming the biological barriers and consequently gene silencing capability was related to the pKa. They demonstrated that LNPs with pKa lower than 5.5 were not successful in gene knockout in in-vitro and in-vivo systems (225).

The idea that conjugation of ionizable lipids can serve as a strong determinant for siRNA pharmacokinetics was first put forth by Biscans, Annabelle, et al. They covalently attached the ionizable lipid, DLin-MC3-DMA, to siRNA and reported enhanced endosomal escape (evidenced by a 51% increase in large foci Gal8+ cells) in cell culture without compromising the siRNA efficacy. They, however, observed non-specific modulation of gene expression in tissues with more than 20 pmol/mg accumulation of DLin-MC3-DMA suggesting the limitations of this approach(111).

To overcome the limitations of ionizable lipids (as well as lipidic and polymeric systems in general), such as cytotoxicity and potential immune activation, hybrid delivery systems have been recently introduced (226). Sanghani et al. introduced pH-sensitive PEGylated CL4H6-

MRTF-B siRNA-loaded LNPs to safely deliver myocardin-related transcription factor B (MRTF-B) siRNA and efficiently into human conjunctival fibroblasts to prevent conjunctival fibrosis after glaucoma filtration surgery. Their near-neutral PEGylated nanoparticles were not cytotoxic at the siRNA concentration of 50 nM while having far superior silencing compared to their highly cationic non-PEGylated counterparts (>80% in vitro), which was attributed to effective endosomal escape (227).

By doing a systematic derivatization study on one of their previously developed lipids (YSK12-C4), they reached a structure-activity relationship (SAR) to assist them in nominating the best pH-sensitive cationic lipid for further investigations. The SAR revealed that the apparent pKa is considerably affected by the structure of the hydrophilic headgroup but not the hydrophobic tail. Thus, the endosomal escape (pKa dependent) and intrahepatic distribution (pKa independent) can be improved by the modifications of the headgroup and the tail, respectively. Notably, the hydrophilic headgroup and the hydrophobic tail minimally interact with one another, allowing for the independent use of both structures to design the desired pH-sensitive cationic lipid. They then formulated an LNP with a potent pH-sensitive cationic lipid CL4H6 (CL4H6-LNPs), which in in-vivo experiments demonstrated more efficiency for endosomal escape, cytosolic release, and the RNA-induced silencing for the complex-loading of siRNAs when compared to the previously reported LNP formulations. It was also superior in terms of biodegradability and compatibility (228).

LNPs' surface charge is an additional feature that can be tailored for targeting abilities. Negatively charged carriers have been utilized for brain disorders therapeutics; in a comparative study, Gabal et al. reported 1.2-fold higher brain targeting efficiency for anionic nanostructured lipid carriers than their cationic counterparts. However, anionic particles experience limitations

due to difficulties in nucleic acid packaging and poor transfection efficiency. Tagalakis et al. showed that PEGylation improves the receptor-mediated transfection efficiency of anionic nanocomplexes. They used cationic targeting peptides as a bridge between the PEGylated anionic liposomes and the pDNA cargo. Not surpisingly, the newly developed structures displayed more resistance to aggregation in both serum and transfected cells. They also demonstrated enhanced tissue penetration and dispersal and more widespread cellular transfection than homologous non-PEGylated anionic and cationic systems (229). Anionic integrin-targeted hybrid nanocarriers were also explored for siRNA treatment of neuroblastoma with reduced systemic and cellular toxicity and minimal clearance by the liver. Anionic receptor-targeted nanocomplexes were as specific and efficient as their cationic equivalent. This was evident in an animal model as well since anionic receptor-targeted nanocomplexes transfected tumours in an integrin-mediated fashion and entered tumours effectively, with little off-target biodistribution (230).

1.1.4.6 Cytotoxicity

A constant theme in the development of LNPs is the incorporation of lipid degradability to improve biocompatibility. Maier et al. sought to further optimize the LNP platform in this regard (e.g., higher capacity to be metabolized, increased in vivo transfection efficacy, and no toxic metabolites/by-products) by probing novel ionizable lipids used in LNPs (135). Following a review of the literature, they established a correlation between certain structural parameters and activity, which served as a roadmap for the development of effective lipids in vivo. Their lipid should be amphipathic, contain a hydrophilic headgroup made up of an ionizable amine, and have long hydrophobic dialkyl chains. Besides, it should also have a pKa between 6.2 and 6.5 and the ability to adopt a “cone” shape in an acidic environment (231–233). They designed the lipid structure so that the bio-cleavable groups were located within the hydrophobic lipid tails in order

to introduce biodegradable functionality in a way that would promote rapid in vivo metabolism into more hydrophilic, water-soluble products while maintaining excellent potency. They used an ester of linoleyl chain (L319) to replace the 9,10-cis double bond in order to keep the structural characteristics of the lipid necessary for in vivo activity. They reported facilitated excretion and elimination, considerable tolerability, and excellent potency in rodents and NHPs (135). Ester linkages were incorporated in LNPs' tails, between C9 and C10 in the linoleyl chain (named L319), as they were easier to hydrolyze by intracellular lipases or esterases. This modification led to results which were on par with those of the highly potent MC3-lipid but with almost complete elimination in a 24 period. The position of the ester bond played a critical role in the functioning and elimination rate: the closer the ester bond was to the head group, the greater its effect would be on the apparent lipid pKa, and the lower its silencing potency in vivo. The further the ester bond was from the headgroup, the more persistent the lipids would be in the liver (212).

Another approach to reduce the persistence of LNPs was the employment of disulfide bonds. Shirazi et al. synthesized a series of degradable multivalent cationic lipids (CMVLn, n=2 to 5), including a disulfide bond spacer between the headgroup and hydrophobic moiety. This spacer can be cleaved by reducing agents such as glutathione in the reducing milieu of the cytoplasm and thus facilitate elimination. The lipids transfected the mouse fibroblasts with comparable efficacy to highly effective non-degradable analogues and standard commercial reagents like Lipofectamine 2000 while being much less cytotoxic (234). Another derivative was developed by Akita et al., who designed a hepatocyte-targeting siRNA carrier by incorporating tertiary amines and disulfide bonds (ssPalmE) in the hydrophobic scaffold. They reported enhanced gene knockdown in the presence of vitamin E in the scaffold (235).

Finally, it must be noted that pH-sensitive ionizable lipids used in common LNP formulations are also advantageous for nucleic acid delivery from the cytotoxicity perspective, given their lower interactions with blood cells' anionic membrane in a neutral state (at the pH of the circulation system) and thus higher biocompatibility (218).

1.1.4.7 Post-administration Reactions

One other roadblock in LNP-mediated delivery of nucleic acids is the undesirable post-administration reactions. The intravenous injection of LNPs can trigger both complement-dependent and complement-independent (236,237) reactions such as mild flu-like symptoms or more severe cardiac anaphylaxis (236). Hypersensitivity reactions may be influenced by numerous physiochemical properties such as surface charge, lamellarity, and cholesterol content of LNPs (238). A combination of corticosteroid immunosuppressant dexamethasone, for instance, antihistamines such as H1/H2 blockers, and oral acetaminophen, along with reduced infusion rate can be used as a pre-dosing action to manage the infusion-related reactions (239). It has also been reported, in the case of Onpattro®, that the severity of the symptoms will subside by repeated administration and exposure to the drug (240). Another approach is to incorporate PFG-lipids to decrease the possibility of LNPs' recognition by MPS and renal filtration clearance (163,241). Some of the mentioned biological barriers in this section have been demonstrated in Figure 1.5.

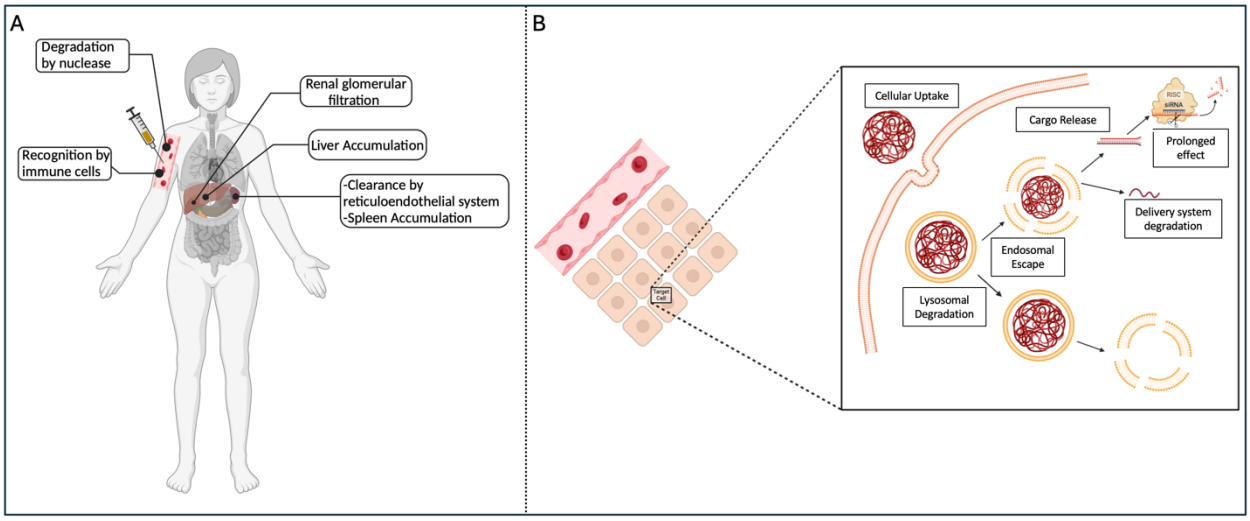

Figure 1.5. Biological barriers for LNP delivery. A. extracellular barriers, B. Intracellular barriers

1.1.5. Scope of Technical Studies

Taken together, an ideal siRNA delivery system must meet multiple requirements such as particle size less than 100 nm to be sufficiently stable enough and capable of successfully passing through liver fenestrae, lack of immunogenicity and toxic effects, apparent ionizable cationic lipid pKa value ~6.4, and around neutral surface charge to avoid sequestration by MPS (171). Considering the aforementioned issues, in the next chapter we report the design of a novel lipopolymer delivery system for the treatment of breast cancer. For this purpose, 1.2 kDa branched PEI polymer was grafted with lauric acid through gallic acid linkers to increase its hydrophobicity and enhance its interactions with the membrane phospholipid bilayer. The result lipopolymer was complexed with siRNA to target specific genes in the MDA-MB-231 breast cancer cell line. Previous data in our lab has shown that contrary to the expectation of anionic additives lowering the interactions with cellular membranes, their incorporation into siRNA polyplexes led to improved cellular uptake (242). In this study, we are introducing new additives and we hypothesized that adding anionic additives to polyplexes and using lower ratios of polymer to

siRNA could respectively increase their cellular uptake and reduce their cytotoxicity. In addition, to explore the impact of ion strength on siRNA polyplexes' efficiency, phosphate buffer was investigated with (pH levels 5.0 to 8.0) as complexation media. The results showed successful delivery of siRNA and efficient gene silencing for both reporter green fluorescent protein (GFP) and endogenous survivin gene.

1.2 Acknowledgement

We would like to thank Marcel Ezra Michailides for his assistance in drafting some of the sections of this chapter and creating Table 1.1.

