SiRNA Therapy with Lipid-modified Polymers in Chronic Myeloid Leukemia

by

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ABSTRACT

BCR-ABL fusion oncogene is the main driver mutation of Chronic Myeloid Leukemia (CML) that controls initiation, maintenance and progression to more aggressive stages of the disease. Current therapies with tyrosine kinase inhibitors in CML involve certain limitations, such as drug resistance and insensitivity. Therefore, RNA interference (RNAi) represents a promising tool for the silencing of over-expressed genes that can regulate unwanted biological effects produced in diseases such as cancer.

CML arises from the acquisition of the BCR-ABL oncogene in normal hematopoietic stem cells that progressively outgrow the normal hematopoietic stem cells in the stem cell niche and subsequently leads to an uncontrolled expansion of immature myeloid cells. Tyrosine kinase inhibitors (TKIs) used to target the ABL tyrosine kinase have shown significantly increased clinical outcomes in CML patients, however, patients in blast crisis phase are more likely to relapse and develop resistance often due to mutations in the TKI domain and innate or acquired insensitivity of CML stem cells. RNAi represents a promising alternative for the treatment of CML as small interference RNA (siRNA) molecules can be applied to the silencing of specific targets that modulate the biological outcome and induce therapeutic effects. However, potent carriers that can overcome delivery barriers of RNAi agents and are effective in difficult-to-transfect cells, such as CML cells, are needed for the progression of siRNA-based therapies towards clinical applications.

In this thesis work, we explored the use of lipid-modified polyethylenimines (PEI) of low molecular weights (0.6, 1.2, 2.0 kDa) in *in vitro* and *in vivo* CML models and evaluated their siRNA transfection efficiency in terms of cytotoxicity, siRNA uptake, internalization, gene silencing and biological effects (i.e, apoptosis, cell growth, and cell colony growth). siRNA transfection efficiency of lipid-modified polymers was evaluated

in CML K562 cell lines grown in suspension and adhering to a fibronectin (FN) surface, in an *in vivo* CML (K562) model, and in CML patient cells. The hypothesis of this thesis is that lipid-modified polymers will facilitate siRNA delivery to CML cells efficiently to obtain functional outcomes that could be potentially used as therapy for CML.

Among the lipid substituents evaluated for PEI modification, palmitic acid (PA), alpha linoleic acid (αLA), and cholesterol (Chol) have proven to be highly efficient in delivering siRNA and silencing of the green fluorescent protein (GFP)- reporter gene in K562 cells grown *in vitro* in suspension (PA, αLA, and Chol) and on K562 cells grown adhering to FN-modified surfaces (αLA). Moreover, BCR-ABL-siRNA transfection produced a significant decrease of BCR-ABL mRNA in K562 cells (PA, αLA, and Chol) and CML primary cells (αLA, and Chol) which subsequently resulted in significant increase of apoptotic cells, cell growth inhibition, and reduced ability to form cell colonies *in vitro* in comparison with control groups. Although a decrease in BCR-ABL mRNA was not evident in CML models *in vivo*, a retardation in the tumor growth was observed in comparison with control groups. The results from this study revealed the potential of siRNA-based drugs and are encouraging for the future design of non-viral delivery system with clinical translation capabilities for the treatment of CML. This thesis work provides ample opportunities for delivery systems that could be useful to silence other target genes in CML and other leukemias (i.e., acute lymphoblastic leukemia and acute myeloid leukemia) for therapeutic purposes.

PREFACE

H. Uludağ, as the supervisor author, was involved with the concept formation, design and thesis composition. Ethics approval for the animal research of this thesis was obtained from the University of Alberta Research Ethics Board, Project Name "siRNA Based Therapies for Cancer Treatment", No. AUP00000423. The studies with human cells were conducted under the approval of the University of Alberta Research Ethics Board, Project Name "Feasibility of siRNA Therapy for Leukemic Cells in Culture", No. Pro00057496. Biosafety approval was obtained under UA file # RES0012356.

Chapter 1 contains portions of published work as a book chapter and a published paper, where the major portions are from the book chapter J. Valencia-Serna, B. Landry, X. Jiang, and H. Uludağ, "Potential of siRNA Therapy in Chronic Myeloid Leukemia" In: A. Prokop, Y. Iwasaki, A. Harada (eds) *Intracellular Drug Delivery II: Fundamentals and Applications*, vol.7, Springer, pp. 435-474, 2014. As the primary author, I was responsible for the literature review, discussion, and composition and writing of manuscript. B. Landry contributed specifically to the Acute Myeloid Leukemia (AML) sections of the book chapter, (these AML sections were not included in **Chapter 1**). X. Jiang, with Chronic Myeloid Leukemia (CML) expertise, contributed to some ideas contained in the book chapter. Some sections of **Chapter 1** were also published as B. Landry, J. Valencia-Serna, H. Gül-Uludağ, X. Jiang, A. Janowska-Wieczorek, J. Brandwein, and H. Uludağ, "Progress in RNAi-mediated Molecular Therapy of Acute and Chronic Myeloid Leukemia", *Mol Ther Nucleic Acids*, vol. 4, pp. e240, May 2015. As a co-author, I contributed with sections mainly related to CML, which were included in different sections throughout the paper.

Chapter 2 is a study published as J. Valencia-Serna, H. Gul-Uludağ, P. Mahdipoor, X. Jiang, H. Uludağ, "Investigating siRNA delivery to chronic myeloid leukemia K562

cells with lipophilic polymers for therapeutic BCR-ABL down-regulation", *Journal of Controlled Release,* vol. 172, pp. 495-503, 2013. As the primary author, I designed, performed experiments, collected and analyzed data, and wrote the manuscript. Mahdipoor performed PCR experiments. X. Jiang provided the K562 cell lines (wild type and GFP-positive) used in this study. Gul-Uludağ and Jiang provided guidance with leukemia expertise. Lipid-polymer used in these studies were synthesized by A. Neamnark and R. KC. V. Somayaji performed the NMR analysis of the polymers. C. Kucharski provided technical help with cell culture. B. Landry provided help with PCR analysis.

Chapter 3 contains *in vitro* and *in vivo* CML studies which have not been published yet. N. Chan, as undergraduate student, performed and analyzed some *in vitro* experiments. Aliabadi, with in vivo expertise, provided expertise in *in vivo* cancer models. X. Yang, H.M. Aliabadi, M.B. Parmar helped with the first two *in vivo* studies, where they performed cell injection, mice weighting, tumor measurements, mice handling, siRNA-polymer injections, and tumor extraction. For the first two studies, I was involved in the study design, cell culture, preparation of the siRNA/polymer complexes, tumor processing, and data analysis. For the third in vivo study, I was involved in the study design, cell culture, mice weighting, preparation of the siRNA/polymer complexes, mice handling, and processing and data analysis, and, Aliabadi helped with study design, cell injection, tumor measurement, siRNA/polymer complexes injections, and tumor extraction. Lipid-modified polymers used in this chapter were synthesized by A. Neamnark and A. Manfrin.

Chapter 4 is a study that forms part of a national research collaboration with Professor G. Laroche at Laval University and it is published as J. Valencia-Serna, P. Chevallier, R. Bahadur KC, G. Laroche, H. Uludağ, "Fibronectin-modified surfaces for evaluating the influence of cell adhesion on sensitivity of leukemic cells to siRNA

nanoparticles", *Nanomedicine,* Vol. 11 No. 9, pp. 1123-1138, May 2016. As the primary author, I designed and performed the experiments, collected and analyzed the data, and wrote the manuscript. Chevallier provided training and guidance with the preparation, modification and analysis of modified polymer surfaces at G. Laroche's Lab. R. KC synthesized the polymers and helped with characterization of siRNA/polymer complexes.

Chapter 5 contains studies with CML patient samples and will be serve as a future manuscript. **Chapter 5** studies form part of a national research collaboration with Professor X. Jiang from University of British Columbia. Training for culture and analysis with CML primary cells was provided at X. Jiang's lab by J. Leung (cell harvesting, mononuclear cell separation, $CD34^+$ cells enrichment) and Dr. M. Chen (experimental design, cell-forming colony assay, flow cytometry). C. Kucharski helped with harvesting of primary cells and RT-PCR studies. CML primary samples were provided by Dr. J. Brandwein (University of Alberta), Dr. X. Jiang (University of British Columbia), and Dr. M. Caligiuri (Ohio State University). I planned, designed, performed the studies, collected and analyzed data and wrote the chapter thesis. Polymers were synthesized by Dr. R. KC.

Overall discussion and conclusions in **Chapter 6** contain sections published as J. Valencia-Serna, H. Gul-Uludağ, P. Mahdipoor, X. Jiang, H. Uludağ, "Investigating siRNA delivery to chronic myeloid leukemia K562 cells with lipophilic polymers for therapeutic BCR-ABL down-regulation", *Journal of Controlled Release,* vol. 172, pp. 495-503, 2013 and J. Valencia-Serna, P. Chevallier, R. Bahadur KC, G. Laroche, H. Uludağ, "Fibronectin-modified surfaces for evaluating the influence of cell adhesion on sensitivity of leukemic cells to siRNA nanoparticles", *Nanomedicine,* Vol. 11 No. 9, pp. 1123-1138, May 2016. **Chapter 6** also contains unpublished literature review, discussion and future studies learnt through my PhD studies.

DEDICATION

To my mom, dad and brother for their unconditional love.

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In regard to the main contributors of my thesis and assistance throughout research training I would like to acknowledge, Dr. Breanne Landry, Dr. Hamid Aliabadi, Dr. Hilal Gül-Uludağ, Dr. Remant KC, and my supervisor Dr. Hasan Uludağ. I thank Dr. G. Laroche and his lab members, as well as Dr. X. Jiang and her lab members for opening the doors of their respective laboratories and for allowing me the opportunity to learn new techniques, perspectives and experiences from you and your research labs.

Special gratitude goes out to all members of Uludag lab specially, former members Dr. Charlie Hsu, Dr. Laura Rose, Dr. Breanne Landry for setting up a great example. Dr. Hamid Aliabadi for guidance, support, and current members Cezary Kucharski for fun conversations, Deniz Meneksedag, Eleni Tsekoura, Manoj Parmar for friendship and

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To my supervisor, Dr. Hasan Uludağ for providing me the opportunity to join his team. Dr. Uludağ's encouragement, continuous guidance and, insightful advice has assisted me in many ways along my careers and for this I am thoroughly grateful. My final acknowledgement is that left to my family and friends for their support and encouragement to help me pursue my dreams: to Amanda Serna, Vidal Valencia, Mauricio Valencia, Diana Martinez and Jonathan Quong.

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LIST OF ABBREVIATIONS

Ab	Antibody
ABL	Abelson murine leukemia viral oncogene homolog 1
AHI-1	Abelson helper integration site 1
ASO	Antisense oligonucleotide
ATP	Adenosine triphosphate
AGO	Argonaute
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myelogenous Leukemia
BCR	Breakpoint cluster region
BCR-ABL	Breakpoint cluster region – Abelson murine leukemia viral oncogene homolog 1
BFU-E	Burst-forming unity erythroid
BM	Bone marrow
BSA	Bovine serum albumin
CA	Caprylic Acid
CD	Cyclodextrin
Cer	Ceramide
CFC	Colony-forming cell
CFU-GEMM	Colony-forming unit granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	Colony-forming unit granulocyte/macrophage
Chol	Cholesterol
CML	Chronic Myeloid Leukemia
CPP	Cell penetrating peptide
CTCL	Cutaneous T-cell Lymphoma
CXCR	Chemokine receptor
DMA	1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane
DMEM	Dulbecco's Modified Eagle's Media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DODAB	Dioctadecyldimethylammonium bromide
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
dsRNA	Double-stranded RNA
ECM	Extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EDC	1-ethyl-3-(3-dimethyl-amonipropyl) carbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
FAM	Carboxyfluorescein
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
FN	Fibronectin
f-SWNT	Functionalized single wall carbon nanotubes
GA	Glutaric anhydride
GAPDH	Glyceraldehye 3-phosphate dehydrogenase
GFP	Green fluorescent protein
G-CSF	Granulocy colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HA	Hyaluranic acid
HBSS	Hank's balanced salt solution
H-NMR	Hydrogen-1 nuclear magnetic resonance spectroscopy
HSC	Hematopoietic Stem Cell
IL	Interleukin
JAK	Janus kinase
KIT	Tyrosine-protein kinase
LA	Linoleic acid
LNP	Lipid nanoparticle
LSC	Leukemic stem cells
LSM	Laser scanning microscopy
LT-HSC	Long term - hematopoietic stem cell
LTC-IC	Long-term culture – initiating cells
MN	Mononuclear
mRNA	Messenger RNA
miRNA	Micro RNA
M-MLV	Moloney murine leukemia virus
MMP	Metaloproteases
MO	Monoolein
MPA	Mercaptopropionic acid
mRNA	Messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
NaCl	Sodium chloride
NK	Natural killer

NP	Nanoparticle
NOD	Non-obese diabetic
ODN	Oligonucleotides
Opti-MEM	Reduced serum media, eagle's minimum essential medium
PA	Palmitic Acid
PB	Peripheral Blood
PBS	Phosphate buffered saline
pDNA	Plasmid DNA
PDGFR	Platelet-derived growth factor receptor
PEG	Polyethylene Glycol
PEI	Polyethylenimine
Ph	Philadelphia
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-biphosphate-3-kinase
PLL	Poly-L-Lysine
PTFE	Polytetrafluoroethylene
rBMSC	Rat bone marrow stem cell
RGDS	Arginine-Glycine-Asparagine-Serine peptide
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Real time – polymerase chain reaction
SEM	Scanning Electron Microscopy
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDF-1	Stromal cell-derived factor 1
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SMPB	Sulfo-succinimidyl-4-(p-maleimidophenyl)butyrate
SNALP	Stable nucleic acid lipid particle
TAT	Twin-arginine translocation
TLR	Toll-like receptors
SCT	Stem cell transplantation
STAT	Signal transducer and activator of transcription
TFA	Trifluoroacetic acid
TfR	Transferrin receptor
TKI	Tyrosine kinase inhibitor
T315I	Threonine position 315 to isoleucine mutation

VEGFR	Vascular endothelial growth factor receptor
XPS	X-ray photoelectron spectroscopy
αLA	α- Linoleic Acid

SCOPE

The work of this thesis is focused on the non-viral siRNA delivery in chronic myeloid leukemia (CML) cells. Here, I investigate the ability of lipid-modified polymers to deliver siRNA and obtain gene silencing that produce therapeutic effects in CML models. The chosen target gene is the *BCR-ABL* fusion gene as it is involved in the induction, maintenance, and disease progression to more aggressive disease stages in CML. The various lipid-modified polymers used throughout this thesis were chosen based on the use of the most effective polymer available at the time of each study, and as new iteration of polymers generated based on the research findings of this thesis work became available. In **Chapter 1**, a literature review of the most current knowledge of CML, the available treatments and their limitations are presented. Furthermore, the RNAi mechanistic process and a current state of the art of siRNA delivery systems and their therapeutic outcome in CML models are described. This review highlights the potential of RNA interference in CML, and demonstrates the challenges that need to be considered for the design of siRNA delivery agents for difficult-to-transfect cells and for the translation of siRNA therapies into clinics.

In **Chapter 2**, with the aim of designing effective non-viral carriers for difficult-to-transfect and suspension growing cells, the use of lipid-substituted polyethylenimine (PEI) was investigated in K562 cells *in vitro*. In this study, initial transfection comparison studies between attachment-dependent (breast cancer) cells and suspension K562 cells revealed the transfection effect differences between the two cell types and the challenge in transfecting suspension cells. We investigated the use of different lipids for polymer modification, degree of lipid substitutions and polymer molecular weights, and variations in the complex formation to identify suitable characteristics for efficient transfection of K562 cells. siRNA carrier consisting of palmitic acid (PA) substitution on PEI (1.2PEI-PA),

although it induced some cytotoxicity after transfection, it showed effective siRNA delivery and silencing in K562 cells, which decreased the gene target (*BCR-ABL)* mRNA and increased cell death after treatment. This study demonstrated the proof-of-principle for the potential of lipid-substituted polymers for a functional therapeutic outcome in a CML cell line.

In **Chapter 3**, a new carrier based on α-linoleic acid (αLA), PEI1.2-αLA was similarly effective and less cytotoxic than PEI1.2-PA polymer used in **Chapter 2.** Moreover, to evaluate the preclinical relevance of this delivery system, in **Chapter 3** we present a series of siRNA delivery studies in a *in vivo* CML (K562) model. CML mice models treated with BCR-ABL siRNA/polymer complexes showed a tumor growth arrest in comparison with control groups. Here, we also discuss the challenges found with the *in vivo* model used in these studies.

CML cells are not only growing in suspension, and in fact, a certain population of CML cells, grows under an adhesive environment that protects them from the effect of drug therapies. With the aim of evaluating whether suspension K562 cells can be induced to grow in an adhesion state, **Chapter 4** describes the preparation of polymer surfaces modified with a known protein (fibronectin) that can facilitate their cell binding. These studies revealed the possibility of having K562 cells attached to a fibronectin-grafted polymeric surface that allows their binding and growth. Moreover, the effect of the fibronectin-mediated adhesion of K562 on their response to siRNA treatment was evaluated and comparison to cells grown in suspension. The results of these studies show that the siRNA treatment with lipid-modified polymers are similarly effective on K562 cells grown on adhesion and in suspension. These studies present the option that those CML cells that grow in adhesion to fibronectin may be sensitive to siRNA treatment.

With the purpose of evaluating whether lipid-modified polymers may be successfully

translated in to its use in clinics, in **Chapter 5** we evaluated the siRNA delivery and transfection effect (silencing and biological outcome) of these polymers in CML primary cells. Those polymers that afforded higher siRNA uptake in previous **Chapters** were selected for further evaluation. siRNA delivery and cellular internalization were analyzed by flow cytometry and confocal and transmission electron microscopy. Furthermore, BCR-ABL siRNA transfection was explored in different CML patient samples. These studies revealed that the BCR-ABL gene level and the cell survival *in vitro* can be significantly decreased; and that these effects, are comparable to those found with the K562 cell line. Although these are preliminary studies with CML patient cells, these results show further translation potential of these lipid-modified polymers into clinics. Due to constrains with cell numbers, in these studies mononuclear differentiated cells rather than immature and primitive cell portion was used for allowing enough cell numbers for polymer screenings.

Lastly, in **Chapter 6**, we present the overall conclusions of this thesis work and summarize the identified main characteristics needed from the polymeric siRNA carriers to enact an effective gene-mediated biological effect in CML cells. We also mention the challenges that we overcome with our work and the impact these results have in the field, as well as the areas that will require further improvement for the advancement in the development of siRNA therapeutics in CML.

1. POTENTIAL OF SIRNA THERAPY IN CHRONIC MYELOID LEUKEMIA[1]

[1] A version of this chapter was published in: J. Valencia-Serna, B. Landry, X. Jiang, H. Uludağ, "Potential of siRNA Therapy in Chronic Myeloid Leukemia", In: A. Prokop, et al, *Intracellular Delivery II: Fundamentals and Applications,* vol. 7, Springer, pp. 435-474, 2014.

[1] A version of this chapter was included and published in: B. Landry, J. Valencia-Serna, H. Gül-Uludağ, X. Jiang, A. Janowska-Wieczorek, J. Brandwein, and H. Uludağ, "Progress in RNAi-mediated Molecular Therapy of Acute and Chronic Myeloid Leukemia", *Mol Ther Nucleic Acids*, vol. 4, no. 5, pp. e240, May 2015.

1.1 INTRODUCTION

Leukemic cancers arise from genetic alterations in normal hematopoietic stem or progenitor cells, leading to impaired regulation of proliferation, differentiation, apoptosis and survival of malignant cells. The US National Cancer Institute calculated an overall 5-year relative survival (between 2003 and 2009) rate of 56.0% for various leukemias combined [1]. The front line therapy in leukemia is chemo (drug) therapy [2], [3]; current therapeutic approaches include broad-spectrum drugs against fast-proliferating cells and small-molecule inhibitors targeting specific signal transduction pathways, so called molecular therapies [4]. Leukemic cells generally respond well to drug therapy at the onset of the treatment, but the drugs lose their effectiveness over a period of 6-12 months. It is well recognized now that the resistance to conventional (broad-spectrum) therapeutic agents is inevitable, but recent evidence also indicated that even the most advanced molecularly-targeted drugs lose their efficacy because of the development of resistance in a relatively short time. The inherent plasticity of the cells combined with diverse resistance mechanisms make malignant cells naturally adapt by mounting an effective resistance against the drugs. The high relapse rate in leukemia patients has been additionally attributed to existence of a rare population of leukemia stem cells (LSC) resistant to current drug therapies [5], [6].

With a better understanding of molecular changes in malignant transformations, treatments that target tumor-specific changes are expected to lead to more effective therapies as the normal cells transform into malignant cells. Towards this end, a highly specific leukemia therapy can be developed by exploiting the RNA interference (RNAi) mechanism to silence the aberrant protein(s) responsible for the disease [7], [8]. There are several approaches for gene silencing, including a plasmid encoding for short hairpin RNA (shRNA; a single strand RNA with a hairpin loop structure), small interfering RNA (siRNA; a double strand RNA), and antisense oligonucleotides (a single DNA or RNA

strand). Antisense oligonucleotides promote gene silencing either by hybridizing with the target mRNA and forming a heteroduplex that activates RNase H that in turn degrades the mRNA, or by physically blocking the translation [9]-[11]. shRNA are transcribed within the cellular nucleus, transported into the cytoplasm, and then processed by the cellular-machinery into a siRNA-like form, which is finally incorporated by cytoplasm molecules for silencing activity [12] [13]. Expression of shRNA can be continuously synthesized by the host cells, therefore its effect can persist indefinitely [12]. On the other hand, exogenously introduced siRNA delivered to the targeted cell is loaded onto the RNAi machinery for silencing activity, omitting the transcription and processing steps of shRNA. siRNA characteristics such as, transient effect and non-integration into the hosts cell's DNA are important for safety and translational considerations [14]-[16]. This chapter will focus mainly on the RNA form as siRNA. The siRNA essentially acts as a pharmaceutical 'drug' that can inhibit virtually any single protein expression within the cells, contrary to antibodies or tyrosine kinase inhibitors that only target surface antigens and tyrosine kinases, so that the spectrum of possible target molecules is widely broadened. Once in the cytosol, the siRNA duplexes assemble into a pre-RISC (RNA-induced silencing complex) containing specific proteins, including argonaute proteins (AGO1, 3 or 4) [17], [18], which is subsequently guided to target desired mRNA based on complementary base pairing [17]. Endonucleoyltic cleavage and/or translational repression of the mRNA [17], [18] then silences the protein target. Delivery systems, however, are an absolute necessity for effective use of siRNA since the molecules are highly sensitive to serum nucleases and their large (~13 kDa) and anionic nature (due to its phosphodiesterase backbone) prevents the siRNA to traverse cellular membranes. Viral means to deliver siRNA were emphasized initially, but the undesirable side-effects of viral delivery in a clinical setting makes this approach highly risky for therapeutic use. Alternatively, cationic biomolecules as carrier molecules capable of binding and neutralizing the anionic charges of siRNA and packaging the

siRNA into nano-sized complexes offer the safety of non-permanent interactions with genomic materials and make them more likely for clinical deployment [19]. siRNA carriers suitable for cellular delivery include: (i) formulations of multiple lipids and siRNA for the formation of liposomes of solid lipid nanoparticles (NPs); (ii) polycationic polymers that condense siRNA to form NP networks; and, (iii) carriers composed of multiple domains, including cationic, lipophilic, hydrophilic and targeting (e.g. antibody-derivatized) moieties.

In this chapter, I will summarize the attempts reported in the literature to deliver siRNA molecules using non-viral carriers in leukemia. I will focus on a type of blood cancers, namely chronic myeloid leukemia (CML); which is one of the major classes of leukemia that it is well understood at the molecular level, and constitutes the focus of my thesis work. Most of the work of siRNA treatment in leukemia has been done in *in vitro* cell models using cell lines, with fewer studies in primary cells and animal models, and only one case in the clinical setting for CML treatment. The literature review in this **Chapter** will be focused on preclinical studies exploring the potential of siRNA therapy in CML. In conjunction, the technology of siRNA delivery will be explored, investigating the critical issues pertinent to effective siRNA delivery.

1.2 CHRONIC MYELOID LEUKEMIA (CML) AND CURRENT DRUG THERAPIES

Myeloid leukemias (46% of all leukemias) affect the myeloid cells of the bone marrow, which normally go on to form the blood cells. Thirteen percent of those cases account for the CML. Approximately 350,000 people worldwide are diagnosed with leukemia annually, with ~250,000 death resulting from leukemia each year. Most leukemia occur in the elderly and peak between the ages of 75 and 79 [20]. CML is a

myeloproliferative disease initialized at the hematopoietic stem cells that is thought to arise due to translocation of chromosomes 9 and 22, which results in a fusion between *ABL* and *BCR* genes, or in the so-called Philadelphia (Ph) Chromosome [21]-[23]. Once the normally regulated tyrosine kinase of the ABL protein is permanently activated by the juxtaposition of the BCR sequence, it leads initially to a chronic phase characterized by myeloid cell expansion, while allowing differentiation of expanded cells in the peripheral blood. As the disease progresses, either by increased *BCR-ABL* expression or activation of other pathways, patients enter a more aggressive disease phase (blast crisis), which is characterized by a progressive loss of the capacity of hematopoietic cells [24], [25] to differentiate and an increased expansion and accumulation of immature blast cells in the bone marrow and spread to the bloodstream [26], [27]. The Ph chromosome is not specific for CML, since it can also be found in some patients with acute lymphoblastic leukemia (ALL) [present in 2% to 5% of children, 20% to 40% of younger adults, and up to 50% in older adults (>55 years) [28]], and in 0.5% to 3% in patients with acute myeloid leukemia (AML) .

Current therapies for CML are based on the use of tyrosine-kinase inhibitors (TKIs) and allogeneic hematopoietic stem-cell transplantation. Stem-cell transplantation therapy is an option when the treatment with TKIs fails; however, this therapy has substantial risk of mortality due to chronic graft-versus-host disease [29], [30]. TKIs, such as imatinib, have revolutionized CML therapy. Imatinib binds to the ABL kinase domain with the formation of a bond that impedes ATP binding, subsequently blocking or preventing the interaction of the ABL kinase with substrates and therefore from activating its oncogenic pathways [31], [32]. Targeted therapy with imatinib has transformed the survival potential for patients with chronic phase of CML; it has significant impact on patients with accelerated phase but has a minimal impact in those patients at the blast phase stage [33]. However, CML patients, especially those in

advance-stage disease, can develop TKI resistance leading to relapse [30]. This acquired drug resistance could be due to the amplification of *BCR-ABL* gene, and overexpression of *BCR-ABL* mRNA and protein [34]. However, resistance most often results from point mutations in the kinase domain of BCR-ABL protein that affect drug binding to the protein, thereby reducing the ability of imatinib to block the tyrosine kinase activity. More than 50 distinct *BCR-ABL* mutations have been reported to date and the current repertoire of TKIs can cover all known mutations leading to resistance; however, no single drug can prevent all forms of resistance [31], necessitating the use of TKI cocktails to overcome any possible resistance.