2. Chapter 2. Designing Nanomedicines for Breast Cancer Therapy

A version of this chapter was published as:

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2.1 Introduction

Cancer has been defined by the World Health Organization (WHO) as the uncontrolled growth of abnormal cells (1). Breast cancer surpassed lung cancer as the most commonly diagnosed cancer worldwide in 2020, and it is now the second leading cause of death among females worldwide (2). Conventional therapies like surgery, radiotherapy, chemotherapy, endocrine therapy (hormone therapy) and targeted therapy are available for breast cancer. They, however, have major short-term and long-term side effects such as skin problems (e.g., soreness, itching, peeling) in the area of treatment or reduced sensation in breast tissue in the case of radiotherapy or gastrointestinal disturbance, hair loss and depressed immunity in the case of chemotherapy (3), which are mainly due to their inability to specifically target malignant cells. This is why novel treatment approaches are being explored (4). The advent of nanotechnology has been foreseen as a potential candidate for cancer therapy since it can provide confined cytotoxicity to cancerous cells resulting in the targeted destruction of malignant tissues and reduced arbitrary drug distribution (5). Nanoparticles (NPs) benefit from many favourable characteristics such as a lower elimination rate, increased drug-site contact time and decreased drug resistance (3). The NP drug carriers, also known as vectors, comprise two main components: the active drug and the material that forms the NP itself and can be used to improve systemic availability (6,7,8). Through the ease of conjugation of various helping moieties via a linker, NPs provide a framework for tailoring custom therapy designs. Furthermore, they enable the development of a multifunctional platform capable of incorporating multiple therapeutic agents for simultaneous delivery and effective treatment. On the flip side, their small size can cause adverse effects by allowing them to penetrate biological structures, disrupt their normal activity and cause harmful effects such as tissue inflammation and a shift in cellular redox balance toward oxidation, resulting in irregular

functions, immune stimulation or cell death. Yet, when it comes to encapsulating various kinds of drugs, NPs represent incredibly versatile tools (3).

The NPs can be utilized to deliver short-interfering ribonucleic acids (siRNAs). In this strategy, a synthetic double-stranded siRNA is delivered to implement the naturally occurring RNA interference (RNAi) mechanism to silence or downregulate the expression of a desired protein (9). Outside the cell and in circulation, the NPs bearing the siRNA should stay intact. After being delivered to the cell, though, the siRNA needs to be freed from the endosome, then it will incorporate into the RNA-induced silencing complex (RISC) before its passenger strand is removed and the remaining "guide" siRNA strand directs the RISC assembly to targeted mRNA, followed by either cleavage or blockage of the mRNA to inhibit protein translation (10,11). However, due to the fragile (degradable) nature of anionic siRNA in physiological conditions, the efficacy of siRNA delivery is heavily dependent on its carrier to successfully transport it to the cell and protect it against degradation by nucleases (12). Structural motifs, chemical medications and sequence selectivity have been used to design more effective RNAi drugs. Different motifs may have very different functions that will affect the RNAi processing efficiency (13). For instance, it has been shown that in the case of asymmetric siRNA, the bias for guide strand selection to enter the RISC is toward the strand with a 3′ overhang (14). Chemical modifications, excluding ligand targeting, have two primary purposes: improved safety by attenuating activation of immune sensors, and increased potency by enhancing protection against degradation by nucleases. Sugar, base, and backbone modifications can be used in this regard (13). As an example, extensive 2′-O- methyl base modifications on recent RNAi triggers have largely avoided immunogenic reactions that usually result from sensing the double-stranded RNA (dsRNA) by PKR, Toll-like receptor 3 (TLR3) and TLR7 (15). Finally, sequence selectivity has played an

important role in selecting the right strand, the antisense strand, as the guide strand for incorporation into the RISC (13). It has been demonstrated that a strand with weaker base-pairing as its 5′-end will be chosen to enter the RISC (14,16). Both viral and non-viral vectors can also be used to deliver siRNA to the cells. Although viral vectors like adenoviruses, lentiviruses and retroviruses, have high transduction efficiency, they are worrying considering their potential for insertional mutagenesis and unwanted immunogenicity. Non-viral vectors, on the other hand, are preferred by virtue of their easy-to-engineer nature and lower manufacturing costs. Non-viral vectors, additionally, can solve other limitations of their viral counterparts by having unlimited loading capacity and providing compatible transient gene expression (17). Nonetheless, poor transfection efficiency and significant cytotoxicity are still limiting their therapeutic use (18).

Lipidic (e.g., liposomes and niosomes) and polymeric carriers have been explored as non-viral carriers (17). Cationic polymers have been extensively investigated due to their unique physio-chemical properties that can electrostatically form complexes with nucleic acids. They can protect against enzymatic degradation, mediate transfection via nucleic acid condensation and facilitate cellular uptake and endosomal release. Polyethyleneimine (PEI) is one of the most prominent synthetic cationic polymers with primary, secondary, and tertiary amino functions which is synthesized in linear and branched forms in different molecular weights (19), PEI has repeatedly demonstrated high transfection efficiency (20) which is attributed by its ability to facilitate endosomal escape through the so-called “proton sponge” phenomenon (i.e., uncharged Ns acting as sponges during the endosome maturation that leads to swelling of the polymer, Cl- counterion flux into the endosome and endosome destabilization due to osmotic forces) (21). The excess positive charges in high molecular weight PEI (>20 kDa) induce plasma membrane destruction which causes loss of metabolic activity and cell death. The low molecular weight PEI

(<2.0 kDa) is reported to be less toxic and thus is a more suitable carrier for siRNA delivery. We have previously shown that lipidic substitutions on the PEI backbone can improve the uptake of siRNA/PEI complexes, presumably due to their enhanced chemical compatibility with the cell membrane (22,23).

In this study, we explored leading PEI-Lipid conjugates as polymeric non-viral vectors that include the low molecular PEI (1.2 kDa) grafted with three different lipids: linoleic acid (3-18-C), Oleic acid (1-18-C) and Lauric acid (12-C) via gallic acid (GA) as linker, which allows for three lipid conjugates at a single site of PEI (24). We inspected their efficacy for siRNA delivery in breast cancer treatment. Our goals were to achieve high gene silencing via siRNA activity while minimizing nonspecific toxicity through the development of effective lipid-modified PEI carriers (lipopolymers). To address these, we hypothesize that lowering the lipopolymer ratio and adding negatively charged additives to our nano complexes will, respectively, reduce cytotoxicity and improve gene silencing ability by facilitating cellular uptake. To scrutinize our hypothesis, we compared the lipopolymer/siRNA with lipopolymer/siRNA/additives for which we selected a range of additives and investigated their efficiency in various polymer:additive:siRNA formulations. Finally, the effect of different buffers for complex preparation was also explored to provide a better siRNA delivery environment.

2.2 Materials and Methods

2.2.1 Materials

Branched 1.2 kDa PEI (bPEI1.2) was obtained from Polyscience, Inc (Warrington, PA, USA). Methylthiazolyldiphenyl tetrazolium bromide (MTT), Dulbecco's Phosphate Buffered Saline (PBS), Linoleoyl chloride, Lauroyl chloride and Poly(acrylic acid) (PA) [MW: ~2000 Da]

were obtained from Sigma-Aldrich (St. Louis, MO, USA). Green Fluorescent Protein (GFP) positive MDA-MB-231 (MDA-MB-231-GFP+) cell line was prepared through retroviral transfection (25). Dulbecco's Modified Eagle Medium F12 (DMEM F12), dimethyl sulfoxide (DMSO) was obtained from MiliporeSigma (Saint Louis, MO, USA). GFP-siRNA, negative control scrambled siRNA, 6-'(FAM)-labeled scrambled siRNA, and survivin siRNA were obtained from Integrated DNA Technologies Inc. (Coralville, IA, USA) and their sequences are mentioned in Appendix A Table A1. *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (HEPES) (BP 310-500) was purchased from Fisher Bioreagents (Pittsburgh, PA, USA). Trypsin was purchased from ThermoFisher Scientific Inc. (Waltham, MA, USA). Other additives including β-Glycerophosphate Disodium Salt Hydrate (GDS), Potassium Phosphate Monobasic (PPM), Sodium Pyrophosphate (SPP), tri- Sodium Orthophosphate (TSO) and *N*- Lauroylsarcosine Sodium Salt (LS), as well as the 1/15 mole L^{-1} phosphate buffer, 0.1 mol L^{-1} Citrate buffer, and PEI-LA transfection reagent were prepared in-house. To create 3.7% formaldehyde, Hank's Balanced Salt Solution (HBSS) was used to dilute a 37% stock solution, which was purchased from Sigma-Aldrich.

2.2.2 Cell Culture

The green fluorescent protein (GFP) gene derived from jellyfish Aequorea victoria has proven to be a good quantitative reporter for gene expression in eukaryotic cells. GFP-expressing cell lines are generally produced by transduction with viral plasmids that have been genetically modified to express the GFP gene and the insertion of the gene into the genome of the target cell line [Soboleski MR, Oaks J, Halford WP. Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. The FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2005 Mar;19(3):440.]. The MDA-MB-231 breast cancer

cells were cultured in DMEM/F12 medium containing 10% fetal bovine serum (FBS) as well as 100 U/mL of penicillin, and 100 μg/mL of streptomycin and 5 mL sodium pyruvate. The cells were maintained at 37 °C under humidified conditions with 95/5% air/CO2. The cells were routinely examined for mycoplasma contamination using a PCR-based method as explained by Young et al. (26) and validated by STR DNA profiling analysis at the Genetic Analysis Facility, The Centre for Applied Genomics, The Hospital for Sick Children (Toronto, ON, Canada). At around 80% confluency, cells were seeded 24 h before treatment with siRNA complexes as follows. After removing the utilized media and rinsing with 5 mL PBS, 1 mL of 0.05% trypsin was added and the cells were incubated at 37 °C for 2–3 min to promote cell dissociation and were diluted by the addition of 5 mL DMEM. Subsequently, the cells were centrifuged at 600 rpm for 5 min, followed by resuspension in 5 mL fresh DMEM. Finally, 300 μL of cells were seeded in 48-well plates at the density of 60,000 cells/mL for GFP gene experiments and 20,000 cells/mL for survivin gene experiments.

2.2.3 siRNA Complex Preparation

Different PH levels were tested for siRNA complexation. Lower pH levels lead to better protonation of the lipopolymer and hence improved binding to siRNA and more stability of the complex. Neutral pH levels were also tested since they prevent the disruption of the cells and bodily fluids considering the pH level 7.4 of blood circulation. Citrate and phosphate buffer were selected due to their buffering capability at lower and higher pH levels, respectively. Citrate buffer (0.1 M) was prepared by mixing disodium citrate (0.1 M; 1 L solution contains 21.01 g citric acid monohydrate + 200 mL NaOH 1 M) and HCL 0.1 M. Phosphate buffer was prepared by mixing potassium dihydrogen phosphate 1/15 M (9.073 g L^{-1}) with disodium phosphate 1/15 M (11.87

g/L). All the buffers were filtered with a syringe before use. Additives (0.14 ug/uL) were added to the media after siRNA (0.14 µg/µL) had been dissolved in the media and before the transfection reagent (PEI-LA 1 mg/mL or PEI-GA-Lau7 0.5 µg/µL) was added at desired (*w*/*w*/*w*) ratio of transfection reagent to additive to siRNA. The PEI-LA and PEI-GA-Lau7 lipopolymers were prepared as described in our earlier papers. (Figure 2.2.) (24,27). Ca^{2+} (0.14 µg/µL) was then added in the form of $CaCl_2$ if required with the ratio 1:1 to siRNA. The solutions were then incubated at room temperature for 30 min for optimal complexation. Finally, the remaining amount of buffer was added to make 200 µL of the solution with the final siRNA concentration of 50 nM in the tissue culture medium (Figure 2.1.).