Second-generation TKIs such as dasatinib and nilotinib, which are also approved as front-line TKI therapy, are more potent TKIs and produce more rapid declines in CML disease burden than imatinib, which translates into more durable cytogenetic (absence of metaphase Ph^+ cells) and hematological remissions (achievement of normal white blood and platelet cell counts and, no signal of CML symptoms) [34], [35]. Nilotinib is approx. 30-fold more potent than imatinib as an ABL inhibitor. Dasatinib is a potent inhibitor of ABL kinase Src-family kinases, which are known to be involved in multiple intracellular signal transduction pathways including oncogenesis and disease progression [34]. Ponatinib, a third-generation TKI, is a newer drug that has the unique property of inhibiting both the native (un-mutated) and mutated BCR-ABL proteins, especially those including the T315I mutation, which confers resistance to all other CML drugs (including nilotinib and dasatinib) and seems to translate in worst overall survival compared with other mutations found in imatinib-treated patients [34]-[36]. A strategy of combing two or three ABL inhibitors with non-overlapping *BCR-ABL* mutations resistance profiles, such the example of Ponatinib exemplified above, could prevent the emergence of drug resistance [35]. However, it is expected that treatment with these new ABL inhibitors could also lead to new point mutations that overcome the resistance of these new drugs

[34], given the plasticity of leukemic stem cells.

Several clinical trials between 2011 and 2014 that compared new-generation TKIs (bosutinib, dasatinib, nilotinib, and ponatinib) vs. imatinib in CML patients were analyzed. Extracted data revealed that even though there is a higher major molecular response (patients achieving detection levels of ≤0.1% *BCR-ABL1* measured by RT-PCR) of patients initiated on new-generation TKIs at year 1 (44.18% patients treated with a new-generation TKI vs. 27.35% patients treated with imatinib [37]), the mortality rate at 1 year between a new-generation TKI and imatinib is comparable (1.49% patients treated with a new-generation TKIs vs. 2.01% patients treated with imatinib [37])[38].

Reports of adverse effects associated with TKIs have raised concerns about the cardiovascular toxicity of new-generation TKIs. The use of new-generation TKIs was associated with a statistically significant increased risk of vascular occlusive events in comparison with the use of imatinib: vascular occlusive events occurred in 5.88% of patients treated with new-generation TKIs vs. 1.04% of patients treated with imatinib. These events developed specially in patients with pre-existing risk factors [37]. For the case of use of ponatinib as therapy, 27% of patients with or without cardiovascular risk factors treated with this TKI were reported to have vascular occlusive events [37]. Moreover, vascular occlusive events occurred more frequently with ponatinib than with previously approved TKIs [38].

Although all TKIs approved for CML therapy share the activity against BCR-ABL, they differ in their potency and activity against other kinases, including kinases involved in vascular biology such as vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors (PDGFR), and fibroblast growth factor receptors (FGFR) [38]: Imatinib besides inhibiting ABL, inhibits PDGFR and KIT kinases; dasatinib targets KIT, PDGFR and SRC kinases; nilotinib is a potent inhibitor of KIT and PDGFR; and

ponatinib is a potent inhibitor of numerous tyrosine kinases including SRC, PDGFR, and VEGFR [38]. It is likely that vascular toxicities are related to off-target rather than on-target effects as these TKIs are strong inhibitors of VEGFR [38]. More studies will be needed to understand the cause of these adverse effects.

Although the new-generation TKIs are more potent against ABL1 kinase, the evidence of adverse effect will need to be taken into consideration at time of choosing a treatment of a patient. When patients show any of these adverse effects, treatment will be required for secondary effects in parallel with CML, or an alternative TKI therapy could be used to reduce these effects.

1.3 INSENSITIVITY OF CML STEM CELLS TO TYROSINE KINASE INHIBITORS (TKIs)

Although imatinib can inhibit the production of ~99% of differentiated leukemic cells, it fails to deplete the LSCs [34]. Studies have shown that despite the complete depletion of *BCR-ABL* transcript levels in these LSCs with TKIs, the cells remain viable. These data indicate that even in the presence of imatinib, especially in accelerated and advanced states of the disease, CML has progressed and evolved so these LSCs no longer require BCR-ABL activity to maintain their viability [22], [31], [39], [40], and anti-apoptotic and pro-survival signals are provided by alternate pathways. Thus, the drivers of cell proliferation and survival, probably influenced by BCR-ABL in the early stages of CML, now operate autonomously and could lead to CML relapse [41]. Thus, not only BCR-ABL inhibition is needed for the eradication of progenitor CML cells, but BCR-ABL-independent survival mechanisms of LSCs also needs to be targeted for a complete CML eradication.

One characteristic common to all LSCs is that they require the microenvironment of

bone marrow, the stroma, to thrive. The stroma bathes the leukemic cells in growth factors, chemical signals and cell-surface ligands that keep the cells in a dormant phase resistant to drug therapy. Part of what keeps these cells entrenched in the bone marrow is a chemical signal sent by the stroma, called stromal cell-derived factor (SDF-1). This signal binds to a protein located at the surface of the stem cells, called C-X-C chemokine receptor type 4 (CXCR4) [42]. In the case of CML, BCR-ABL protein seems to be involved in the inhibition of SDF-1-induced migration and signaling which allows an abnormal release of immature myeloid cells from the bone marrow into the circulation [43]. On the other hand, it has been shown that under imatinib, CXCR4 expression in $CD34^+$ can be reversibly up-regulated, hence allowing these cells to home to bone marrow, helping them to become quiescent and to become insensitive to TKIs [43], [44]. Down-regulation of CXCR4 expression along with TKIs therapy could therefore enhance the eradication of LSCs in CML.

Researchers are also developing drugs that target a key property of stem cells, namely their self-renewal potential. One signaling pathway that seems to play an important role in self-renewal of CML LSCs hinges on two proteins: Wnt and beta-catenin [44], [45]. In 2012, Armstrong *et. al*. reported that a small molecule that inhibits beta-catenin, given in combination with imatinib, reduces CML survival and eliminates leukemia stem cells in a CML mouse model [42], [46]. *AHI-1* is a newly discovered oncogene that is highly expressed in primitive hematopoietic CML stem and progenitor cells, and whose overexpression has been shown to promote abnormal differentiation and proliferative activity of myeloid cells in CML. Zhou et al. showed that *AHI-1* overexpressing BCR-ABL^+ cells (CML cells transduced with an *AHI-1* construct) showed greater resistance to growth inhibition effects of imatinib in comparison to control cells. Suppression of *AHI-1* by transduction of an AHI-1 silencing construct (AHI-1/sh4) resulted in increased sensitivity to imatinib. AHI-1 was also found to significantly

increase or reduce protein expression and phosphorylation of BCR-ABL, JAK2 and STAT5 once *AHI-1* was overexpressed or suppressed, respectively. Suppression of *AHI-1* in primary $CD34^+$ CML cells was also shown to increase imatinib sensitivity especially in imatinib-resistant and blast crisis patients who express relatively higher levels of AHI-1 [47].

1.4 DOWN-REGULATION OF GENE EXPRESSION BY RNAI IN CML

RNA interference (RNAi) is a process by which double-stranded small interfering RNA (siRNA) induces sequence-specific, post-transcriptional gene silencing [48]. Endogenous RNAi is triggered by the transcription of long pieces of double-stranded RNA (dsRNA), which are cleaved into the smaller (21–23 nucleotides long) fragments by the enzyme Dicer. In practice, siRNA is synthetically produced and then directly introduced into the cell, thus circumventing Dicer mechanics [49]. Once siRNA is present in the cytoplasm of the cell, it is incorporated into the protein complex RISC (RNA-induced silencing complex). Thereafter, Argonaute, a protein contained within RISC, cleaves the sense strand of the siRNA, thereby releasing it from RISC. The now activated RISC, which contains the antisense strand of the siRNA, selectively seeks out and cleaves mRNA that is complementary to the antisense strand [48], [49]. The activated RISC complex is not affected by this reaction and can move on to destroy additional mRNA targets, which further propagates the silencing of gene expression. In mammalian cells, RNAi persists effectively only for an average of 66 h due to its dilution during cell divisions [48], and so repeated administration is necessary to achieve a persistent effect if needed [49].

The shortcomings of current leukemia treatments call for development of new strategies to deliver more efficacious drugs into CML cells. Owing to increasing knowledge of CML at a molecular level, RNAi is a promising approach for leukemia

treatment. To control the expression of *BCR-ABL* and other genes involved in these cellular malfunctioning processes, synthetic small interfering RNA (siRNA) delivered into diseased cells to interact with the target mRNA of aberrant genes and silence their protein expression. However, a delivery carrier that helps these siRNA moieties to reach the mRNA in the cytoplasm is necessary, as these molecules cannot enter the cell on their own. In order to achieve this purpose, carriers need to interact with the siRNA moieties to form siRNA nanoparticles to protect the siRNA from serum nucleases, facilitate their cell membrane interaction and internalization, promote the siRNA release from endosomes into the cytoplasmic environment to ultimately allow the siRNA interaction with the RISC protein complex [50], [51].

1.5 RNAi DELIVERY SYSTEMS FOR CML MODELS

Existing transfection and delivery methods are more suitable for attachment-dependent cells (e.g., breast cancer cells) rather than attachment-independent cells. Physical treatments such as electroporation on the other hand, although helpful to investigate the biology and the effect of gene depletion by RNAi -especially on difficult-to-transfect cells such as primary or suspension-growing cells [52]-[54]-, cannot be translated *in vivo* because of the significant toxicity they induce to cells after transfection, and because they have been designed for an *in vitro* setting only [55], [56]. Electroporation (and related 'nucleofection' physical methods) is the most common method to deliver siRNA for experimental purposes. Viral vectors have been effectively used but these present significant safety risk due to their host's genome integration or cause lethal immune responses and inflammation [51], [57]. This review will focus on the use of nonviral biomaterials that interact with siRNA molecules to form nanoparticles as delivery carriers in CML.

1.5.1 Mechanism of Uptake and Intracellular Processing of siRNA Nanoparticles in Leukemic Cells

Cell membrane is the first interface that siRNA nanoparticles need to interact with for internalization. The lipid bilayer acts as an impermeable membrane to entry of unwanted materials from the external environment (including siRNA nanoparticles) and as selectively permeable, by the control of protein channels and pores, to the entry of nutrients and exit of metabolites [48], [49], [58]. The heterogeneous lipid composition and distribution of hundreds of lipid species present in cell membrane influences the degree of lipid diffusion in the membrane as well, as the thickness and shape (architecture) of the cell membrane. These characteristics are not conserved among cells and are dependent on the cell type, cellular activity and constant changes in signaling with the external environment [58], [59]. The heterogeneity of these components and the affinity among some of them, such as the affinity between cholesterol and sphingolipids, lead to the formation of clusters along the membrane that are known as lipid rafts [60], [61]. These lipid rafts have their own charge, which can make a siRNA nanoparticle more or less interactive with specific regions on cell surface, leading to different type of interactions along the membrane. The successful integration of the siRNA nanoparticles with the membrane will depend on the ability to interact with this area by charge affinity or to move to another area of more affinity [62]. These factors make the cell membrane a dynamically uneven surface with unstable characteristic features for interaction with siRNA nanoparticles [62].

1.5.2 Differences in Delivery Between Suspension-growing vs. Attachment-dependent Cells

The delivery of siRNA nanoparticles is challenging when the target cells are suspension-growing (i.e., attachment-independent) cells. The interaction of suspended particles with cells growing in suspension is expected to be different from cells attached to other cells or to tissue culture plastic. siRNA nanoparticles need to be designed in

such a way that they can bind to suspended cells and promote their entrance into the cells for delivery of nucleic acids.

Uptake of siRNA nanoparticles in suspension-growing cells such as leukemic cells is known to be more difficult to achieve in comparison with attachment-dependent cells [63]. Initial studies performed in our lab compared siRNA uptake between the attachment-dependent breast cancer MDA-MB-231 and suspension-growing K562 CML cells with a generally effective lipid-modified polymeric carrier. A 15-fold reduction in siRNA uptake was found in K562 cells in comparison to MDA-MB-231 cells, showing a considerable reduction in the siRNA delivery efficiency. In addition, despite a 29-fold increase in the siRNA uptake with these carriers (in comparison to non-carrier groups), silencing of the green fluorescent protein (GFP) in GFP-positive K562 cells was proven to be ineffective [63]. The relatively small amount of siRNA may have not reached its target (RISC for degradation of the mRNA), possibly due to incomplete internalization or endosomal entrapment of the particles [63]. Similarly, Lorenz et al. evaluated the interaction of polymeric particles with different cell types and found that when the amount of amino groups of the particles was increased, a greater amount of particles interacted with cell membranes [64]. Moreover, although it was found that the interaction between the cells and nanoparticles was the same with all the cell lines tested, whether attachment-dependent (HeLa and mesenchymal stem cells (MSC), or suspension-growing (KG1a as a model for CD34+ hematopoietic stem cells and Jurkat as model for T cells) cells, the internal location of these particles differed among the cells: particles that interacted with MSC and HeLa (attachment-dependent) cells were located in intracellular compartments, most likely located inside endosomes; while particles that interacted with KG1a and Jurkat (suspension-growing) cells were found at the cell membrane or periphery of the cells [64], suggesting that these particles were not able to overcome the cell membrane barrier and therefore were not internalized. An

active endocytosis was perhaps limited in the suspension-growing cells. Zhao et al. also compared the oligonucleotide uptake between leukemic and the different types of normal human hematological cells and found that the uptake differed among the hematopoietic cell types: uptake was the highest in myeloid/macrophages, followed by B-cells, T-cells, and the lowest in neutrophils [65]. On the other hand, human leukemic cells were also found to take up more oligonucleotides than normal or residual cells from the same patient and, this uptake was increased or decreased in leukemic and normal cells upon cell growth factor stimulation and cell growth inhibition, respectively. This led the authors to conclude that the uptake in leukemic cells was probably greater due to their higher cell growth and activation [65]. Another factor for less effective transfection of suspension-growing cells may be the composition of their cell membrane; Labat-Moleur et al. suggested that the poor transfection ability of cationic vectors in lymphocytes, and other non-adherent cells might be attributed to weak interaction of these vectors due to the lack of Ca^{2+}-dependent cell surface extracellular matrix (ECM) ligands, such as proteoglycans and cadherins, that are only present in adherent cells [66]. Moreover, He et al. found a correlation of expression of caveolins and the easiness to transfect certain leukemic cells. The harder to transfect the cells were, such as the case of AML KG1 cells, the lower the expression of Cav1 and Cav2 were. Conversely, easier to transfect cells, such as AML Molm13 cells, had higher expression levels of Cav1 and Cav2 [67].

These observations indicated that the uptake of nucleic acid nanoparticles is dependent; not only on the cell type (i.e., attachment-dependent vs. suspension-growing), but also on the internalization pathway, which could be different among different suspension-growing cells, and the composition of the membrane of a certain cell type. Thus, not only cationic charges seem to be important for high affinity interactions with cell membranes of suspension-growing cells, but also there might be a

need of specific ligands or moieties in the materials (as will be discussed below) of particles that display direct interaction with cell membrane components that lead to a complete nanoparticle cell internalization.

1.6 NONVIRAL BIOMATERIALS FOR SIRNA DELIVERY FOR *IN VITRO* CML MODELS

Of all the studies reviewed here for siRNA delivery for CML, 32% of them used electroporation as the siRNA delivery method, whereas 45% used a transfection reagent available in the market. These delivery methods are excellent options for the study of the biology of CML and have eased the identification of potential new therapeutic genes targets (**Table 1.1**). In the review below, 22% of the delivery systems are non-commercial, non-viral carriers (**Table 1.1**). The latter carriers have the potential for translation so they can be used in CML *in vivo* models and could also be used with further development in a clinical setting in the future.

1.6.1 Lipid-based nanoparticles

Cationic lipids for siRNA delivery have been broadly explored for electrostatic interactions with siRNA molecules in cancer applications. One specific type of lipid nanoparticles (LNPs) used in CML is the dioctadecyldimethylammonium bromide (DODAB):monoolein (MO) siRNA-lipoplexes particles. Coating of DODAB:MO-siRNA lipoplexes with PEG-Ceramide (PEG-Cer) has been explored to improve the stability of the lipoplexes. In comparison with LNPs without pegylation, the pegylated LNPs released less siRNA in human serum, protected the siRNA from displacement by serum proteins and prevented lipoplexes aggregation in serum [68]. Another type of LNPs explored for transfection in leukemia cells is the stable nucleic acid lipid particles (SNALPs). SNALPs consists of siRNA molecules surrounded by a lipid bilayer containing the cationic lipid

(1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane, DMA) and the neutral lipid (such as cholesterol), which are coated in the final step of their preparation with polyethylene glycol (PEG)-lipids. The use of alkylated DMA/DMA as cationic lipids improved the LNPs transfection efficiency in comparison to DMA only in AML (Molm13, THP1) and CML (K562) cell lines, yielding more than 90% silencing. The silencing in more challenging to transfect (KG1) cells was ~20%. These alkylated DMA-containing LNPs had low toxicity and good stability. [67].

1.6.2 Conjugate-based nanoparticles

Cell penetrating peptides (CPPs) can be up to 30 amino acids long and are inherently able to translocate cell membranes. Overall cationic charge of CPPs confers them the ability to interact electrostatically with the phosphate backbone of nucleic acids to form stable nanoparticles, while allowing them to interact with cell membranes. Arthanari et al. used the cationic Tat-derived CPPs (aminoacids 49–57 of HIV-1 TAT protein) covalently attached to cationic membrane active peptide LK15 (Tat-LK15 peptide) for the delivery of siRNA in K562 cells [57]. The combination of these two peptides increased the transfection efficiency by two folds in comparison with Tat peptide alone in several cell lines. With doses ranging from 1 to 30 μg of siRNA in 1 ml (24 to 729 nM based on our calculation), there was a ~70% reduction in the expression of p210 BCR-ABL 48 h post-transfection for all concentrations. High density of positive charges of siRNA nanoparticles led to cytotoxicity ranging from 0% (10 μg siRNA) to 30% cell death (30 μg siRNA) [57]. Eguchi et al. on the other hand, generated a carrier composed of a TAT-peptide transduction domain (PTD) and double-stranded RNA-binding domain (PTD-DRBD). GFP siRNA delivered with PTD-DRBD (siRNA concentration of 100 to 400 nM) in Jurkat T-cells containing an integrated GFP reporter gene resulted in a reduction of the mean GFP fluorescence of 90% and a mRNA silencing of 90%, while Lipofectin (100 nM for Lipofectamine® 2000 and 10-50 nM with Lipofectamine® RNAiMAX) resulted in a

mean fluorescence reduction of 40-50% and a mRNA levels silencing of ~50%. Transfection of primary human umbilical cord vein endothelial cells (HUVEC) with PTD-DRBD/siRNA nanoparticle resulted in no cell toxicity [69].

The use of functionalized single wall carbon nanotubes (f-SWNTs) for siRNA has also been explored in CML [70]. Cyclin A2, involved in cell cycle regulation and associated with proliferation in leukemic cells was targeted by siRNA with f-SWNTs in K562 cells. This treatment (25 nM) led to a ~80% reduction of cell numbers of up 60 h after treatment in comparison with cells treated with f-SWNTs and control siRNA. No significant toxicity was found with cells treated with f-SWNTs alone or in combination with control siRNA. A significant reduction of the Cyclin A2 protein expression correlated with a 70% reduction in the colony formation assay [70].

1.6.3 Polymer-based nanoparticles

Cationic polyamines, polyethylenimines (PEIs) with various molecular weights (MWs) and modifications are capable of forming stable nanoparticles with siRNA and have been used for transfection of nucleic acids in different cell lines and live animals [48], [71], [72]. The high transfection efficiency of PEI is attributed to its "proton-sponge" effect, by which PEI once in the endosome attracts ions that lead to swelling and bursting of the endosome [49], which results in the release of the nucleic acids into the cytoplasm. This high transfection efficiency is mostly seen with high MW (~25 kDa) PEI where cellular delivery of nucleic acid cargo is efficient (unlike low MW PEIs). However, excessive endosome rupture leads to cell toxicity, thus limiting the dose of siRNA that can be delivered [71], [73]. Moreover, an inverse relationship between cytotoxicity and transfection is observed in PEI, such that low MW (2-5 kDa) PEIs are considered to have better safety profiles due to non-toxicity, but are ineffective for nucleic acid delivery [74]. By using the amine groups of the PEI that allow conjugation with other ligands, the Uludag group has investigated the effect of lipid substitutions on low MW (0.6 – 2

kDa) PEIs to increase the polymer interaction with the cell membrane and nucleic acids delivery (**Figure 1.1**). It was found that the relatively nontoxic but ineffective PEI2 polymer carrier can be transformed into an effective delivery agent by grafting a lipid molecule onto the polymer [71], [74]. Although generally effective, the gene delivery efficiency of these modified polymers can vary among leukemic cell lines [71], [75]-[77]. Our recent studies on AML cells indicated that caprylic acid (CA)-substitution, and to a slightly less extent linoleic acid (LA) substitution, sustained most silencing among the lipid-substituted 2 kDa PEIs for down-regulation of Green Fluorescent Protein (GFP) reporter gene and the C-X-C chemokine receptor type 4 (CXCR4) in THP-1 cells [78] and down-regulation of CXCR4 in AML primary cells [78], [79]. siRNA-mediated silencing of hyaluronic acid receptor CD44 was possible using LA-substituted 2 kDa PEIs (PEI2-LA) in CD34+ KG-1 and KG-1a cell lines and CD34+ AML primary cells [80]. For the case of CML cells we found a polymer (1.2 kDa PEI) substituted with a relatively high amount of palmitic acid (PEI1.2PA; 2.0 PA per PEI1.2) to be effective. The ability to deliver siRNA intracellularly was found to be dependent on the molecular weight of PEI and the amount of lipid substitution, explaining its relative efficiency. The oncogene *BCR-ABL* was also effectively silenced with this polymer, resulting in the expected apoptosis induction in the targeted cells as will be discussed in **Chapter 2** [63]. It is presently not known if this is a unique combination, or other molecular weight PEIs and/or lipids can substitute for its efficiency.

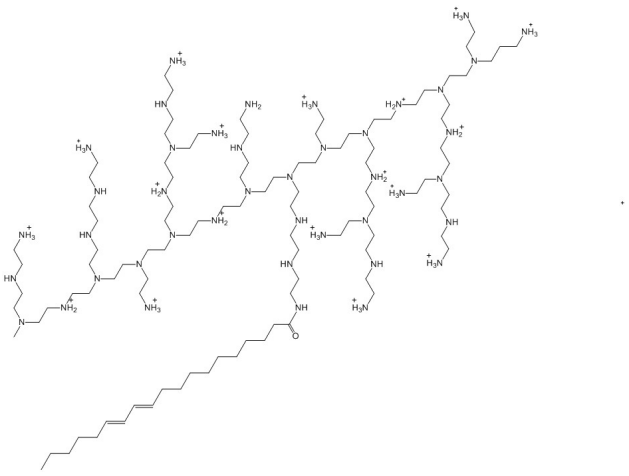

Figure 1.1. Linoleic acid-substituted 2 kDa PEI

Another polymer-based carrier explored for siRNA delivery in K562 cells was a biodegradable, charged polyester-based vector (BCPV) [81]. BCPVs are a class of cationic polylactides where the fraction of charged moieties could be precisely controlled. Experiments showed low cell toxicity (10%) at polymer concentrations up to 160 µg/ml. The siRNA cell association was 13-fold higher with BCPV in comparison with Lipofectamine® 2000. BCR-ABL mRNA decreased to ~21% when delivered by BCPV in comparison to ~76% when delivered with Lipofectamine® 2000. In correlation with the silencing results, a 50% decrease in cell viability was seen with BCR-ABL siRNA/BCPV treatment (vs. 17% with Lipofectamine®), and an apoptotic effect of 12.4% (vs. 5% with Lipofectamine® 2000). These results showed a favorable material for siRNA delivery to induce siRNA-mediated BCR-ABL silencing and modulate cell proliferation and apoptotisis in suspension K562 cells [81].

1.6.4 Specific-binding siRNA nanoparticles

Among the most effective specific-binding interactions are those ligands or antibodies coupled onto nanoparticles that allow them to interact with complementary

molecules (or receptors) on cell membranes [62], [82]. These interactions result in either receptor-mediated endocytosis or receptor-mediated direct penetration in the absence of endocytosis, for example when gold nanoparticles and cell-penetrating peptides are used as delivery carriers [62]. For nanoparticle adherence and engulfment to take place at an adhesion site, ligand-receptor interactions need to overcome the resistive forces that prevent the nanoparticle uptake. Examples of these resistive forces are the memory of the cell membrane to return to its original form and the hydrophobic exclusion of polar surfaces by the cell membrane [62].

Transferrin receptor (TrfR) is a cell membrane-associated glycoprotein that promotes endocytosis once its ligand transferrin (Trf) is bound at the cell surface. Also since TrfRs are known to be overexpressed in cancer cells, they are being exploited in targeted delivery. A TrfR-targeted SNALPs composed of Chol/DSPC/DODAP/PEG-Cer has been developed to encapsulate BCR-ABL siRNA [83]. K562 and LAMA-84 cells were transfected twice every two days with siRNA concentrations ranging from 100 to 2000 nM. siRNA association with LAMA-84 cells increased more than 8 folds when SNALPs were coupled with TrfR in comparison with non-targeted SNALPs. A dose-dependent toxicity (58% with 2 μM of siRNA) was seen with scrambled siRNA with LAMA-84 cells, but to a much lower extent with K562 cells. Levels of *BCR-ABL* mRNA were reduced in a dose-dependent manner up to 1 μM (~60%) with TrfR-SNALPs in LAMA-84 cells, whereas no reduction was found with non-targeted SNALPs. Similar results were found at the protein level, except that with the scrambled sequence there was a non-specifically reduction at the protein levels at the highest siRNA concentration used (2 μM) [83]. High toxicity and off-target effects in this system were probably due to the high siRNA dose used (2 x 1-2 μM of siRNA) and the highly cationic charge of the carrier.

Immuno-targeting with antibodies by themselves does not necessarily correspond to high internalization since the carrier needs to also play an important role with cell

membrane interactions and internalization. Immuno-polyplexes were constructed using 25 kDa PEI-based polyplexes attached via a streptavidin bridge to biotin-labeled antibodies that target different cell membrane proteins. A significant selectivity in delivery was observed: an anti-CD3 immunopolyplex was functional only in Jurkat T-cells (CD3+/CD19-), while an anti-CD19 immunopolyplex was functional only in Granta B-cell line (CD3-/CD19+). However, only ~11% of Jurkat cells and ~2% of Granta cells were transfected, showing a dependency on the transfection efficiency on the cell type and carrier used. Transfection of a mixture of Jurkat and J.RT3/T3.5 cells (a CD3-/CD19- T-cell line) with anti-CD3 immunopolyplexes showed that >80% of transfected cells were CD3+, indicating the selectivity of the delivery system in a heterogeneous cell population. Toxicity studies showed a decrease in cell viability to 50% for Jurkat cells and to 90% for J.RT3/T3.5 cells [84], which shows an association of transfection with significant cytotoxicity of 25 kDa PEI. Poorer transfection was shown with naked polyplexes (5% siRNA-positive cells) in comparison with anti-CD3 immunopolyplexes (10% siRNA-positive cells). Antibody-mediated attachment does not necessarily induce internalization of nanoparticles. An antibody-coupled CPP (oligo-arginine$_9$) complex was also developed as delivery system to target JL1-positive T-cell leukemias [85]. Uptake studies showed a higher binding affinity of JL1-CPP nanoparticles for JL1-overexpressing cells than for JL1-lowexpressing Jurkat cells (96% and 5.7% siRNA-positive cells, respectively). No toxicity studies or silencing experiments were performed [85].

Therefore, using an antibody seems to increase selectivity and enhance the efficacy of the carrier in the cases described. However, this targeted system seems to be limited by the efficacy of the carrier used; therefore, a more efficacious carrier could enhance targeted transfection even further and reduce the need of using high siRNA concentrations.