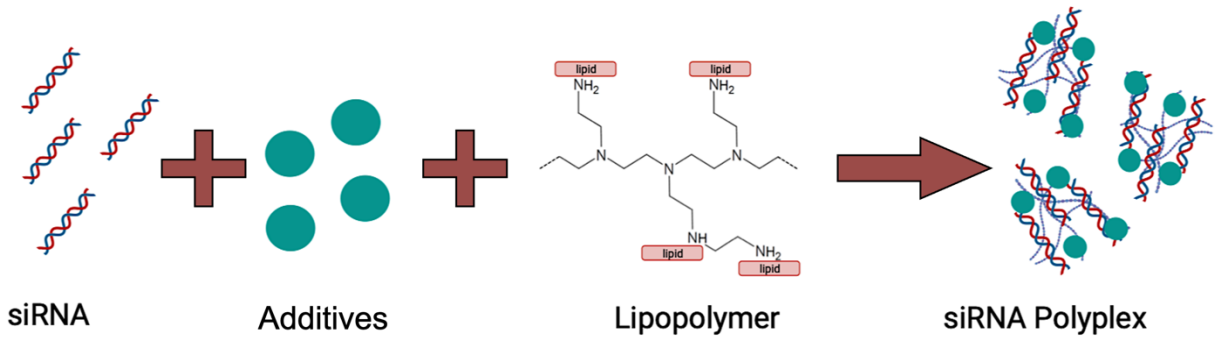


Figure 2.1. siRNA complex formulation

The specific concentrations used are indicated in Figure legends. Table 2.1. and Table 2.2 show a list of additives that were initially selected and a sample design for the siRNA complex preparation, respectively.

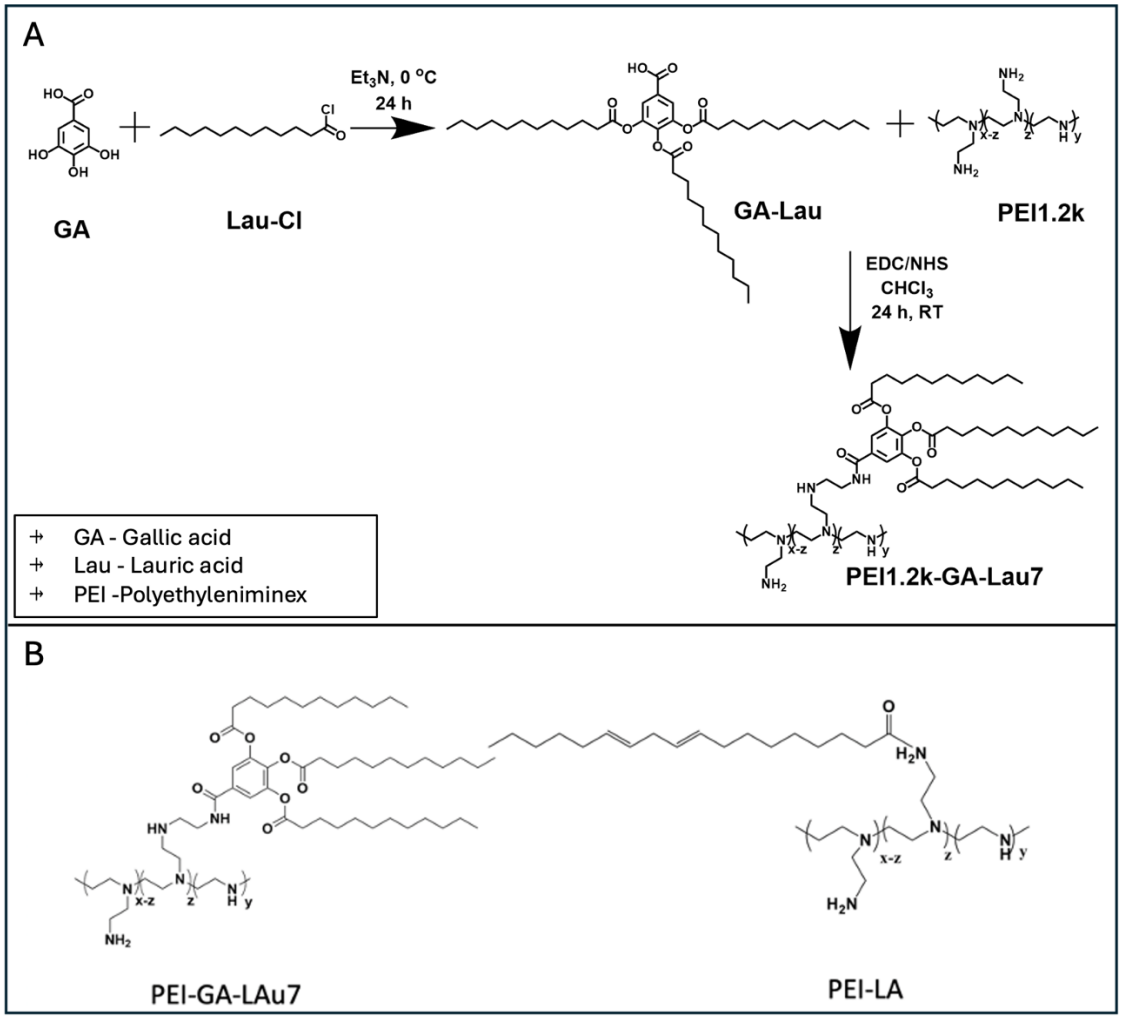


Figure 2.2. PEI-GA-Lau7 and PEI-LA. A. PEI-GA-Lau7 synthesis process, B. Chemical structure of PEI-GA-Lau7 and PEI-LA

Table 2.1. List of additives. These additives were selected based on being anionic and not being present in the cell culture medium in high volumes.

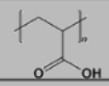
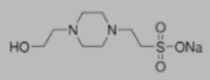
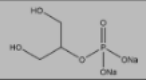
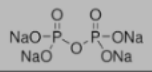
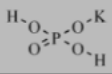
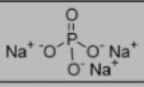
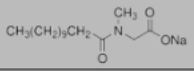
Abbreviation	Full Name	Chemical Structure
PA	Poly Acrylic acid	
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]	
GDS	B-Glycerophosphate Disodium Salt Hydrate	
SPP	Sodium Pyrophosphate	
PPM	Potassium Phosphate Monobasic	
TSO	Tri-Sodium Orthophosphate	
LS	N-Lauroylsarcosine Sodium Salt	

Table 2.2. Example of a study design and corresponding siRNA complex preparation (for duplicate wells). Components were added in the order they appear in the chart (left to right), except for the media which was added in two parts (10 uM siRNA concentration solution for complex preparation and final concentration of 50 nM of siRNA in culture medium were used). NT: No Treatment which indicates the cells treated with only media as the negative control. Specific (W/W/W) ratios used for polymer/additive/siRNA are mentioned in each experiment separately. The ratio column indicates the ratio of transfection reagent to siRNA.

Carrier	Ratio (w/w)	Media (uL)	siRNA (uL)	Additive (uL)	Transfection reagent (uL)	Total Volume (uL)
NT	0	200	0	0	0	200
Buffer + siRNA	0	196	4	0	0	200
PEI-LA/SiRNA5.0	5	193.2	4	0	2.8	200
PEI-LA/SiRNA5.0-PA1.0	5	186.4	4	4	5.6	200
PEI-LA/SiRNA7.5	7.5	191.8	4	0	4.2	200
PEI-LA/SiRNA7.5-PA1.0	7.5	183.6	4	4	8.4	200
PEI-LA/SiRNA10.0	10	190.4	4	0	5.6	200
PEI-LA/SiRNA10.0-PA1.0	10	180.8	4	4	11.2	200
PEI-GA-Lau7/SiRNA1.0	1	194.88	4	0	1.12	200
PEI-GA-Lau7/SiRNA1.0-PA1.0	1	189.76	4	4	2.24	200
PEI-GA-Lau7/SiRNA1.0-HEPES1.0	1	189.76	4	4	2.24	200
PEI-GA-Lau7/SiRNA1.0-GDS1.0	1	189.76	4	4	2.24	200
PEI-GA-Lau7/SiRNA1.0-SPP1.0	1	189.76	4	4	2.24	200
PEI-GA-Lau7/SiRNA1.0-PPM1.0	1	189.76	4	4	2.24	200
PEI-GA-Lau7/SiRNA1.0-TSO1.0	1	189.76	4	4	2.24	200
PEI-GA-Lau7/SiRNA1.0-LS1.0	1	189.76	4	4	2.24	200

2.2.4 Physiochemical characterization of siRNA polyplexes

The particle size and surface charge (ζ-potential) of siRNA polyplexes were determined by Litesizer 500 (Anton-Paar). The complexes were prepared in 200 µL as described above and were diluted in 1 mL of their corresponding complexation media before measurement. For heparin dissociation experiments, the heparin sodium salt was added to complexes in concentrations ranging from 0 to 10 U/mL for 1 h incubation with the complexes. The complexes were then added to 96-well plates that already had an equal volume of 2X SYBR Green to reach a total volume of 500 uL. The amount of fluorescence was measured with Fluoreskan Ascent 2.5 (Thermo Labsystems) at excitation/emission 485/527 nm. SYBR Green I was used to quantify pure siRNA. Pure siRNA and siRNA+heparin were used as negative and positive controls, respectively.

2.2.5 GFP Silencing

The amount of fluorescence in cells was measured as an indication of GFP gene expression. For this, the cells were rinsed with PBS (1X) after the removal of the media, trypsinized, fixed in 3.7% formaldehyde and then transferred to black 96-well black plates for fluorescence measurement by Fluoroskan Ascent 2.5 (Thermo Labsystems) at excitation 485 nm/emission 527 nm.

2.2.6 Flow Cytometry

To determine the delivery efficiency of siRNA complexes, MDA-MB-231 cells were transfected with FAM-labeled scrambled siRNA at the same concentration either 24 or 48 h after seeding. On day 3 post seeding, the media was removed, and cells were washed with PBS. Then, they were detached and fixed in 3.7% formaldehyde. The no-treatment samples were used as the negative control. The FAM-labeled siRNA positive population and mean fluorescence were quantified using BD Accuri C6 Plus flow cytometer using FL2 channel (10,000 events/sample). The FAM-labeled siRNA-positive population was set as 1% in this case.

2.2.7 RNA Extraction and Quantitative Reverse Transcription PCR (qRT-PCR)

For the qRT-PCR experiment, to quantify the amount of silencing achieved by desired siRNA, MDA-MB-231 cells were first seeded 24 h prior to survivin siRNA transfection. Next, 72 h later, total RNA was extracted by the addition of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and transferred to cDNA using SensiFAST cDNA Synthesis Kit (Meridian Biosciences Inc., Cincinnati, OH, USA) according to the manufacturer's instruction. For amplification, 3 µL of cDNA was added to 7 µL of the master mix which itself includes 5 µL of SensiFAST SYBR Hi-

ROX reagent (Meridian Biosciences) and 1 µL of each of the forward and reverse primers for either survivin gene or β-actin as the housekeeping gene (Integrated DNA Technologies Inc., Coralville, IA, USA). The sequences for the mentioned primers are as follows: β-actin primers and survivin primers (Appendix A, Table A2). The samples were amplified by StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a denaturation stage (95 °C, 20 sec) followed by 40 cycles at 95.0 °C for 3 sec (denaturation) and annealing and elongation at 60 °C for 30 sec. The results were analyzed using $2^{-\Delta\Delta CT}$ and presented as a relative quantity of no treatment.

2.2.8 MTT Assay

To perform the assay, 100 µL of filtered MTT solution (5 mg/mL) was added to the 48-well plates at 1 mg/mL. The cells were incubated at 37 °C to convert the yellow MTT to purple formazan crystals using NAD(P)H-dependent oxidoreductase enzyme (28). After 2 h, the media were removed, and the crystals were dissolved in DMSO for 5–10 min. The solutions were then transferred to 96-well plates and their absorbance was measured at 570 nm by the multi-well spectrophotometer.

2.2.9 Statistical Analysis

The data are presented as mean ± s.d. The results were analyzed by homoscedastic one-tailed distribution *t*-test, where the asterisks (*), (**) and (***) represent significantly different groups with $p < 0.05$, $p < 0.005$ and $p < 0.0005$ in figures in comparison with no treatment and circles (°), (°°) and (°°°) indicate the significant silencing of targeted mRNA transcripts by specific siRNA compared to that of control siRNA with the above-mentioned *p* values.

2.3 Results

2.3.1 PEI-GA-Lau7 Can Deliver GFP-siRNA Better than PEI-LA to MDA-MB-231 Cells

To determine if the newly developed PEI-GA-Lau7 could be advantageous over the previously optimized PEI-LA, both polymers were compared at polymer:siRNA ratios of 5, 7.5 and 10 with/without PA as an additive in the DMEM complexation medium. GFP-positive MDA-MB-231 cells were analyzed 72 h after transfection. The silencing activity was similar (40–43%) all ratios of PEI-LA and the PEI-GA-Lau7 polymer were as effective as PEI-LA, but the latter polymer showed more toxicity under these conditions (>50% cell death; not shown). Citrate (pH levels from 1 to 5) and phosphate (pH levels from 5 to 8) buffers were examined as complexation media. Lower ratios of 1 and 2 for PEI-GA-Lau7 were used to lower its toxicity, whereas previously optimized ratios of 5, 7.5 and 10 for PEI-LA were used. Several additives were tested to PEI-GA-Lau7 and the GFP fluorescence was measured 72 h after treatment (Figure 2.3.). Cells could not survive the treatment with complexes which were formed in citrate (not shown); thus, this buffer was not tested any further. Regarding the phosphate buffers, the silencing with PEI-LA was similar in pH 5.0 and 8.0 buffers, but cytotoxicity appeared to be higher in the latter buffer. The silencing with the PEI-GA-Lau7 was best in the pH 8.0 buffer with no apparent cytotoxicity at the polymer:siRNA ratio of 1:1. At the higher ratio, the cytotoxicity of PEI-GA-Lau7 generally increased. PA was not particularly beneficial in improving the silencing, but other additives appeared to increase the silencing efficiency to some extent (10–20%), with no obvious candidates among the HEPES, GDS, SPP, PPM, TSO, and LS emerging as a clear-cut choice.

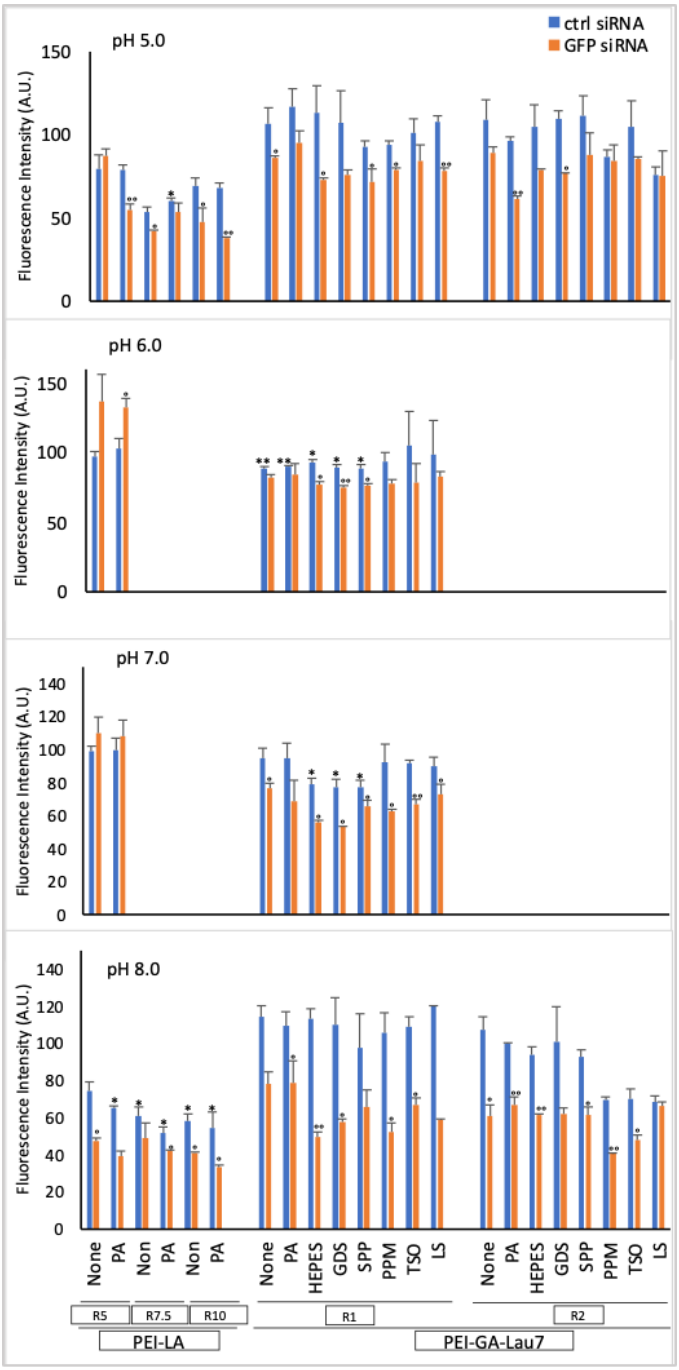


Figure 2.3. Green fluorescence measurement (microplate reader) in MDA-MB-231 GFP+ cells as an indication of gene expression. The expression of the GFP gene was measured 3 days after delivery of 50 nM siRNA polyplexes at weight ratios 1:1:1 and 2:1:1 of PEI-LA or PEI-GA-Lau7:additive:siRNA which were prepared in phosphate buffer (**A**) pH 5.0, (**B**) pH 6.0, (**C**) pH 7.0 and (**D**) pH 8.0 to MDA-MB-231 cells. NT: No Treatment. The asterisks indicate the significant toxicity of GFP siRNA treatment (*) $p < 0.05$ and (**) $p < 0.005$ compared to no treatment. The circles represent the significant silencing of GFP transcripts by specific siRNA treatment compared to that of control siRNA, (°) $p < 0.05$, (°°) $p < 0.005$.

2.3.2 Ratio 1 of Additive Is Better than Higher Ratios in MDA-MB-231 GFP+ Cells

To further optimize the formulation, higher ratios of additives to siRNA (1:1, 2:1 and 3:1) in phosphate-8.0 were tested while the PEI-GA-Lau7:siRNA ratio was kept at 1:1 (Figure 2.4.). When compared to PEI-LA, PEI-GA-Lau7 caused less cytotoxicity than before. Moreover, the polymer:additive:siRNA ratio of 1:3:1 appeared to be toxic in most cases. PA, which performed best when combined with PEI-LA, underperformed when combined with PEI-GA-Lau7. When inspecting the PEI-GA-Lau7 results, on average, the majority of the selected additives had fairly comparable silencing abilities, HEPES (~45%), GDS (~48%), and PPM (~47%), whereas LS demonstrated the highest silencing (~60%) consistent with Figure 2.3. results. Apart from PA, ratios 1 and 3 of additives showed the highest and lowest GFP silencing activity, respectively.

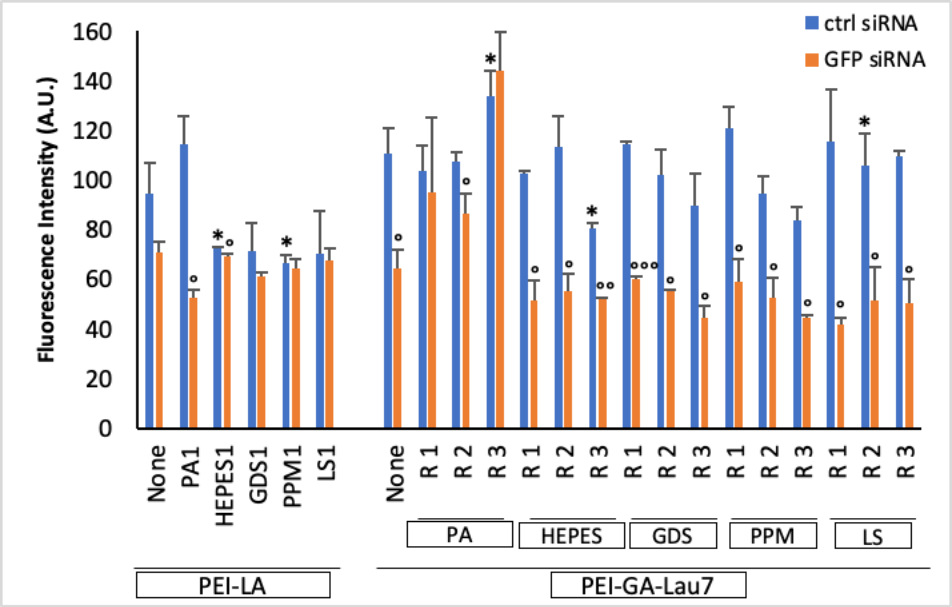


Figure 2.4 The expression of the GFP gene was measured by a microplate reader. MDA-MB-231 cells were analyzed 3 days after treatment with in-phosphate pH 8.0 siRNA polyplexes. Ratio 1 of PEI-LA or PEI-GA-Lau7 polymer was used in combination with *w/w/w* ratios 1, 2 or 3 of additives or PA to siRNA for PEI-GA-Lau7 and in the case of PEI-

LA, only ratio 1 of additives was used. siRNA was used in a final concentration of 50 nM. NT: No Treatment. The asterisks indicate the significant toxicity of GFP siRNA treatment (*) $p < 0.05$ compared to no treatment. The circles represent the significant silencing of GFP transcripts by specific siRNA treatment compared to that of control siRNA, (°) $p < 0.05$, (°°) $p < 0.005$ and (°°°) $p < 0.0005$.

The impact of calcium in the formulations was also investigated, following the work of Dick et al. (29) which found a beneficial effect of Ca^{2+} on plasmid DNA (pDNA) complexes and transfection efficiency. The Ca^{2+} was added to the complexes in phosphate-8.0 and cells were examined 3 days following the treatment. No noticeable improvement in toxicity resulted from the incorporation of Ca^{2+} and no beneficial effect of Ca^{2+} could be seen on the silencing efficiency (not shown). Incorporation of Ca^{2+} was deemed non-beneficial and not pursued any further in the case of siRNA delivery.

2.3.3 Physiochemical Characteristics of siRNA Polyplexes

To characterize the physical properties of the particles, their size and zeta potential were measured after the complexes were made in phosphate buffer with different pH with or without additives as usual and were then diluted in the same buffer to reach the total volume of 1 mL (Figure 2.5.A). The particle sizes varied between 200 nm and 1.6 µm. However, most of them were between 500 and 750 nm. When no additive was added, the smallest particle (551 nm) and largest particle (644 nm) were seen in pH 7.0 and 5.0, respectively. pH 6.0 and 8.0 gave similar particle sizes, which were ~600 nm. Regarding the additives effect, contrary to complexes in pH 8.0, additives appeared to increase the particle sizes in most of the cases in pH 5.0–7.0. PA, however, led to the smallest particle size in all pH levels. The polydispersity index (PDI) of the complexes is summarized in Appendix A. Figure A1.