1.7 NONVIRAL BIOMATERIALS FOR NUCLEIC ACID DELIVERY *IN VIVO* RELATED CML APPLICATIONS

I am aware of one *in vivo* study that focused on siRNA delivery in a CML model using non-viral vehicles. G3139 antisense oligonucleotide against Bcl-2 gene was delivered by transferrin receptor (TfR) targeted lipopolyplexes (LPs) to K562 *in vitro* and K562 tumors grown in athymic mice [11]. As expected, *in vitro* Bcl-2 protein expression after Tf-LP G3139 treatment decreased to 40% while it decreased to 48% and 42% approx. with LP-G3139 and free G3139, respectively, so that nanoparticles did not have a benefit in this regard *in vitro.* For *in vivo* studies, Tf-LP G3139 (5 mg/kg) was administered by IV injections every other day for a total of seven doses. Pharmacokinetic studies showed a longer circulation time of Tf-LP G3139 in comparison with free G3139. G3139 was delivered into tumors more efficiently when it was loaded on Tf-LP than when it was in its free form (2.4 folds). Tf-LP G3139 treatment showed a significant reduction in tumor size (slightly greater than tumor reduction with free G3139) in comparison with untreated tumors. Tf-LP G3139 treatment resulted in increased mice survival in comparison with free G3139 and control groups. Unexpectedly, treatment with free G3139 decreased the Bcl-2 protein expression to 30% of the tumors, while treatment with Tf-LP G3139 decreased the protein expression to 60% [11]. Poor penetration of Tf-LP G3139 molecules to the inner layers of tumors could explain the lower protein down-regulation with these particles. Based on the significant increase in the IL-12 levels in serum and enlargement of the spleen of tumor bearing mice treated with Tf-LP G3139 (no IL-12 increment in mice treated with free G3139 or empty Tf-LP), the increased antitumor activity and survival of mice treated of these mice can be explained by a combination of the effects of Bcl-2 down-regulation and the capacity of the Tf-LPs to induce immunostimulation [11].

In the first and only non-viral clinical siRNA study, a recurrent Ph (+), CML patient

resistant to imatinib and chemotherapy after allogeneic hematopoietic stem cell transplantation was treated with BCR-ABL siRNA liposomes (without discontinuing therapy with imatinib, ARA-C and immunosuppressive medication) and with three IV injections (10, 30, 10 μg/kg) to evaluate the safety and feasibility of the siRNA non-viral treatment. After the first injection (10 μg/kg) of BCR-ABL siRNA liposomes a transient effect was noticed with complete haematological response (reduction of peripheral blood leucocytes with disappearance of immature cells) and absence of Ph chromosome. Nine days after first the siRNA administration, BCR-ABL mRNA levels decreased from 6.6% to 0.053%; however, at day 11 BCR-ABL mRNA expression had reached 16%. On day 21, a second siRNA with higher dose (30 μg/kg) was administrated: leucocytes remained constant and BCR-ABL mRNA decreased again to 7.8% four days later. On day 28, a third dose of 10μg/kg was administrated and leukocyte counts remained low for 2 days but on day 39 leukemia progressed as was evident by the presence of 70% blasts in peripheral blood and increased levels of BCR-ABL mRNA to 50%. Mild reduction or no reduction of BCR-ABL mRNA may suggest emergence of siRNA resistance or transfection failure (i.e. due to induction of serum RNase). The IV administration of siRNA was well tolerated without any clinically adverse events [86]. Authors concluded that siRNA administration is feasible, safe and has potential for development of non-viral siRNA-based therapies, and suggested that more studies and more patients may be worth pursuing and studied to explore its effects when siRNA sequences and delivery carriers are optimized [86]. It may be worth noting that the patient treated in this study had a point mutation in the ABL kinase at the ATP-binding pocket, which is likely to explain the resistance to imatinib. Moreover, effective silencing of BCR-ABL mRNA after BCR-ABL siRNA liposomes treatment although initial showed that BCR-ABL may be targeted by siRNA when TKIs are ineffective.

1.8 RNAi TARGET GENES FOR THERAPEUTIC APPLICATIONS IN CML

Several potential targets have been pursued for siRNA therapy of CML cells (**Table 1.1**). Silencing specific targets has been used as a tool to elucidate their functional role in CML and the biological outcome upon depleting the selected target. The main aim of these studies was identification of novel targets that decrease proliferation rates and induce apoptosis that could be potentially used in combination with conventional drugs to improve drug sensitivity.

Table 1.1. siRNA targets shown to be beneficial in CML.

Ref	Target (Additional Role)	Rationale & Related Outcomes	siRNA Carrier (therapeutic in vitro conc.)/ CML cells Model	siRNA Silencing Outcome
			Role: BCR-ABL Fusion Gene	
[87]	BCR-ABL	Compare efficiency of cell killing by Imatinib to that of silencing of *BCR-ABL* with siRNA	Oligofectamine™ (unknown) K562 cells	40% reduction of mRNA and almost complete reduction protein were found. Apoptosis rate of anti-BCR-ABL siRNA treated cells was at the same level as cells treated with Imatinib or ~5 times more than control cells.
[88]	BCR-ABL	Demonstrate therapeutic effect of BCR-ABL down-regulation by siRNA delivery	Electroporation (0.5 µg/100µl, 357 nM Est.) K562 and CML primary cells	mRNA level in K562 cells decreased to 28% and in primary CML cells to 40%. Reduction of viable cells by 75%. No proliferation inhibition in primary CML cells.
[89]	BCR-ABL	Inhibit *BCR-ABL* expression and evaluate sensitization to imatinib	Electroporation (200-800 nM) BCR-ABL+23Dp210, M07p210, and CML primary cells	Decreased cell viability and sensitization of imatinib-resistant CML cell lines to imatinib.
[90]	BCR-ABL	Compare effects of two pathways of BCR-ABL suppression (siRNA for inhibition of protein synthesis and Imatinib for inhibition of already synthesized protein).	Lipofectamine® 2000 (180 nM, 3 times every 2 d) K562 cells	82% reduction at the mRNA level and a 50% reduced cell proliferation capacity.

[56]	BCR-ABL	Study anti-leukemic properties of BCR-ABL by RNAi	Electroporation (1 μg per 5 x 10^5 cells) K562 cells	For K562 cells, 90% reduction of *BCR-ABL* mRNA expression after 12h. Slight increase of apoptosis. 2-fold increase of DNA fragmentation. Caspase-7 and -9 activated.
[57]	BCR-ABL	To assess efficacy of Tat-LK15 peptide in delivering siRNA to target *BCR-ABL*	Tat-LK15 peptide: fusion of HIV-Tat-derived peptide to cationic peptide LK15 (1 to 30 μg siRNA/mL – 71 - 2142 nM, Est.) K562 cells	Expression of p210 BCR-ABL was reduced for all concentrations. Cytotoxicity due to siRNA nanoparticles ranging from 0% (10 μg) to 30% (30 μg). No cell decrease was detected after 48 h in comparison with control siRNA group.
[83]	BCR-ABL	Encapsulate BCR-ABL siRNA and Transferrin-liposomes and assess efficacy	Transferrin receptor-targeted DSPE-PEG-MAL liposomes for silencing demonstration (200 – 2000 nM) K562 and LAMA-84 cells	~35% *BCR-ABL* mRNA and protein down-regulations and up to 20% decrease in cell viability in LAMA-84 with double siRNA dose. 50% decrease in cell viability in K562 cells with one dose of siRNA.
[81]	BCR-ABL	Demonstrate carrier efficacy in down-regulating BCR-ABL	Biodegradable charged polyester-based vectors (2.5 μg siRNA in 1 ml – 176 nM, Est.) K562 cells	75% *BCR-ABL* mRNA down-regulation 48 h after transfection, and 50% reduction of viable cells and 10% increase in apoptotic cells 74 h after treatment.
[68]	BCR-ABL	Pegylation of lipoplexes for improved stabilization of siRNA nanoparticles	DODAB:MO/PEG-ceramide (100 nM) K562 cells	50% *BCR-ABL* mRNA down-regulation, and 78% cell survival with pegylated nanoparticles, and 80% down-regulation and 64% survival with unpegylated nanoparticles.
		Role: Stem/Progenitor Cell function		

[91]	HIF-1α (transcriptio n factor)	Up-regulated in CML primary cells vs. healthy donors	Lipofectamine® 2000 (unknown) K562 cells	HIF-1α overexpressed in CML primary cells. HIF-1α silencing followed by down-regulation of p21. Significant decrease in proliferation and ability to form colonies.
[92]	Leukotriene B4 receptor (BLT2)	Role of BLT2 in CML and drug-resistant CML cells.	Dharmafect® (100 nM) KCL2 cells	BLT2 receptor is significantly overexpressed in CD34+ stem /progenitor CML cells. 60% decrease in cell growth and 50% increase in apoptosis.
[93]	SNAIL	To identify pathway by which CML exosomes enhance adhesion on stroma cells	Lipofectamine® RNAiMax (2 pmol siRNA in 24-well plates –600 µl of final vol. according to manufacturer. 3 nM, Est.–) LAMA84 cells	CML exosomes promote survival and adhesion to stromal cells by activation of EGFR signalling in stroma cells, through increased expression of SNAIL, and its targets MM9 and IL8.
[94]	Nucleostemi n (self-renewal)	Investigate effects of nucleostemin silencing in K562 cells.	HiPerfect™ (200 nM) K562 cells	Reduced cell growth, G1 cell cycle arrest followed by apoptosis.
[95]	Gli1	To inhibit nuclear mediator of Hedgehog signalling pathway (self-renewal)	Electroporation (30 nM) K562 and KU-812 cells	50% knockdown of *Gli1* mRNA. 90% cell viability inhibition.
		Role: Cell Cycle/ Mitosis		
[70]	Cyclin A2	Deliver cyclin A2 siRNA with SWNTs and evaluation of cyclin A_2 role upon doxorubicin treatment.	Ammonium functionalized single wall carbon nanotubes (f-SWNTs) (25 nM) K562 cells	A positive correlation between down-regulation of cyclin A_2 and decreased colony formation.
[67]	KIF11	Lipid nanoparticles for siRNA in leukemic cells	Alkylated DMA/DMA lipoplexes coated with PEG-lipids (50 and 250 nM) K562 cells, Molm13, THP-1	90% silencing of KIF11 mRNA.
		Role: Apoptosis/ Autophagy		
[96]	MCL1	Antileukemia effect of MCL1 silencing and	Lipofectin® (200 nM)	Decreased proliferation and synergistic effect with Imatinib.

		synergistic effect with imatinib in CML	K562 and KU812 cells	
[97]	BCL-X_L	Identification for anti-apoptotic BCL-2 family members in TKI-mediated apoptosis	Interferin® (10 nM) K562 and KCL22 cell lines	Down-regulation of BCL-X_L alone (and BCL2 to a lesser extent) resulted in apoptosis and potentiated TKI-mediated apoptosis, while normal patient cells remained unaffected.
[98]	ATG5 and ATG7	Targeting autophagy induced after IM treatment	Electroporation (3571 nM) K562, BV173, CML primary cells	Co-treatment of imatinib and silencing of ATG5 or ATG7 genes resulted in enhanced cell death in K562 while normal stem cells were much less affected.
		Role: Transcription Factor Related		
[99]	Wilms' tumor 1 (WT1) and BCR-ABL	Anti-leukemia additive effect of co-silencing of BCR-ABL and WT1 transcription factor	TransMessenger® (0.8 µg siRNA in 24-well plates – 200 µl of final vol. according to manufacturer. 286 nM, Est.-) K562 and CML primary cells	Additive effect in the inhibition of cell growth and in the increase of apoptosis in comparison with transfection of either siRNA alone in K562 cells and blast crisis CML patient cells. WT1 siRNA on its own also included apoptosis and decreased proliferation.
[100]	Growth-factor-independent 1B gene (GFI1B)	Evaluation of *GFI1B* expression in some types of leukemias	TransMessenger® (800 ng siRNA in 24-well plates [200 µl according to manufacturer, 286 nM, Est.]) K562 cells	Silencing induces reduction in proliferation and increases in apoptosis unlike healthy cells.
[101]	BCR-ABL and GFI1B	Anti-leukemic additive effect of co-silencing of BCR-ABL and GFI1B	DOTAP, liposomal transfection (175 pM for GFI1B and 54 pM for BCR-ABL) K562 and CML primary cells	Additive effect in the inhibition of cell growth and in the increase of apoptosis in comparison with transfection of either siRNA alone.
[54]	STAT5A	Effects of STAT5A siRNA knockdown on cell growth and apoptosis induction	HiPerFect™ (unknown) K562 cells	~75% suppression of *STAT5A* mRNA. Resistant K562 cells became ~4 times more sensitive to imatinib. An increase in caspase-3 activation was seen.
[102]	Zinc finger protein, X-linked (ZFX)	Elucidate the functional mechanisms of ZFX in CML	Electroporation (unknown) K562 cells	Silencing of ZFX reduced cell proliferation by inducing cell cycle arrest and apoptosis, and enhanced sensitivity to imatinib by inactivating the PI3K/Akt signalling pathway.
[103]	eIF4E	Study activation of eIF4E pathway during treatment with dasatinib	Dharmafect®	Silencing of eIF4E lead to 60% cell growth inhibition and 30% increase in apoptosis. Cells became more sensitive

			(100 nM) K562 cells	to dasatinib with co-treatment with EIF4E-siRNA
		Role: Tyrosine Kinase		
[104]	Lyn	Study effect of Lyn ablation in CML blast crisis cells	Electroporation (0.5 µg siRNA in 100 µl. 357 nM, Est.) K562, EM-2, EM-2, LAMA Mo7e (BCR-ABL-), normal CD34+, blast crisis CML primary cells	Lymphoid CML blasts underwent induction of apoptosis (~100%) after 72-96 h, whereas absence of Lyn had no effect on normal CD34+ cell viability.
[52]	Syk and Axl	Identify downstream effectors of Lyn involved in resistance to nilotinib	Electroporation (200 nM) K562 and CML primary cells	Silencing Lyn's downstream effectors Syk and Axl restored capacity of nilotinib to inhibit cell proliferation.
		Role: Others		
[53]	Preferentially expressed antigen in melanoma (PRAME)	Investigate function of PRAME in CML progression by RNAi in K562 cells	Electroporation (1500 nM) K562 cells	70% knockdown of *PRAME* mRNA. Significant inhibition of cell proliferation and decrease of clonogenic growth. 60% of apoptotic cells in comparison with 15% of controls.
[105]	PPP2R5C (protein phosphatase)	Effect of PPP2R5C down-regulation in imatinib-sensitive and –resistance CML cells	Electroporation (3 µg/100 µl, 2140 nM, Est.) K562, 23D-Bcr-Abl, CML primary cells	Inhibition of the proliferation of CML cells. Rendered imatinib-resistant cells more sensitive to TKIs.
[106]	GCS or MDR1	Relation of GCS and MDR1 to regulation P-gp gene expression and function activity in drug retention	Lipofectamine® 2000 (unknown) K562 cells	Silencing of GCS can affect MDR1 expression and inhibit P-gp efflux. Silencing of GCS or MDR1 sensitized drug-resistant cells to chemotherapy and increased drug retention.
[107]	Heat shock protein90 (Hsp90)	Explore inhibitory effect on Hsp90 in imatinib-resistant CML cells	Lipofectamine® 2000 (50 nM) K562 cells, IMR-K562 cells and CML primary cells	Double silencing Hsp90 and BCR-ABL significantly enhanced the cell proliferation inhibition (80% at 96 h) in CD34+ CML cells in comparison with cells treated with either siRNA alone (60% to 70% at 96 h).