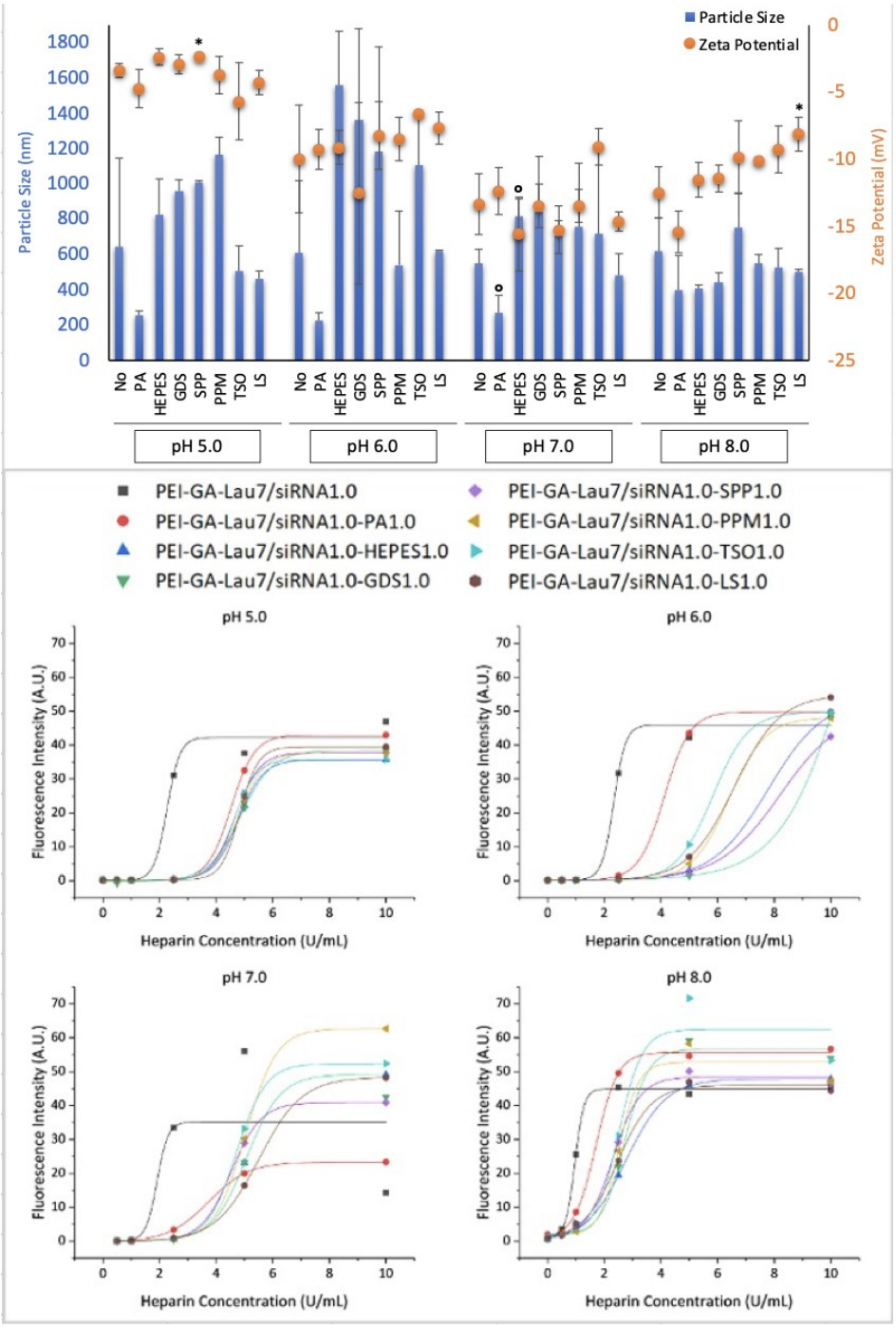


Figure 2.5. Physiochemical characteristics of additive polyplexes. (**A**) size and ζ potential, and (**B**) stability against heparin for siRNA complexes were measured in different pH levels either with or without additive incorporation at the ratio 1:1:1 of PEI-GA-Lau7:additive:siRNA. The circles represent the statistical significance of specific additive formulations compared to without additive formulation in the corresponding buffer media, (°) $p < 0.05$, (°°) $p < 0.005$ and (°°°) $p < 0.0005$.

The zeta potentials of the complexes were all between −15 and −2 mV. The complexes prepared in phosphate buffer-5.0 had less negative zeta potentials on average (approximately −4), which was followed by pH 6.0 (~9), 8.0 (~11) and 7.0 (~13). In most of the cases, additives led to a lower value of the zeta potentials.

To further elucidate the characteristics of polyplexes, the stability of the polyplexes against heparin displacement was investigated as a function of increasing heparin concentrations (Figure 2.5.B). The polyplexes were formed by ratio 1:1:1 of PEI-GA-Lau7:additive:siRNA. A lower half-maximal dissociation concentration (DC_{50}) was observed at higher pH levels for PEI-GA-Lau7 (i.e., DC_{50} of 1.9 U/mL in pH 8.0 and 4.3 U/mL in pH 5.0). Most of the additive formulations behaved similarly; the additives led to more stable complexes against heparin (at all pH values tested), which was indicated through higher DC_{50}compared to complexes without additives.

2.3.4 siRNA-Mediated GFP Silencing Is Persistent for at Least 6 Days in MDA-MB-231 Cells

The activity of siRNA complexes was then explored for a period of 6 days (Figure 2.6.). When the 2 polymers were compared at various time points without the additives, the PEI-GA-Lau7 silencing ability was higher on days 1 (15%), and 3 (50%), but that of PEI-LA was higher on day 6 (70%). However, more overall silencing was accomplished in combination with additives for PEI-GA-Lau7, with 84% being the highest in the case of PEI-GA-Lau7 + LS on day 3. Interestingly, even with the additives, a similar pattern in performance was visible, with PEI-LA performing at its peak on day 6 and PEI-GA-Lau7 performing more or less similarly from day 3 onward.

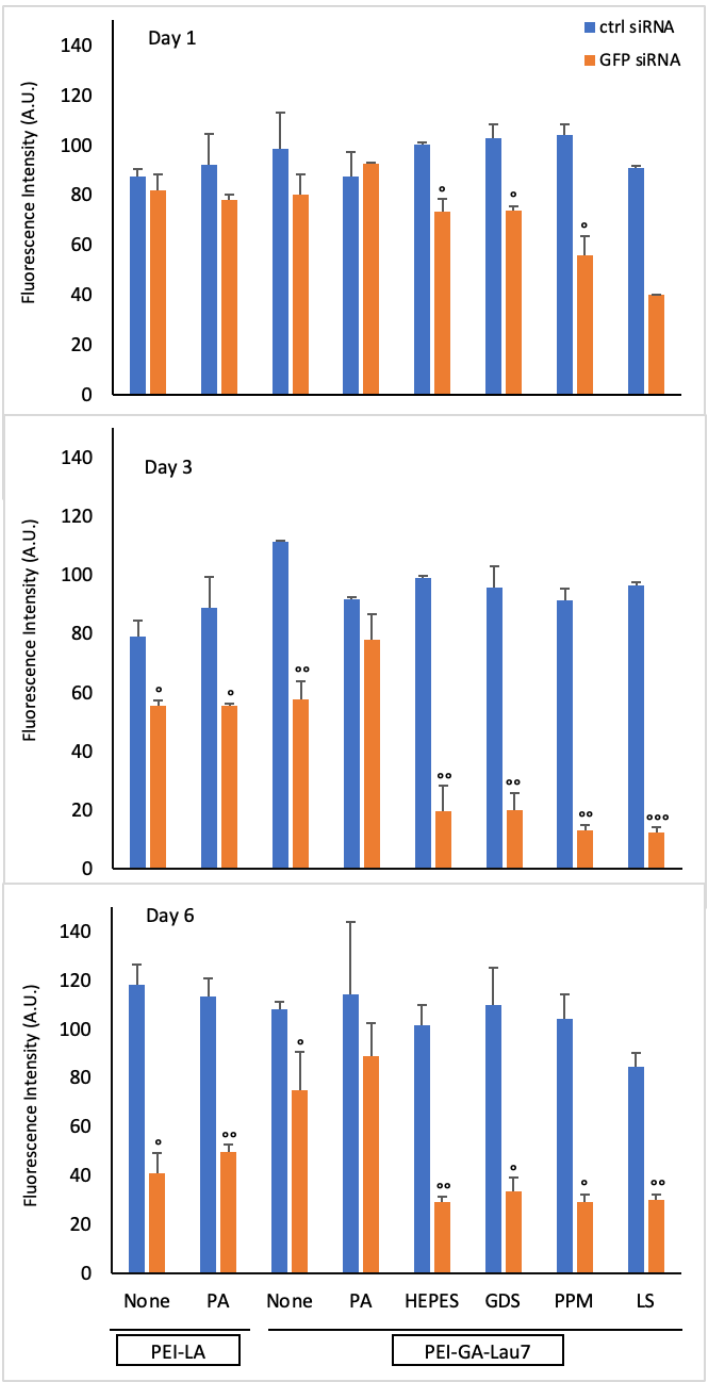


Figure 2.6. Time course study to reveal the persistence of the GFP silencing effects of pre-optimized siRNA polyplexes. MDA-MB-231 cells were treated with siRNA complexes at the weight ratio 1:1:1 of PEI-LA or PEI-GA-Lau7: additive or PA: siRNA which were prepared in phosphate 8.0. The amount of green fluorescence was measured by microplate reader 1-, 3- or 6-days post-transfection. A total of 50 nM concentration of siRNA was used. The circles represent the significant silencing of GFP transcripts by specific siRNA treatment compared to that of control siRNA, (°) $p < 0.05$, (°°) $p < 0.005$ and (°°°) $p < 0.0005$.

2.3.5 Optimized Polyplexes Showed ~95% Cell Uptake in MDA-MB-231 Cells

Flow cytometry was then performed to investigate the cellular uptake of different formulations (Figure 2.7.). The cells were seeded and treated either 24 or 48 h later with the phosphate-8.0 formulated FAM-labeled siRNA at ratios 1:1:1, 3:1:1 or 5:1:1 of PEI-LA or PEI-GA-Lau7:siRNA:additive. Most PEI-GA-Lau7 complexes gave >90% siRNA-positive cells and only 2 of the PEI-GA-Lau7 formulated polyplexes had less than 90% cellular uptake (~80% and ~70% for ratio 1 of PEI-GA-Lau7 to siRNA without additive or with PA as the additive, respectively). However, only 2 of the PEI-LA formulated polyplexes had >90% cellular uptake (ratio 5 of PEI-LA to siRNA when combined with either HEPES or LS). With PEI-LA polyplexes, the uptake was generally increased after 48 h, whereas an opposite trend was observed with PEI-GA-Lau7 polyplexes.