DMA: (1,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane. DODAB:MO: dioctadecyldimethylammonium bromide (DODAB):monoolein (MO). DSPE-PEG-MAL: 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[maleimide (polyethylene glycol)2000] ammonium salt. IMR: imatinib-resistant, PEI: polyethyleneimine. Est.: estimated

1.8.1 BCR-ABL Fusion Gene Targeting

In one of the first studies to explore siRNA therapy *in vitro*, Wilda and co-workers performed BCR-ABL siRNA transfection (using Oligofectamine™) in K562, which decreased the mRNA level to 40%, and the protein level was almost completely abolished in comparison with non-treated cells. Moreover, apoptosis level was similar to treatment with imatinib (8-fold higher than controls). In this case, no additive effect was found when cells were co-treated with imatinib and BCR-ABL siRNA [87]. In another study, BCR-ABL mRNA expression after BCR-ABL siRNA transfection by electroporation decreased to 28% in K562 cells, and to 40% and 36% in CML mononuclear cells and CML CD34+ cells, respectively [88]. Viability of K562 cells was reduced by 75%, however no significant inhibition of cell proliferation or colony formation was observed in primary CML cells; whereas treatment with imatinib reduced significantly the viable cell numbers and the colony numbers from CML CD34+ cells [88]. It is likely that the delivery system (electroporation) was not effective in primary cells or the siRNA was not specific enough for BCR-ABL silencing. Wohlbold and co-workers targeted *BCR-ABL* expression in *BCR-ABL*-transduced cells by electroporation (200 - 800 nM siRNA). This siRNA treatment resulted in a significant reduction of BCR-ABL protein, which led to a reduced regulatory effect of its substrates, reducing the expression of antiapoptotic Bcl-X_L protein and increasing the expression of cell cycle inhibitor p27. BCR-ABL silencing led to a significant reduction of cell viability in a dose-dependent manner (10% and 70% decrease in viability with 200 and 800 nM siRNA, respectively). In *BCR-ABL*-transduced cells transfected with *BCR-ABL* siRNA, a significant drop in the IC_{50} values of imatinib (3.4-fold drop) and a significant increase in apoptotic cells (6-fold increase) was observed in comparison with cells treated with imatinib only [89]. Multiple BCR-ABL-siRNA doses have also been explored by Zhelev *et al*: siRNA dose was composed of 3 applications at 60 nM every 2 day. The effects of this treatment were compared with 3 applications of imatinib at 180 nM every 2 days [90]. Treatment of BCR-ABL siRNA with Lipofectamine®

in K562 cells reduced the level of BCR-ABL mRNA to 18%, the protein level to 36%, and the cell proliferation to ~50%. On the other hand, imatinib treatment showed a lower decrease at the protein level (20%) and a 54% reduction in the cell proliferation activity, showing very similar therapeutic effects of BCR-ABL siRNA and imatinib [90]. Effective *BCR-ABL* silencing was also obtained by Rangatia *et al.* using electroporation*,* where a ~90% decrease at the mRNA level led to a two-fold increase of sub-G1 cell population as well as to an increase of DNA fragmentation and mitochondrial-induced apoptosis. Although only a transient mRNA reduction was seen with siRNA treatment, a long-term effect was observed in proliferation of K562 cells: cells were unable to actively divide for at least 2 weeks in comparison with untreated cells. This reduction in proliferation was explained by the cell cycle arrest in the G1 phase, which was confirmed by a decrease in cyclin D1 and increase in p21 and p27 cell cycle inhibitors [56]. Arthanari *et al.* used the Tat-LK15 fusion peptide to deliver siRNA (71 to 2142 nM concentration, Est.) and showed a ~70% reduction in $p210^{BCR-ABL}$ 48 h post-transfection for all concentrations. Surprisingly, no significant decrease in the cell viability was detected between cells treated with BCR-ABL and control siRNA after 48 hours [57]. The effect of transferrin-receptor BCR-ABL siRNA/liposomes was evaluated on LAMA-84 and K562 cells (siRNA doses between 200 and 2000 nM) [83]. Double BCR-ABL siRNA dose showed a 40% decrease at the mRNA level and 30% decrease at the protein level in LAMA-84 cells, and one single BCR-ABL siRNA dose led to a ~20% decrease in the cell viability in the same cells; while there was a 20-50% decrease in the cell viability of in K562 cells in comparison with control siRNA with one single dose of BCR-ABL siRNA. No silencing and biological effects were found of liposomes with no receptor, which may explain the inefficiency of the liposome on its own. The fact that expression of BCR-ABL in K562 cells is almost 2 times more than that in LAMA-84 cells may explain the greater decrease in cell viability on the former [83]. Yang *et al.* used biodegradable charged polyester-based vectors (BCPVs) for BCR-ABL siRNA delivery (176 nM, Est.) in K562 cells, which

resulted in 75% down-regulation of BCR-ABL mRNA, 50% reduction of cell viability and 10% increase in the apoptotic cells in the presence of serum. Low citotoxicity levels were found with these BCPVs polymers [81]. Oliveira, *et al.* used pegylated dioctadecyldimethylammonium bromide (DODAB):monoolein (MO) lipoplexes for BCR-ABL siRNA delivery. At a siRNA concentration of 100 nM, pegylated siRNA-lipoplexes significantly decreased BCR-ABL mRNA expression by 50% and decreased cell survival by 22%. However, non-pegylated nanocarriers achieved higher silencing levels (75% decrease) and decreased further the cell viability by 36% [68]. This decreased effect with the pegylated lipoplexes was explained due to the presence of PEG at the lipoplex surface, which may block the siRNA release from the endosome by impeding the destabilization and close interaction of the lipoplexes with the endosome membrane [68].

Conditions such as siRNA concentration, siRNA delivery method, degree of silencing effect, and number of doses applied have a proportional effect on the biological outcome of CML cells that translates into decrease cell proliferation, percentage of apoptotic cells. The effects of BCR-ABL siRNA transfection have been found comparable to the effect of imatinib treatment. These initial studies show proof of concept that siRNA transfection against BCR-ABL can lead to therapeutic outcomes and open the door for RNAi as a potential alternative for CML management and give room for improvement of current therapies.

1.8.2 Stem/Progenitor Cell Function Targeting

Hypoxia-inducible factor-1α (HIF-1α) was significantly expressed in CML patient samples of the bone marrow (approx. 6 times) than normal donor samples. Transfection of HIF-1α-siRNA with Lipofectamine® 2000 in K562 led to an mRNA reduction to 25% that translated into a significant reduction of the cell proliferation and inhibition of the colony formation (50%). This study suggested that the over-expression of HIF-1α,

through up-regulation of p21 expression, maybe be involved in the CML pathogenesis by increasing the proliferation and colony formation of CML cells [91].

The role of BCR-ABL-mediated transformation in activating the expression of the leukotriene B4 receptor 2 (BLT2) was found to be essential in promoting leukemogenesis through suppression of tumor suppressing p53 signalling pathway, especially in the leukemic CD34+ stem/progenitor cell population. The high expression level of *BLT2* mRNA was correlated with increased *BCR-ABL* expression in BCR-ABL transduced cell lines and CD34+ CML patient cells. Inhibition of BLT2 by siRNA and Dharmafect® in KCL22 cells showed a 60% decrease in cell growth and a 50% increase in apoptosis. Consistent with the cell line results, the treatment of CD34+ CML cells with a small drug molecule selective antagonist of BLT2 receptor (no siRNA used) induced apoptosis in a dose-dependent manner and impaired the ability to form colonies. When BLT2 inhibitor was used in combination with dasatinib, it showed an enhanced apoptotic effect and abolished the colony formation with CD34+ CML cells [92].

Studies from Corrado, *et al.* [93] proposed that there is an exosome-mediated bidirectional crosstalk between mesenchymal stromal cells and CML cells that leads to the activation of the epidermal growth factor receptor (EGFR) pathway and consequently, the production of IL8 in stromal cells that sustains the survival of CML cells in the bone marrow niche [93]. The authors found that CML cell lines and primary cells secrete exosomes containing amphiregulin (AREG) that activate the EGFR signalling in bone marrow stromal cells. Pre-treatment of stromal cells with CML exosomes activated the EGFR signalling pathway leading to an increased gene expression of the transcriptional factor SNAIL, and a consequently increase of the expression of MMP9 and IL8 and the protein binding membrane annexin A2 in the stroma cells. This cascade of events promoted the adhesion of leukemic cells to the stromal monolayer, as well as proliferation and survival of CML cells. Finally, treatment with SNAIL siRNA and

Lipofectamine® RNAiMax reduced IL8 and MMP9 expression in stromal cells [93]. Reduction of SNAIL expression mediating siRNA could therefore have a therapeutic potential in decreasing the adhesion and proliferation of CML in the bone marrow cells and may make these cells more sensitive to TKI therapies.

Nucleostemin is a protein localized in the nucleolus of stem and tumor cells that regulates their self-renewal and cell cycle progression. Nucleostemin-siRNA electroporation in K562 showed that a 55% decrease of the Nucleostemin mRNA led to a 36% cell growth inhibition, an increase in the apoptotic cells, and a cell cycle arrest in the G1 phase for 3 consecutive days after the siRNA treatment [94].

A study suggest that smoothened (SMO) and Gli, essential downstream activators of the Hedgehog pathway, may play important roles in the survival and maintenance of LSC in CML. siRNA-mediated silencing of Gli by electroporation in K562 cells showed a 50% Gli mRNA silencing and a 90% decrease of cell viability [95]. This would be a suitable target to control the growth and self-renewal capacity of CML stem cells.

1.8.3 Cell Cycle/Mitosis Targeting

Cyclin A2, a cell cycle mediator, was targeted by RNAi using ammonium functionalized single wall carbon nanotubes (f-SWNT) in K562 cells. Silencing cyclin A2 in doxorubicin-treated K562 cells led to a significant decrease in the percentage of cell in S phase, growth inhibition, apoptosis induction and increased erythroid differentiation. This suppression also caused a small fraction of K562 cells to differentiate along megakaryocytic and monocyte-macrophage pathways upon doxorubicin treatment. A positive correlation between the ability of doxorubicin to induce apoptosis in K562 cells and the downregulation of cyclin A_2 was seen; the lower the cyclin A_2 expression, the higher the sensitivity to doxorubicin was. These results indicated a pro-apoptotic role of cyclin A_2 and its ability to regulate cell differentiation in CML [108].

He *et al.* targeted the expression of KIF11, a kinesin essential for bipolar spindle formation, using alkylated DMA-containing lipid nanoparticles (50 and 250 nM) in an array of adherent and suspension cell lines, including Molm13, THP-1, K562, and KG1 leukemia cells. For the K562 cells in particular a 90% silencing (at both concentrations) at the KIF11 mRNA level was found 24 h after transfection, while no cytotoxic effect was seen caused by the transfection [67]. Similar silencing effect was seen with Molm31 and THP-1 acute myeloid leukemia cells (80-90% silencing). For the case of KG1 cells, a milder silencing of 20-30% was found. However, not biological effect was evaluated *in vitro* with this gene target [67].

1.8.4 Apoptosis/Autophagy Targeting

MCL-1 expression was highly over-expressed in bone marrow CML patient cells in comparison with normal cells. Use of single-stranded antisense oligonucleotides (ASO) and siRNA to down-regulate expression of MCL-1 with Lipofectin® in CML cell lines was explored. Targeting of MCL-1 by ASO almost completely abolished the protein expression, which led to a substantial increase of dead and apoptotic cells. Expression of MCL-1 was also down-regulated in imatinib-resistant K562 cells which was also associated with increased dead and apoptotic cells. Co-treatment of MCL-1 ASO and imatinib gave a substantial synergistic effect in comparison with each treatment alone. Similar results were found when an MCL-1 siRNA was used. Targeting of MCL-1 in CML can be a strategy to promote CML cell dead in imatinib sensitive and insensitive CML cells [96].

High levels of CIP2A expression in CML increase the risk of imatinib resistance and disease progression. An increased expression of the anti-apoptotic protein *BCL-X_L* in CML primary samples with high levels of CIP2A was found [97]. Silencing of *BCL-X_L* by siRNA using Interferin® resulted in apoptosis and potentiated TKI-mediated apoptosis in K562 and KCL22 CML cell lines. Although these results with siRNA were not confirmed in CML

primary cells, the use of drug inhibitor of *BCL*-X_L showed a significant apoptotic effect in CD34+ cells from high CIP2A CML patient samples as a single agent or in combination with TKIs. On the contrary, mononuclear cells from health volunteers treated with the same inhibitor remained insensitive [97].