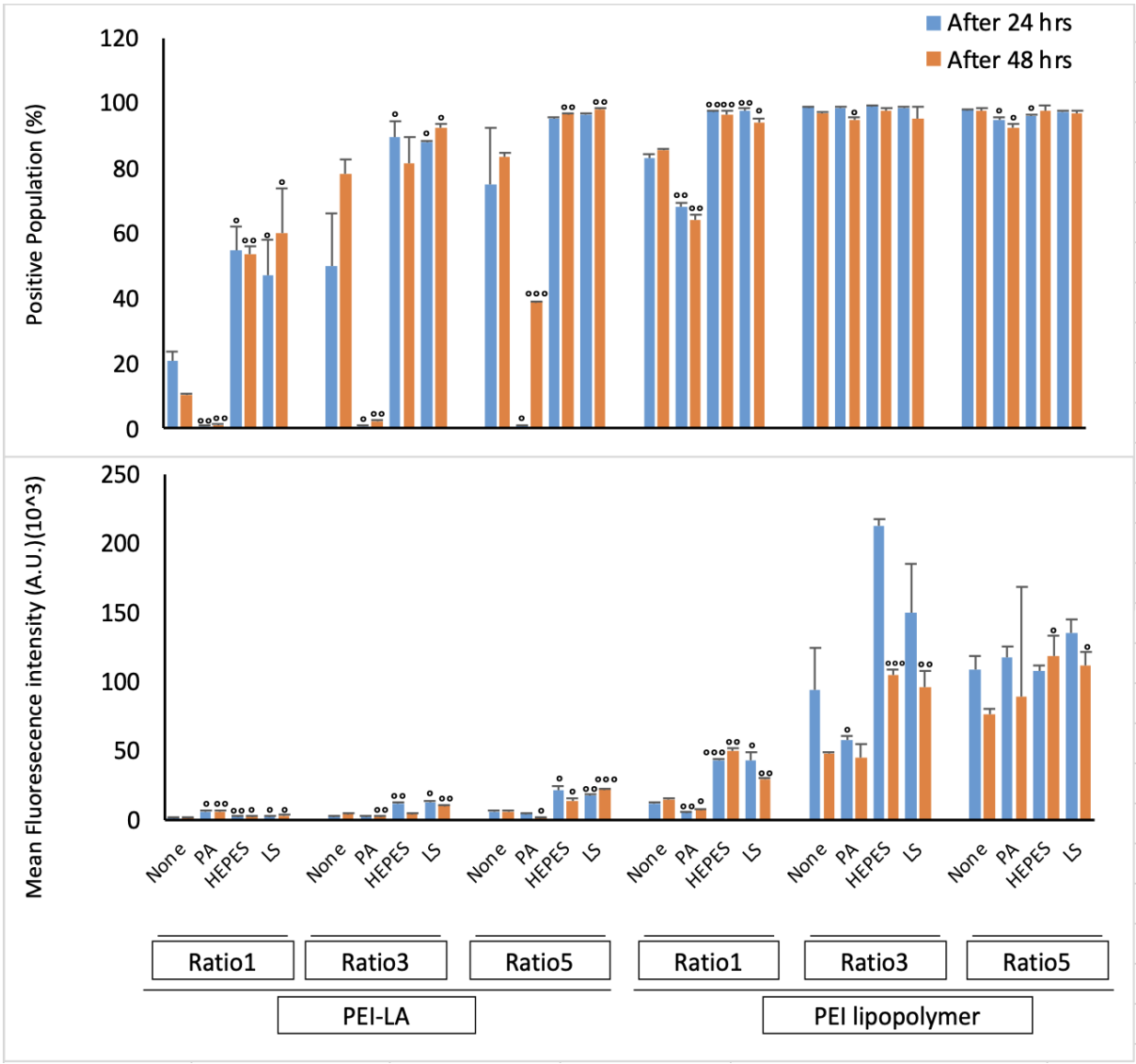


Figure 2.7. Cellular uptake of siRNA/polymer polyplexes in MDA-MB-231 cells. Cells were transfected with 50 nM of FAM-labeled siRNA at 1:1:1, 1:3:1 or 1:5:1 PEI-GA-Lau7 or PEI-LA:siRNA:additive or PA (*w*/*w*/*w*) ratios either 24 h or 48 h after seeding. The complexes were made in phosphate pH 8.0. (**A**) Mean FAM-labeled siRNA uptake (mean + SD) (**B**) Fam-labeled siRNA positive cells (mean + SD). The circles represent the significant siRNA uptake/mean fluorescence intensity by specific siRNA treatment compared to that of without additive formulation, (°) $p < 0.05$, (°°) $p < 0.005$ and (°°°) $p < 0.0005$.

2.3.6 Polyplexes Can Silence Endogenous Genes with High Efficiency and Low Cytotoxicity

To probe the efficacy of our optimized polyplexes to silence an endogenous gene, survivin was chosen as the target. Wild-type MDA-MB-231 cells were treated with 50 nM survivin siRNA complexes in phosphate-8.0 buffer using either PEI-GA-Lau7 or PEI-LA in ratios 1, 3 or 5 with or without PA, HEPES or LS as the additive to treat. Then, the qRT-PCR assay was performed on day 3 post-treatment (Figure 2.8.). Comparing different ratios of PEI-GA-Lau7, the most amount of silencing was observed with the incorporation of HEPES to ratio 3 of PEI-GA-Lau7:siRNA. Yet, when the same formulation was used in ratio 1 of PEI-GA-Lau7:siRNA, >90% silencing was achieved. For LS-formulated polyplexes; however, ratio 1 proved to be the best after ratio 3 and ratio 5 regarding survivin gene silencing. Except for ratio 1 in which LS and HEPES polyplexes similarly showed minimum toxicity (~−20%), in the other two ratios of PEI-GA-Lau7 to siRNA, HEPES was clearly less toxic (~−50% for HEPES vs. ~35% for LS in ratio 3 and ~6% for HEPES vs. ~35% for LS in ratio 5). In terms of PEI-LA versus PEI-GA-Lau7 comparison, in ratio 1, ~10% and ~40% of toxicity and silencing on average were seen with PEI-LA, both of which were improved with PEI-GA-Lau7 (~−5% toxicity and ~80% silencing). Adding HEPES or LS to PEI-LA polyplexes reduced the toxicity from about 10% to almost −10% in ratio 1 of PEI-LA to siRNA.

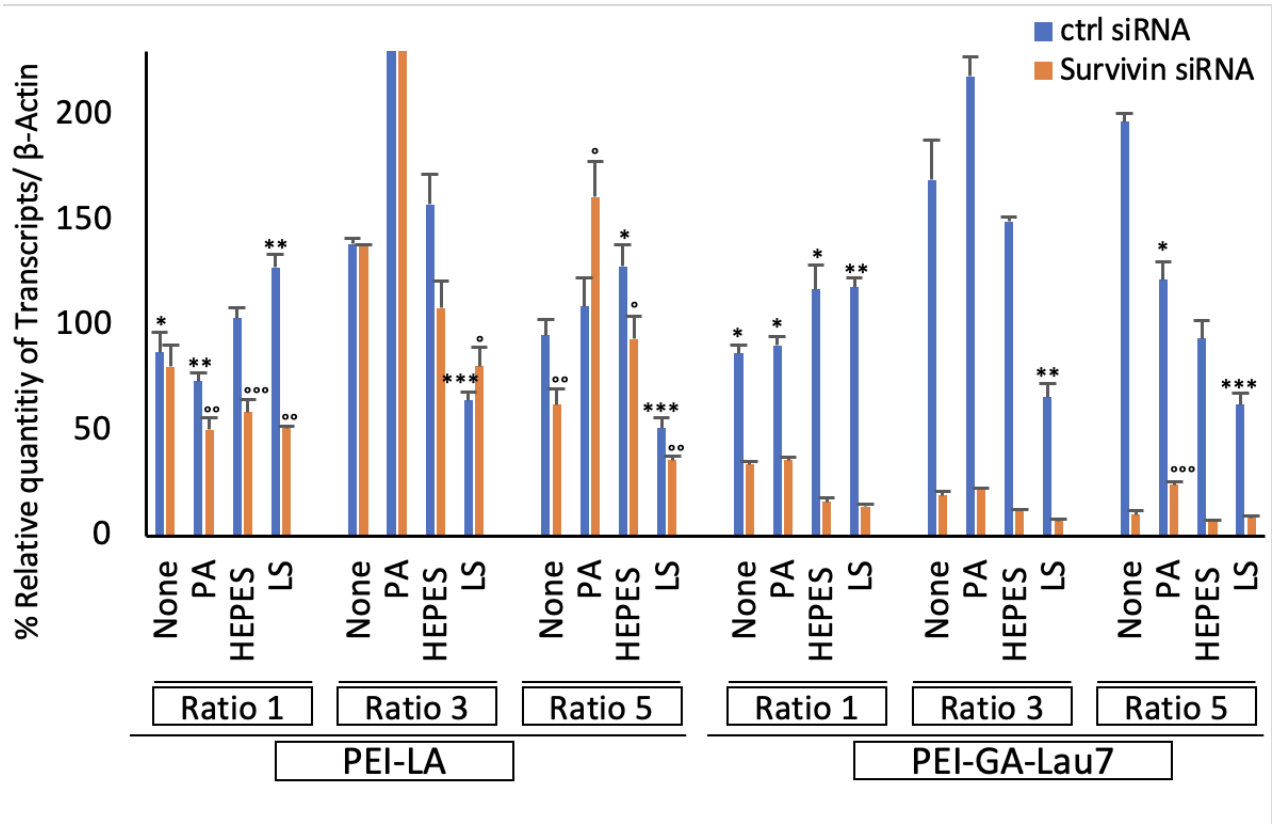


Figure 2.8. siRNA complexes' efficiency was examined using qRT-PCR. In total, 300 uL of MDA-MB-231 cells with 20 K per mL confluency were cultured in 48-well plates. After 24 h, 100 uL of survivin siRNA polyplexes were added to the cells. Polyplexes were synthesized in phosphate pH 8.0 and the final concentration of 50 nM of siRNA was used. Ratio 1 of PA, HEPES and LS to siRNA was used as additives. The RNA was extracted 3 days post-treatment. NT: No Treatment. The asterisks indicate the significant toxicity of GFP siRNA treatment (*) $p < 0.05$, (**) $p < 0.005$ and (***) $p < 0.0005$ compared to no treatment. The circles represent the significant silencing of survivin transcripts by specific siRNA treatment compared to that of control siRNA, (°) $p < 0.05$, (°°) $p < 0.005$ and (°°°) $p < 0.0005$.

2.3.7 Polyplexes Could Effectively Inhibit Cell Growth in MDA-MB-231 Cells

The MDA-MB-231 cells were treated with synthesized polyplexes 24 h after seeding. Ratios 1, 3 and 5 of PEI-LA or PEI-GA-Lau7 and ratio 1 of additives to siRNA were employed in phosphate-8.0 buffer. The cell viability was assessed 72 h after treatment (Figure 2.9.). The best cell-killing activity (>90%) was achieved by the incorporation of HEPES into ratio 1 of PEI-GA-

Lau7:siRNA. Treatment with ratio 1 of PEI-GA-Lau7 + LS killed ~70% of the targeted cells. More toxicity was observable in higher ratios of PEI-GA-Lau7 in control siRNA samples. PEI-LA was less toxic in higher ratios with polyplexes formulated with control siRNA. The superior specific killing ability of the PEI-GA-Lau7 alone to PEI-LA can also be observed in all the ratios, especially in ratio 1 (~5% for PEI-LA vs. ~98% for PEI-GA-Lau7).

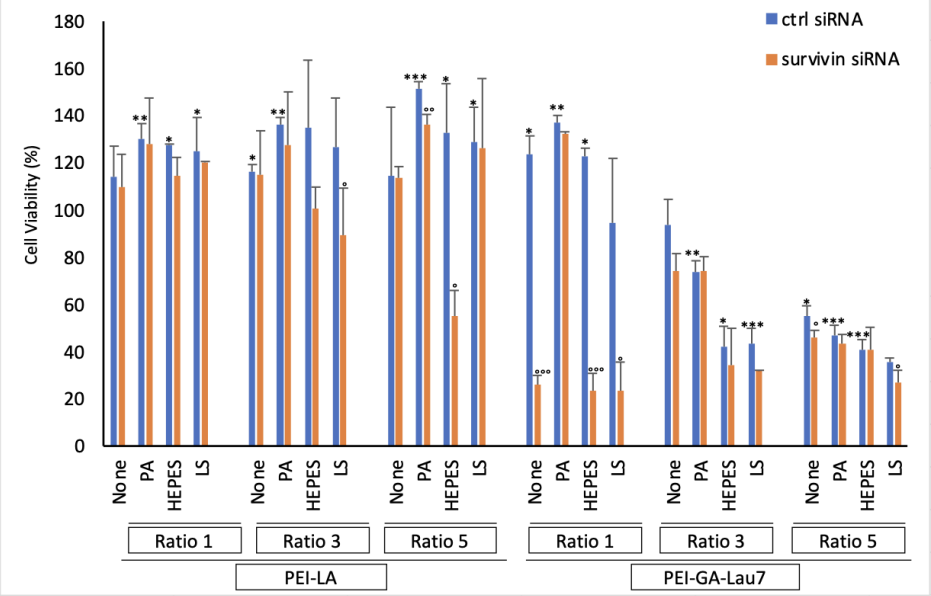


Figure 2.9. The viability of MDA-MB-231 cells was assessed after the delivery of survivin siRNA in MTT assay. A total of 50 nM siRNA was delivered to the cells 24 h after seeding and the MTT solution (1 mg/mL) was added to the cells 72 h after treatment. Lastly, the absorbance at wavelength 570 nm was measured with a spectrophotometer on the same day. Ratios 1, 3 and 5 of PEI-LA and PEI-GA-Lau7 and ratio 1 of additives to siRNA were used. Phosphate pH 8.0 was used as the complexation media. The results are represented as a relative percentage to NT which was put as 100%. NT: No Treatment. The asterisks indicate the significant toxicity of survivin siRNA treatment (*) $p < 0.05$ compared to no treatment. The circles represent the significant viability of cells after treatment by specific surviving siRNA formulation compared to that of control siRNA, (°) $p < 0.05$, (°°) $p < 0.005$ and (°°°) $p < 0.0005$.

2.4 Discussion

Nucleic-acid-based therapeutics have been investigated as promising approaches for targeted therapies for several cancers including breast cancer (30,31,32). siRNAs allow the targeting of specific therapeutic markers and are easy to synthesize as a therapeutic. They may also have a short development time allowing them to readily switch siRNAs that can target different biomarkers. However, until better carriers are created to protect and deliver them intact into the cytoplasm, they will not be able to fulfill their perceived potential for therapeutic outcomes (33). Carrier development is challenging; they ought to be safe and nontoxic and determine if they can be deployed to other nucleic acid therapeutics (27). The transfection reagents that have been used here are based on nontoxic low molecular weight (1.2 kDa) bPEI that has been modified with hydrophobic moieties and has previously been studied for pDNA delivery (24). The membrane destabilization has been suggested as the mechanism for both cytotoxicity and cargo delivery (34,35); hence, the hydrophobic moieties are expected to improve interactions between the cell membrane and polymeric carrier which in turn will result in higher cellular uptake and, unfortunately, more cytotoxicity (27). To address this issue, in the present study, one of our main interests was to achieve the highest gene silencing while lowering the non-specific cytotoxicity. We decided to look into lower transfection reagent to siRNA ratios for this purpose. The N/P ratio refers to the ratio of nitrogen atoms in PEI to phosphates in nucleic acid. It has been estimated that in physiological pH, 1 in 5 or 6 Ns of bPEI is protonated and these are the only amines that interact with nucleic acids. Since the pKa of each individual nitrogen cannot be determined, the N/P ratio is used to describe the amount of present polymer in polyplexes and along with zeta potential has been demonstrated to greatly impact the complex effectiveness. Higher ratios will have more interactions with the cell membrane due to their excess positive charges and will lead to more

cellular uptake (36,37). On the other hand, they are reported to form more stable complexes which can act as a barrier inside the cells for siRNA release (38). In this context, after comparing our newly developed polymer PEI-GA-Lau7 with PEI-LA as our leading breast cancer transfection carrier in previously optimized ratios, we initiated our studies with further optimization of complex formulations. The results showed considerably improved nontoxicity (on average 40% in PEI-LA vs. −10% in PEI-GA-Lau7) as well as enhanced silencing ability (60% for with-HEPES or LS formulated PEI- polyplexeS) for PEI-GA-Lau7 complexes formed at ratios 1 and 2 to siRNA. The flow cytometry also showed improved uptake for the PEI-GA-Lau7 polyplexes and is most likely the underlying basis of improved GFP silencing.

Moreover, it has previously been shown that anionic additives in polyplexes can be beneficial not only for pDNA delivery but also for siRNA (27,39). It has been reported that contrary to the likelihood of the excess anionic charges hampering the interactions between complexes and cell membrane (40), adding PA to PEI-LA increased the cell uptake (39). Furthermore, more anionic complexes can also benefit from lower cytotoxicity (41) as well as protecting the siRNA from enzymatic degradation (42). Parmar et al. have also shown that adding hyaluronic acid (HA), poly(acrylic acid) (PA) or dextran sulfate (DS) to polyplexes as additives can result in more robust siRNA release due to lower polyionic complexation and increased siRNA availability inside the cells. They also observed improved retention time for siRNA particles inside the cells thanks to additive incorporation (27). Although these carriers were able to deliver their cargo efficiently, there is always a demand for better carriers. That is why one of our objectives for this study was to identify new additives for siRNA complexation. In this regard, we first selected 6 potential additives (HEPES, GDS, SPP, PPM, TSO, LS) in addition to PA. After a series of experiments, LS and HEPES repeatedly demonstrated better performance and were thus chosen

for further studies. Considering the combination of PEI-GA-Lau7: siRNA ratio and the additives' functionality, using ratio 1 of PEI-GA-Lau7 could achieve 48% silencing on average while half of the selected additives showed >50% silencing. In head-to-head comparisons in these experiments, PEI-LA showed relatively lower potency for siRNA mediated silencing. Cytotoxicity was also improved considerably after utilizing lower ratios of PEI-GA-Lau7 (see Figure 2.3.). In addition, the ratio of additives was also optimized for these formulations in an experiment in which siRNA polyplexes were formulated with either ratio 1, 2 or 3 of additives to siRNA (Figure 2.4.). Ratio 1 had the most average silencing (~60%) after ratio 2 (~50%) and ratio 3 (~42%). Although none of the complexes were more than 10% toxic, a similar trend was seen as the ratios of additives went up from 1 to 3.

Another objective of our study was to explore various buffers and systematically see if the ion strength will influence the efficacy of siRNA polyplexes. Different solutions such as HEPES (43,44), water (45), PIPES buffer (20 mM PIPES, 150 mM NaCl) (46), HBG buffer (20 mm HEPES, 5% *w/v* glucose, pH 7.4) (47) and Tris-EDTA (48) have been investigated for nucleic acid complexation. Within this framework, we experimented with water, citrate and phosphate buffers and DMEM as the complexation media, with Ca^{2+}as a special additive due to the previously observed benefit of this ion on nucleic acid complexes. Comparing different pH levels of phosphate buffers, pH 7.0 (~13%) and 8.0 (−8%) were the most and least toxic but phosphate-8.0 showed by far the highest silencing activity compared to other phosphate buffers. The most silencing (~60%) was observed with the inclusion of either HEPES or LS as the additive with the ratio 1 of PEI-GA-Lau7 to siRNA in pH 8.0.

The physiochemical characteristics of particles can dramatically affect their efficacy. Therefore, we probed the effects of pH and additives on particle size and zeta potential. The smallest average size of particles was achieved at pH 8.0. Since larger particles cause adverse effects like microinfarctions (49), pH 8.0 was selected for further studies. The fact that PA resulted in the smallest particles in all four pH levels can be explained by PA being the only polymer among all the additives so it can form more interactions with the PEI polymer to better compact the complexes. Additives, on the other hand, increased the size of the particles in most cases except at pH 8.0. To elaborate, in pH 8.0, the no-additive complex was ~619 nm while the particles with PA, HEPES and LS were ~402, ~412 and ~505 nm, respectively. The particles with other additives were larger.

Zeta potentials were all negative which can be beneficial in the sense that they may cause less toxicity and irreversible damage to the cell (50). This can, at the same time, decrease the cellular uptake given the negative charge of the cell membrane. However, considering the flow cytometry results, it seems that the anionic charge did not impede the cellular uptake for these complexes. pH 5.0 showed the average zeta potential of ~−4 mV which increased to ~9 mV in pH 6.0, ~−13 mV in pH 7.0 and ~−11 in pH 8.0. This might be because the more acidic the environment is, the more positive charges it has that can push the negatively charged complexes toward the positive side. In pH 8.0, however, the additives show an effect with LS (~−8 mV) pushing the zeta potential to the positive side more than the rest of the additives. PA with ~−15 mV, on the other hand, showed the most negative charges. These patterns are consistent with previous studies (51).

The lower DC_{50} in pH 8.0 can be explained by the abundance of negative charges in more alkaline pH, which will loosen the siRNA-lipopolymer binding and make it easier for heparin to dissociate it. The fact that additives increased DC_{50} was a reflection of additives making more stable complexes in the sense that they could shield the complexes and make it more difficult for heparin to access the polymer. From the dissociation study, it was shown that the PEI-GA-Lau7 complexes in pH 8.0 have a tendency for good dissociation in lower levels of heparin (DC_{50}), compared to other pH levels so it can be a better candidate for gene delivery.

We then explored survivin as a target to determine if the obtained results with a reporter gene could be reproduced with the endogenous biomarker. Apoptosis is a cell death programmed with distinct biochemical and morphological characteristics that have been conserved throughout evolution (52). Impairments in apoptosis lead to many diseases including cancer (53). It may also adversely impact cancer cells' response to chemotherapy and radiation, contributing to treatment resistance (54). Apoptosis induction, whether by stimulating apoptotic pathways or inhibiting antiapoptotic pathways, is being investigated as a cancer therapy strategy. Survivin has been found to be involved in a variety of intracellular mechanisms, all of which promote cell survival (55). Survivin belongs to the inhibitor of apoptosis proteins (IAP) family, which prevents apoptosis by inhibiting caspase activation (56). It is the smallest IAP and was first discovered in 1997 (57,58). Its gene of 14.7 kb which spans on the chromosome 17 telomeric part and is localized to q25 band (57), encodes a 16.5 kDa protein consisting of 142 amino acids. Unlike other mammalian IAP members that usually have two or three Repeats of Baculovirus IAP Repeat (BIR) domain, an essential Cys/His-based zinc finger for apoptosis inhibition, the survivin protein has only one copy of the BIR domain (59). Contrary to embryonic and fetal organs with strong expression of survivin, in most of the differentiated normal tissues (60), survivin is undetectable, which makes it a perfect

target to reduce cell growth solely in malignant cells without affecting normal cells (27). Overexpression of survivin has been observed in many cancer models and its nuclear expression is associated with its cell division role via controlling the stability of microtubules of the normal mitotic spindle (61). Survivin has previously been reported as a viable target for breast cancer treatment (62). Shepherdin (63), YM155 (64), and terameprocol (65,66) are examples of small molecular weight antagonists for survivin that have been investigated for cancer therapy. Nevertheless, due to their poor selectivity as small organic entities, these agents have the potential to have undesirable effects [38]. Several studies have successfully induced apoptosis via survivin knockdown using various RNAi techniques (67,68,69,70). qRT-PCR revealed >90% silencing in wild-type MDA-MB-231 cells by the inclusion of HEPES and LS in ratio 1 of PEI-GA-Lau7 to siRNA. Whereas LS could only silence ~50% of survivin transcripts in higher ratios. The previously seen low cytotoxicity was also evident in this experiment with ~−3% for PEI-GA-Lau7 and ~10% for PEI-LA on average in ratio 1 to siRNA. Further confirmation of survivin silencing was based on the cell viability of MDA-MB-231 cells after survivin targeting. PEI-LA at its best formulation, PEI-LA/siRNA5.0-HEPES1.0, could achieve ~78% cell death while its other formulations with PEI-LA showed <40% cell killing activity. PEI-GA-Lau7, on the other hand, showed ~98% cell death on its own and ~99% and ~71% when combined with HEPES or LS in the ratio 1 to siRNA (optimal formulationS). Responses in non-malignant breast cells have been compared to cancerous cells in our previous paper. Significant cell growth inhibition by similar formulations in breast cancer MDA-MB-231 cells was reported but not in the MCF-10A cells as the non-malignant breast cells line. Although the non-malignant MCF-10A cells were in fact transfected by the complexes due to morphological similarities, the siRNA uptake was lower compared to malignant cells (27).

It is also noteworthy that a concentration of 50 nM of siRNA was used in all the experiments which is in the concentration range for therapeutic silencing (71,72). The cell line, carrier and other factors can change this range due to changes in siRNA bioavailability, expected treatment effects and other reasons. Different concentrations of siRNA are being used. Persengiev et al. reported a concentration-dependent specificity for siRNA in HeLa cells. In their study, by using expression profiling, using a range of 0 to 200 nM of luciferase siRNA, they reported 100 nM as the threshold where non-specific effects happen, which can be crucial when the treatments will not lead to cell killing. Lowering the siRNA concentration, however, reduced the silencing efficiency. They finally observed the best efficiency between 25 and 50 nM. Their results indicate the importance of optimizing the siRNA concentration in a way to achieve efficient silencing with the lowest siRNA concentration (72). Finally, PEI1.2 KDa was excluded as a control in these studies because historically it has been tested in the same cell line in our lab and has shown no transfection efficiency (73).