Autophagy was another survival mechanism triggered by inhibition of the BCR-ABL tyrosine kinase after imatinib treatment in the CML cell lines K562 and BV173 and CML primary cells; co-treatment of imatinib and silencing of autophagy genes ATG5 or ATG7 by siRNA (electroporation) resulted in sensitizing effects of imatinib in K562 and primary CML cells (significant reduction in the number of colonies), while normal mononuclear and $CD34^+$ cells were much less affected [98] [109].

1.8.5 Transcription Factor Targeting

Wilms' tumor 1 (WT1) is an aberrantly overexpressed transcription factor in different leukemias in comparison with normal hematopoietic stem cells. WT1 is involved in hematopoiesis and regulates proliferation and differentiation of blood cells [110]. WT1 siRNA-mediated silencing reduced mRNA levels to 43% in K562 cells, and to 58% and 83% in 2 CML patient cells. Transfection with WT1-siRNA using TransMessenger® in K562 cells showed a 12% decrease in cell proliferation and a 6% increase in the apoptotic cells and; in CD34+ CML patient cells, it showed a 4% decrease in cell proliferation, whereas the apoptotic cells did not change. In combinatorial treatment of WT1 and BCR-ABL siRNAs in K562 cells, an additive effect was seen with a 69% decrease in cell proliferation whereas in CML patient cells the co-treatment showed 68% decrease in proliferation. An additive effect was also evident by an increase in the apoptotic cells in both cell types [99].

Growth factor independent-1B (*GFI-1B*) is a transcription factor that controls the development and differentiation of erythroid cells and megakaryocytes at the erythro-

megakaryocytic progenitor stage [111]. *GPI1B* mRNA expression was overexpressed in leukemic cells, and siRNA-mediated silencing of GFI1B with TransMessenger® showed a reduction in cell proliferation and increased apoptosis in K562 cells [100]. Koldehoff *et al.* investigated whether anti-leukemic effect of *BCR-ABL* silencing could be further increased by co-silencing of *GFI1B* using DOTAP as the delivery carrier [101]. A significant drop in cell viability was evident with the combination of *GFI1B* and *BCR-ABL* siRNAs, as well as *BCR-ABL* mRNA levels after co-silencing. An additive induction of apoptosis after co-silencing was also observed. Similar results of the inhibition of mRNA levels of *BCR-ABL* and *GFI1B* were found in advance CML patient cells. The co-silencing led to a significant reduction of *MDR1* (P-gp) and *c-Myc* mRNA levels, suggesting that *BCR-ABL* and *GFI1B* may be connected to other critical mediators involved in cancer transformation [101].

Kosova *et al.* studied the effect of STAT (signal transducer and activator of transcription) knockdown in apoptosis and proliferation in imatinib-sensitive- and imatinib-resistant K562 cells. STAT5 is involved in the development of myeloproliferative diseases, while STAT3 is implicated in malignant transformation; both STAT5 and STAT3 are constitutively expressed in haematological malignancies [112], [113]. Quantification of mRNA levels revealed a significant increase in *STAT5B, and STAT5A* (>50%), but not in *STAT3* in imatinib-resistant cells (only 4%) as compared to imatinib-sensitive cells. Transient knockdown of STAT5A by siRNA with HiPerFect^{TM} increased the sensitivity to imatinib treatment in imatinib-resistant and imatinib-sensitive cells, 4.5- and 1.2-folds, respectively [54]. When imatinib-resistant cells were treated with 5 μM imatinib, cell viability decreased by ~20%, while treatment with the same concentration of imatinib and STAT5A-siRNA decreased the cell viability by ~60% [54].

Zinc finger protein X-linked (ZFX), a transcriptional regulator of hematopoietic stem cell regeneration, was significantly overexpressed in CML patient cells and K562 cells in

comparison with healthy donor bone marrow cells. siRNA silencing of *ZFX* using electroporation reduced mRNA and protein expression to 20% in K562 cells, which resulted in significant reduction of proliferation and ability to form colonies, arrested cells in the G0/G1 stage, and induced apoptosis. Combinatorial treatment of ZFX-siRNA with imatinib in K562 cells resulted in significant decrease in the cell viability and colony formation in comparison with imatinib treatment alone. The significant reduction of p-Akt levels in ZFX-silenced CML cells, suggested that the therapeutic effect of ZFX siRNA treatment was possible by inactivation of the PI3K/Akt signalling pathway [102].

Treatment with dasatinib induced phosphorylation of the oncogene eIF4E (a gene associated with transformation and tumorogenesis) in a time-dependent manner in K562 cells. Depletion of eIF4E by siRNA with Dharmafect® resulted in a cell growth inhibition of 60% and a 30% increase in apoptosis. Combinatorial treatment of eIF4E-siRNA and dasatinib enhanced the therapeutic effects of dasatinib in K562 cell by reducing cell growth to 20% and inducing apoptosis to 90% [103]. Treatment with pharmacologic eIF4E inhibitors in TKI-resistant CD34+ CML patient cells also showed a significant reduction of proliferation, colony formation and self-renewal capacities and, induction of apoptosis. Combination of dasatinib with of eIF4E-siRNA enhanced these therapeutic effects even further [103].

1.8.6 Tyrosine Kinase Targeting

Lyn kinase is activated by BCR-ABL [114] and is over-expressed in blast crisis CML cells (treated with TKIs) in comparison with early stage CML cells (before TKI treatment) [115]. Lyn expression in K562 cells and CML patient cells was silenced by Lyn-siRNA delivery by electroporation (357 nM, Est.) [104]. Reduction of Lyn protein was between 80 and 95% in K562 cells, normal CD34+ bone marrow cells, and lymphoid and myeloid CML blast crisis primary cells. K562 cells showed a significant reduction of cell viability after 2 and 4 day after transfection (up to 70% decrease) and a 40% increase of

apoptotic cells 3 days after transfection in comparison to the control siRNA group [104]. Silencing of Lyn had no effect on cell viability of normal $CD34^+$ cells, while this silencing in lymphoid CML blast crisis cells showed almost a 100% decrease of cell viability between 3 and 5 days after transfection in comparison with cells treated with control siRNA. Similar reduction on cell viability was found in TKI-resistant lymphoid CML blast crisis cells. For the case of myeloid CML blast crisis cells, Lyn silencing also reduced cell viability but to a lesser extend (50% decrease). These results showed that Lyn silencing increases apoptosis and decreases cell survival of CML blast crisis cells, especially when lymphoid, while not affecting the normal hematopoietic cells [104].

Along the same lines Gioia *et al.* investigated the role of Lyn kinase signalling as a mediator of resistance to nilotinib and found that Lyn was overexpressed 8-fold more in nilotinib-resistant K562 cells in comparison with nilotinib-sensitive K562 cells. The proteins spleen tyrosine kinase (Syk), UFO receptor tyrosine kinase Axl, and the adaptor protein CDCP-1 were found to be mediators of Lyn signalling pathway in nilotinib-resistant cells. Inhibition of Syk, Axl and CDCP by siRNA electroporation increased (or restored) the sensitivity to nilotinib. Consistent with these results, an overexpression of Lyn, CDCP-1 and Axl was also detected in nilotinib-resistant $CD34^+$ patient cells. The role played by Syk and Axl in the nilotinib resistance identifies these genes as potential targets as a combinatorial therapy for CML [52].

1.8.7 Other Gene Targeting

The expression of the preferentially expressed antigen of melanoma (PRAME) gene was explored in leukemia. PRAME acts as a repressor of retinoic acid receptor (RAR) signalling and thus, the functional repression of PRAME was investigated in K562 cell line in the absence of retinoic acid. PRAME-siRNA electroporation treatment showed ~70% knockdown of *PRAME* mRNA and complete inhibition of the protein expression, which resulted in a significant inhibition of the proliferation and clonogenic growth.

PRAME knockdown also led to a significant accumulation of cells in G_0/G_1 phase and a proportional decrease of cells in S phase, which suggests a relationship between PRAME and cell cycle arrest in the G_0/G_1 phase. This cell cycle arrest was followed by a gradual increase in apoptotic cells and caspase-3 activation. Overexpression of PRAME was also found to prevent the cells from erythroid differentiation [53].

Protein Phosphatase 2, Regulatory Subunit B', Gamma (PPP2R5C) levels were over-expressed in peripheral blood mononuclear cells from chronic phase CML patients, and *PPP2R5C* expression was significantly decreased in patients undergoing remission [116]. *PPP2R5C* plays an important role in cell proliferation, differentiation, and transformation based on its induction of the de-phosphorylation of p53 at various residues, which negatively modulates apoptosis thus promoting cell survival [116]. It was possible to reduce *PPP2R5C* mRNA and protein levels in K562 and resistant-K562 cells with specific siRNA by electroporation treatment. *PPP2R5C* mRNA levels in CML primary cells was also reduced with specific siRNA treatment, leading to reduced proliferation rate in both K562 and CML primary cells. An increase in apoptosis rate in K562 cells was also evident. These results indicate that down-regulation of *PPP2R5C* could significantly inhibit the proliferation of CML cells and more importantly, could render imatinib-resistant cells sensitive to TKIs [105].

The sphingolipid ceramide plays an important role in apoptotic signalling in response to anticancer drugs. Intracellular levels of pro-apoptotic ceramide increase when cells respond to drugs, contributing to the anti-cancer efficacy of the drugs. However, multidrug-resistant cells accumulate ceramide due to an enhanced activity of glugosyceramide synthase (GCS), which converts the available ceramide in glucosylceramide (GlcCer). This conversion impedes ceramide from being involved in the activation of apoptosis [117], [106]. Although controversial, down-regulation of GCS has been shown to down-regulate expression of P-glycoprotein (P-gp) [106], an efflux

pump that decreases intracellular levels of drugs. With the aim of decreasing the multi-drug resistance in doxorubicin-resistance K562 cells, Zhang and co-workers targeted GCS or P-gp by siRNA using Lipofectamine® 2000. Upon silencing GCS or P-gp with specific siRNAs, the transporter activity was significantly decreased, suggesting a linkage between GCS and P-gp expression, and providing potential therapeutic targets in CML therapy [106].

Hsp90 is a constitutively expressed chaperone that facilitates folding of client proteins such as BCR-ABL, and when the latter contains mutations, its dependency to Hsp90 in further increased [118]. Moreover, Hsp90 has been shown as a potential target for the reduction of proliferation and survival of leukemia stem CML cells [118]. The effect of silencing of Hsp90 by siRNA was evaluated in K562, CML CD34+, using Lipofectamine® 2000 as the delivery agent (50 nM siRNA concentration) [107]. Protein reduction of Hsp90 (no quantification analysis performed) gave a significant cell viability reduction of K562 cells between 1 and 4 days after transfection (~40% on day 3), while combinatory siRNA silencing of HsP90 and BCR-ABL gave a further inhibitory effect (~60% on day 3). Similar effect was seen in CML CD34+ cells, where silencing of Hsp90 gave an inhibitory effect from 1 to 4 days, with an inhibition of 40% on day 4 and 75% when both genes were targeted [107]. This shows that a combinatory inhibition of Hsp90 and BCR-ABL by siRNA can serve as a method to decrease the proliferation and survival of hematopoietic stem CML cells.

Taken together, it is evident that several promising protein targets are available for siRNA-mediated silencing. Effective functional responses have been obtained, in the form of reduced proliferation, apoptosis induction as well as sensitization to CML drugs, after targeting individual or combination of the appropriate targets. However, these studies reveal that current transfection methods are highly effective on cell lines, but when the same strategy is applied to patient samples, their silencing efficacy and

therapeutic effects are much lower. Therefore, more efficacious siRNA non-viral carriers are needed especially in the case of primary and stem cells so increase their translation potential and their effects are like those seen with inhibitors or TKIs. Whether this approach could be applied clinically remains to be seen.

1.9 PERSPECTIVE ON SIRNA THERAPY IN CML

New functional carriers that promote efficient delivery of gene-based agents (i.e., siRNA) in a controlled and non-toxic way are motivating researchers to find physiological solutions for treatment of CML. A better understanding of the clues that lay behind the uptake and intracellular trafficking of siRNA nanoparticles in the challenging suspension-growing leukemic cells will further help in this endeavour. The effect of carrier characteristics such as molecular size, degree of substitution (or modification) and optimal balance of the lipophilic-cationic moieties should be better understood not only on siRNA delivery efficiency, but also on toxicity, intracellular trafficking and cell specificity. This together with the identification of novel siRNA targets that can be used in combination with classical siRNA targets in CML, such as *BCR-ABL*, to silence gene combinations involved in the activation of different survival pathways in CML should prove beneficial. The combinational delivery, where multiple targets are silenced simultaneously, is likely going to yield more efficacious therapy, and possibly more specific outcomes. Irrespective of the target, however, non-viral siRNA delivery is more likely to be the clinically acceptable approach, given the relatively safe nature of such a delivery mode. The siRNA therapy could act in conjunction with the drugs currently employed to improve their effectiveness or re-sensitize the cells to current drugs. However, the siRNA therapy could also serve as a stand-alone therapy if LSC could be specifically targeted. There is no reason why the delivery methods used for CML cells could not be applied to other types of leukemias, but this will most likely require a

different set of biomaterials effective in a particular type of leukemia. Very little information exists on the molecular details for effective carriers in different leukemias so that this should be a fruitful avenue of exploration in the future.