2.5 Conclusion

In conclusion, we report a robust siRNA delivery system, PEI-GA-Lau7, which can effectively downregulate the expression of the survivin gene in MDA-MB-231 cells. This was accomplished by the incorporation of two newly identified anionic additives, HEPES and LS, into the polyplexes which enhanced the siRNA uptake by the cells. The cytotoxicity was decreased to a minimum by lowering the ratio of PEI-GA-Lau7 to siRNA and the complexation media was also optimized by choosing a phosphate buffer with a pH level of 8.0. Our polymer has proven to be a promising non-viral vector for gene therapy in breast cancer. Nevertheless, similar studies should be conducted in animal models to further validate the beneficial effect of formulated polyplexes. Since ‘stealth’ features might be critical in impacting blood pharmacokinetics favourably and

achieving better disease tissue targeting through improved circulation, further engineering of our nanoparticles might be needed for animal studies (74).

2.6 Acknowledgement

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3. Chapter 3. Overall Conclusions and Future Directions

3.1 Overall Conclusions

This thesis investigated the potential of nucleic acids and their application as a treatment strategy for breast cancer. Nucleic acid-based therapy offers an alternative approach to traditional chemotherapy in cancer treatment. The complex interplay among signalling pathways and compensatory mechanisms within cancer cells has prompted a shift in drug development focus toward nucleic acids (1–7). These molecules target specific pathways and proteins implicated in the disease. However, nucleic acids face challenges such as their inability to penetrate cells and susceptibility to degradation by serum endonucleases. Therefore, the aim of this dissertation, outlined in Chapter 2, was to develop polymeric nanocarriers tailored for gene delivery to breast cancer. Through meticulous polymer engineering, effective non-viral gene delivery agents have been designed for targeted applications (8,9).

Several separate investigations have explored a diverse array of cationic polymers, including high molecular weight polyethyleneimine (PEI), poly(L-lysine), and poly(amidoamines), for gene delivery purposes (10). Among these, PEIs have been widely recognized for their efficacy in both laboratory and clinical settings. Notably, branched 25 kDa PEI, with its high molecular weight, has been acknowledged as an effective gene delivery vehicle. However, the considerable cellular and systemic toxicities associated with the high cationic charge density of high molecular weight PEI restrict its practical application. Conversely, low molecular weight PEI exhibits reduced toxicity and can be easily eliminated from the body. Unfortunately, despite its lower toxicity, and low molecular weight PEI is not as effective as a gene delivery agent, as it tends to form unstable complexes that struggle to traverse the cell membrane. In order to overcome this constraint, we have strived to augment the gene delivery efficiency of low molecular weight PEI (1.2 kDa) by integrating a hydrophobic component, Lauric acid, via a gallic acid linker

that enables three substitutions at each site to enhance hydrophobicity. While lipid substitutions have been documented previously, the utilization of the GA as linker and a variety of anionic additives represented novel aspects of this study. The newly formulated polymer exhibited superior performance compared to the previously employed polymer, particularly at lower polymer:siRNA ratios. Employing lower ratios is crucial in clinical settings to mitigate toxicity, as corroborated by our in vitro investigations.

Moreover, our research revealed that integrating anionic additives enhances the delivery of siRNA to MDA-MB-231 breast cancer cells by facilitating their cellular uptake. A wide variety of anionic additives were tested. The variability in the effectiveness of the additives emphasizes the necessity of refining formulations tailored to specific cell types and polymers employed.

3.2 Future Directions

Our ultimate goal remains to focus on the development and optimization of nucleic acid-based therapies for the treatment of cancer. Building upon the foundation laid by our current research endeavour, several avenues for future investigation present themselves, each poised to contribute significantly to the field's advancement.

First and foremost, more in vitro studies can be done to further explore the efficacy of these particles. For instance, western blot or other protein expression assays can be conducted to confirm the reduction in protein expression as a result of the mRNA level decrease observed in qRT PCR experiments. Furthermore, the phenomenon of achieving over 100% cell viability in the control siRNA samples as observed in the MTT assay suggests a potential cellular response triggered by the treatment with control siRNA, thereby modulating metabolic pathways to enhance cell survival. Similarly, the qRT-PCR results revealing more than 100% silencing may be attributed to

the upregulation of survivin, an antiapoptotic gene, promoting cell survival. The heightened survivin expression in the control samples could lead to a comparative increase in silencing when contrasted with survivin knockdown, resulting in values exceeding 100%. Nonetheless, comprehensive investigations are imperative to elucidate the precise mechanisms underlying these anomalies. Future research endeavours should focus on delineating the intricate molecular pathways involved in these phenomena to broaden our understanding of their implications in therapeutic interventions.

Additionally, the translation of promising in vitro results into clinically relevant outcomes necessitates validation through in vivo studies utilizing animal models. Animal studies serve as crucial platforms for elucidating the pharmacokinetic and pharmacodynamic profiles of the developed formulations within a physiological context with consideration of regulatory pathways and signalling mechanisms. These studies not only validate the efficacy observed in vitro but also provide insights into potential systemic effects and safety profiles, thus facilitating the eventual transition to clinical trials (11).

Furthermore, the continued refinement of drug delivery systems remains paramount. Exploring alternative lipid substitutions, diverse linker chemistries (12), novel additives like hyaluronic acid, poly(acrylic acid), dextran sulfate, and methyl cellulose (13). Due to the observed behavioural difference with polymeric additives in our study, it seems logical that a more systematic study can be done with a focus on the relationship between the structure of the additive and its impact on cellular delivery and gene silencing efficiency. Moreover, various pH levels (14) hold promise for optimizing the performance and biocompatibility of the delivery platforms. It has been observed that materials with pKa values around 6.5 are ideal for non-viral nucleic acid delivery because they maintain a positive charge during formulation at acidic pH and a near neutral

charge at physiological pH 7.4 (15,16). Additionally, fine-tuning the ratio of siRNA to carrier molecules offers a potential avenue for mitigating cytotoxicity while maintaining therapeutic efficacy (17). Studies have revealed that the therapeutic effectiveness of nanoparticle distribution changes with the weight ratio (w/w ratio) of PBAE to siRNA (18).

Expanding the scope of targeted genes represents another avenue for exploration. In addition to traditional oncogenes and tumour suppressor genes, targeting surface glycoproteins such as CD44 (19), which plays a crucial role in cancer cell adhesion, invasion, and metastasis, holds promise for disrupting key tumorigenic processes. Similarly, genes involved in cell cycle regulation, such as MPS1 and CDC20 (20), present intriguing targets for intervention, offering the potential to induce cell cycle arrest and inhibit tumour growth.

Moreover, capitalizing on the versatile nature of polyethyleneimine (PEI)-based polymers, exemplified in our investigation, holds promise for advancing gene-editing approaches using Clustered Regularly Interspaced Palindromic Repeats (CRISPR)-Cas9 technology, thus delineating a compelling frontier. CRISPR/Cas9 has catalyzed a paradigm shift in biomedical research, facilitating rapid, cost-effective, and relatively facile genome manipulation. Notably, it has demonstrated efficacy in rectifying genetic aberrations in murine models, effectively ameliorating hereditary disorders. Additionally, reports underscore its potential for similar modifications in human embryos.

CRISPR/Cas9 operates via two indispensable components: guide RNA, which matches the target gene, and Cas9—an endonuclease that induces double-stranded DNA breaks, facilitating genomic alterations. Clinically, this technology holds promise for correcting monogenic disorders (e.g., cystic fibrosis, Duchenne's muscular dystrophy, and hemoglobinopathies), combating

infectious diseases (e.g., HIV), and ex vivo engineering of somatic cells for subsequent therapeutic reinfusion (e.g., T-cell genome engineering for hematological malignancies or pluripotent stem cell genome editing for β-thalassemia mutation correction).

Yet, several challenges impede the clinical translation of CRISPR/Cas9's potential into efficacious bedside treatments. A key obstacle revolves around delivering gene-editing machinery to specific cells, particularly in vivo settings. To achieve this safely, without eliciting toxicity, an appropriate vector is imperative. Ongoing investigations into non-viral delivery systems necessitate further optimization to ensure efficient and safe delivery. By harnessing the precision targeting capabilities of CRISPR-Cas9 alongside the efficient delivery facilitated by lipopolymer carriers, the prospect of sustained gene editing and therapeutic intervention is poised for exploration (21).

In summary, the pursuit of these avenues for future investigation holds significant promise for advancing the development of nucleic acid-based therapies for breast cancer and beyond. By addressing key challenges in delivery optimization, target selection, and long-term efficacy, we can inch closer toward realizing the full therapeutic potential of this innovative approach.

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Appendix A: Supplementary information for Chapter 2

Table A1. siRNA sequences

siRNA Name	Sense Sequence	Antisense Sequence
Control	5’-GCGUAUUAUACGCGA UUAACG-3’	5’-CGUUAAUCGCGUAUAAUACGC-3’
GFP	5’-GAACUUCAGGGUCAGCUUGCCG-3’	5’-GCAAGCUGACCCUCUUGUUCAU-3’
FAM-labeled	5’-/56-FAM/CAGUCGCGUUUGCGACUGGUUTT-3’	5’-AACCAGUCGCAAACGCGACUGTT-3’
Survivin	5’-AGACAGAAUAGAGUGAUAGGAAGCG-3’	5’-CGCUUCCUAUCACUCUAUUCUGUCUCC-3’

Table A2. Primer sequences

Primer Name	Forward Sequence	Reverse Sequence
Survivin	5’-TGAGAACGAGCCAGACTTGG-3’	5’-ATGTTCCTCTATGGGGTCGT-3’
β-Actin	5’-CCACCCCACTTCTCTCTAAGGA-3’	5’-AATTTACACGAAAGCAATGCTATC-3’

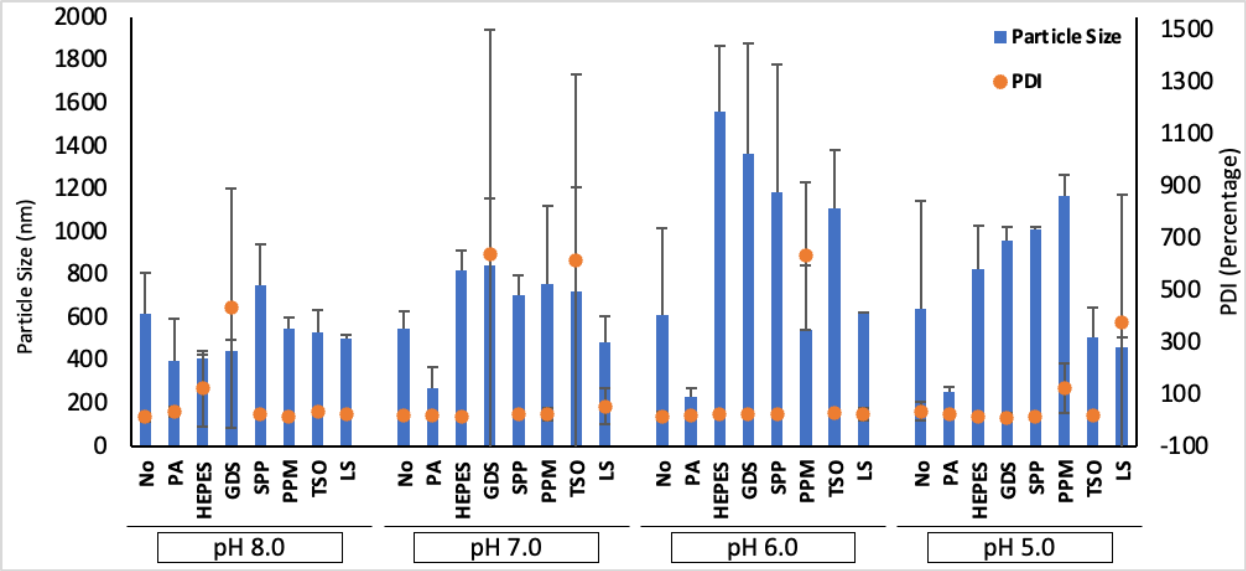

Figure A3. Polydispersity index (PDI) and particle size of the formulations formed in phosphate buffer with four pH levels with ratio 1:1:1 of PEI-GA-Lau7 : additive : siRNA