Since the suspension-growing cells tend to be more difficult to transfect than the attachment-dependent cells, added pressure exists for non-viral delivery to be functional for leukemic diseases. The siRNA nanoparticles need to be effective at a 20-50 nM range in culture for a practical translation to preclinical animal models. It is typical for reported delivery system to employ concentrations beyond this range, including our own work [63]. Concerted effort to lower efficacious doses will be beneficial in this regard. In addition to efficacy, specificity to target the cells of interest is important so that critical genes in normal physiology are not disrupted. Given the cationic nature of these nanoparticles, they could theoretically bind to a multitude of cells *in vivo*. However, 'biochemical' targeting could alleviate this limitation to some extent: only those genes that are aberrantly expressed in CML cells, such as the *BCR-ABL* or other supporting mediators, could be the target of RNAi, so that nanoparticles penetrating 'normal' cells might not lead to silencing important targets. On the other hand, to increase the specificity of siRNA delivery, carriers could be coupled with CML-specific ligands, such as antibodies, to deliver the siRNA to only certain cell populations. For example, carriers could be coupled with an anti-CD34 antibody to target at least most of the CML stem cell portion. However, these antibodies need to be chosen with care so the delivery system is not too limited to certain cell populations. These antibody ligands need to be also exclusively or substantially over-expressed in the target cells to minimize nanoparticle binding to normal cells. A modular design could be envisioned where a delivery system optimized for general cellular uptake is further functionalized with leukemic cell specific cell surface binding molecules.

Finally, little information is available on siRNA delivery to primary cells, either healthy

or malignant cells from CML patients. It is critical not only to evaluate the efficacy in human cells, but also to evaluate the off-target effects of the siRNA delivered and cytotoxic effect of the carriers. While cell lines are preferred (due to practical reasons) in the design and optimization of carriers, characteristics such as, endocytosis rate and intracellular trafficking pathways are expected to be significantly different in leukemia primary samples. Misleading directions could be avoided by employing primary cells early on in the development process.

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2. INVESTIGATING SIRNA DELIVERY TO CHRONIC MYELOID LEUKEMIA K562 CELLS WITH LIPOPHILIC POLYMERS FOR THERAPEUTIC BCR-ABL DOWN-REGULATION[2]

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2.1 INTRODUCTION

Chronic Myeloid Leukemia (CML) is a cancer of the hematopoietic stem cells arising from chromosomal translocation of chromosomes 9 and 22, which results in a fusion between the Abelson murine leukemia viral oncogene homolog 1 (*ABL)* and breakpoint cluster region (*BCR)* genes [1]-[3]. Once the normally regulated tyrosine kinase of the ABL protein is consistently activated by the juxtaposition of the *BCR* sequence, it activates multiple signal transduction pathways, alters cell adhesion to bone marrow stroma, increases cell proliferation and reduces apoptosis, leading to over-population of myeloid cells in the hematopoietic system [3], [4]. Current therapies for CML are based on the use of small molecular drugs tyrosine-kinase inhibitors (TKIs) and stem cell transplantation. TKIs such as imatinib mesylate had a major impact on treatment of chronic phase CML; however, TKI monotherapies are not curative and initial and acquired TKI resistance, as well as relapse, remain significant challenges [3]-[5]. There are multiple mechanisms that confer TKI resistance, including increased expression of *BCR-ABL* and its tyrosine kinase activity, and/or point mutations in the tyrosine kinase domain that affect drug binding to its target [2], [6]. In addition, primary CML stem cells, including primitive quiescent cells, are not effectively targeted or eradicated by TKIs and hence constitute a critical population of cells in setbacks upon IM discontinuation and in generating IM-resistant clones [3], [4]. Stem-cell transplantation therapy is an option when treatment with TKIs fails; however, this therapy has a substantial risk of mortality due to chronic graft-versus-host disease [4], [5].

The shortcomings of current treatments call for alternative and more efficacious therapies for CML. Owing to increasing knowledge on molecular changes in CML, gene-based therapy is becoming a promising approach since it can specifically address the underlying cause of the disease. Synthetic small interfering RNA (siRNA) delivered into cytoplasm of transformed cells can interact with a desired mRNA for down-regulation of

specific proteins involved in processes such as cellular over-growth and inactivation of apoptosis. Although numerous potential molecular targets have been identified for siRNA delivery in CML cells [7]-[10], a functional carrier is needed for effective intracellular delivery of siRNA [11], [12], since the anionic siRNA is incapable of traversing plasma membrane on its own. Physical methods, such as electroporation, result in high cell death [13], [14] and cannot be practised *in vivo.* While viral vectors have been effectively used for manipulation of leukemic cells, they represent a significant safety risk because of their capacity to integrate to the host's genome and/or cause lethal immune responses and inflammation [12], [15]. Cationic polymers, which are actively explored for siRNA delivery [16], are a safer alternative to viruses, especially considering that they are readily amenable for engineering to match the needs of the application. Lipid-substituted polyethyleneimines (PEIs) have been developed for this purpose and showed that they could be tailored to deliver plasmid DNA as well as siRNA to a variety of cell types. Unlike high molecular weight (MW) PEI, which acts as an effective "proton-sponge" for endosomal escape [17] but displays excessive toxicity, we focused on low MW PEIs due to the low cytotoxicity of these polymers [18]. By employing the amine groups of PEI for substitutions, we found that the relatively nontoxic but ineffective 2 kDa PEI (PEI2) polymer could be transformed into an effective nucleic acid carrier as a result of lipid substitution on these groups [18], [19].

In this study, we explored the efficacy of lipid-substituted PEIs for siRNA delivery to CML cells for the first time. By using K562 cells as a CML model, we investigated the structural features of lipid-substituted PEIs that influenced the siRNA delivery. Since the physicochemical properties of the polymers were reported previously in Reference [20], we focused on siRNA delivery and the resulting silencing activity in the chosen cell model in culture. The silencing activity was explored based on a reporter gene target (Green Fluorescent Protein, GFP) that was virally incorporated into CML cells, and the

endogenous *BCR-ABL* oncogene.

2.2 METHODS

2.2.1 Materials

Branched PEIs with MWs of 0.6 (PEI0.6) and 1.2 kDa (PEI1.2) were purchased from Polysciences (Warrington, PA). PEI with MWs of 2 (PEI2) and 25 kDa (PEI25), anhydrous dimethylsulfoxide (DMSO), *N,N*-dimethylformanide (DMF), linoleyl chloride (C18:2 9Z,12Z; 99%), trypsin/EDTA and were obtained from Sigma-Aldrich (St. Louis. MO). Stearoyl chloride (C18; >98.5%) was obtained from Fluka. Caproyl chloride (C8; >99%), palmitoyl chloride (C16; 98%) and octanoyl chloride (C18:2 9Z; 99%) were purchased form Aldrich. Unlabeled scrambled siRNA, 5'-carboxyfluorescein (FAM)-labeled scrambled siRNA and M-MLV reverse transcriptase were purchased from Invitrogen (Burlington, ON). A GFP siRNA (GFP-22) was from Qiagen (Toronto, ON). A custom-synthesized BCR-ABL siRNA (5'-GCAGAGUUCAAAAGCCCTT-3' and 3'-TTCGUCUCAAGUUUUCGGG-5') was obtained from IDT (Coralville, IA), while two other BCR-ABL siRNAs were obtained from Allele Biotechnology (San Diego, CA; catalog numbers: ABP-Ri-VAsi-D09 and ABP-Ri-VAsi-D10). The RPMI Medium 1640 medium with L-glutamine, low-glucose DMEM, Opti-MEM® I reduced serum medium, penicillin (10000 U/mL), streptomycin (10 mg/mL) were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from PAA Laboratories Inc. (Etobicoke, ON). Lipofectamine® 2000 and Lipofectamine® RNAiMAX Reagent are from Invitrogen, Metafectamine Pro from Biontex (San Diego, CA), FuGENE HD from Roche (Laval, QC) and HiPerFect Transfection Reagent from Qiagen (Mississauga, ON). Annexin V-FITC apoptosis detection kit I was purchased from BD Biosciences (San Jose, CA). RNAeasy Mini Kit was from Qiagen (Toronto, ON).

2.2.2 Synthesis and Characterization of Lipid-Substituted Polymers

In this study, we used two different lipid-substituted polymer libraries that were previously synthesized and characterized in-house. For the first library, PEI2 was N-acylated with lipids of varying carbon chains: caprylic acid (CA), myristic acid (MA), palmitic acid (PA), stearic acid (SA), oleic acid and (OA) and linoleic acid (LA), as originally described in [18]. In brief, lipid chlorides individually dissolved in 1 mL of DMF were added drop-wise to 100 mg of PEI in 1 mL of DMSO. Three different lipid:PEI amine ratios (0.066, 0.1 and 0.2) were used during synthesis to control the level of substitutions. After 24 h at room temperature under argon, polymers were precipitated and washed with excess ethyl ether and vacuum-dried at room temperature. The polymers were analyzed by ^{1}H-NMR in D_2O using the characteristic proton shifts of lipids (δ ~ 0.8 ppm; -C$\underline{H}_3$) and PEI (δ ~ 2.5–2.8 ppm; NH-C$\underline{H}_2$-C$\underline{H}_2$-NH-) to calculate the lipid substitution levels. **Table 2.S1** provides a summary of the degree of lipid substitutions, also published in Reference [18].

For the second library, PA substitutions were performed on 0.6, 1.2 and 2 kDa PEIs [20]. Briefly, 60 mg of PEI0.6, 120 mg of PEI1.2 and 200 mg of PEI2 were dissolved individually in 200 mL of chloroform and 160 µL of 1.15 mmol of triethylamine was added. Three different amounts of palmitoyl chloride were added drop-wise to each polymer solution and stirred at room temperature for 12 h. The final products of the three different lipid:PEI amine feed ratios (1, 2 and 4) used for each were precipitated and washed with excess ethyl ether. Based on ^{1}H-NMR analysis, **Table 2.S2** provides a summary of degree of lipid substitutions on these polymers, also published in Reference [20].

2.2.3 CML Cell Model

K562 cells, a *BCR-ABL* positive cell line established from a CML patient in blast crisis [21], was used as the CML model. A GFP-expressing K562 cell line (GFP-K562)

was generated by transduction of cells with a retroviral vector containing the Green Fluorescent Protein (GFP) gene [22] and used as the silencing model due to convenience of assessing GFP silencing. K562 and GFP-K562 cells were maintained in RPMI medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin under incubation (37 °C, 5% CO_2). Every third day in culture, spent medium was removed by centrifugation (600 rpm, 5 min) and cells were diluted 10 times (or 1 x 10^6 cells) in 25 mL of fresh medium for cell expansion or seeded at 1 x 10^5 cell/mL in multi-well plates one day before prior testing. MDA-MB-231 cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and maintained at 37 °C, 5% CO_2. When the cells were confluent (~80% of the plate surface covered), they were de-attached from the surface by HBSS-washing and a 5-minute incubation with Trypsin/EDTA at room temperature. The suspended cells were then collected by centrifugation and sub-cultured at a 10% concentration of the original count.

2.2.4 siRNA/Lipid-Modified Polymer Complex Preparation

For the preparation of siRNA/polymers complexes, an aliquot of siRNA stock solution (10 µM in RNAse-free water) was first dissolved in a 150 mM NaCl solution in polypropylene sterile tubes. Typically, ~0.25 µg of siRNA was added to 150 mM NaCl for a final siRNA concentration of 36 nM in cell suspension. The polymers (dissolved at 1 mg/mL in ddH_2O) were then added to the siRNA solution to give the desired polymer:siRNA weight ratios (2:1, 4:1, 8:1 and 12:1), bringing the final volume to 60 µL. After a 30-min incubation at room temperature, complexes (20 µL/well) were added in triplicate to the cells seeded on 24-well plates one day before. A similar procedure was used to prepare the Lipofectamine® 2000 complexes, except that Lipofectamine® 2000 was diluted separately and then mixed with the siRNA solution at specific siRNA:carrier ratio; the buffer used for Lipofectamine® 2000 complexes preparation was either 150 mM NaCl or Opti-MEM (the recommended medium for Lipofectamine® 2000

formulations). The 2:1, 4:1, 8:1 and 12:1 polymer:siRNA ratios used for polymer complexes corresponded to 13.3:1, 26.7:1, 53.3:1 and 80.1:1 N:P ratio, respectively (assuming 43 Da for PEI single unit, 22 bp for siRNA with 2 phosphates per base pair).

For silencing studies with PA-based polymers, complexes were prepared as described above but the siRNA and polymers were diluted in 300 µL (triplicate) of RPMI medium. 100 µL of complex solution were added to empty wells and a cell suspension of 100,000 cells in 500 µL of complete medium was added on the top of the well with the complexes.

2.2.5 Delivery of siRNA to K562 Cells

One day prior to transfection, 0.5 mL of K562 cells (at 1 x 10^5 cells/mL) was seeded in 24-well plates. K562 cells were transfected with complexes prepared with FAM-labeled and non-labelled scrambled siRNA (as negative control) as described above. At the indicated times, cells were transferred to tubes, centrifuged (1400 rpm for 5 min), washed twice with HBSS and re-suspended in a fixed volume of 3.7% formalin. The cell-associated FAM-siRNA was quantified by flow cytometry (Cell Lab Quanta SC; Beckman Coulter) using the FL1 channel and calibrating the instrument so that the negative control (i.e. no-treated cells) gave ~1% of positive cells as the background. siRNA delivery in K562 cells was determined by measuring the mean fluorescence of cells and percentage of FAM-siRNA positive cells. The cell concentration of the total population was also determined.

2.2.6 GFP Silencing in GFP-K562 Cells

Silencing effect of complexes was evaluated by quantifying the reduction of the GFP fluorescence in the GFP-K562 cells. Complexes were prepared with scrambled (as negative control) and GFP siRNA at the indicated siRNA concentrations and polymer:siRNA ratios. At the indicated time after transfection, cells were transferred to

tubes, centrifuged (1400 rpm, 5 min), washed twice with HBSS and re-suspended in 3.7% formalin. GFP silencing as well as cell concentration were assessed by flow cytometry using the FL-1 channel. The GFP-expressing cell population is shown on the third quadrant under the FL1+ region of the histograms, while the GFP-negative cell population can be seen shifted towards the left when there is a silencing effect. Percent decrease in mean fluorescence was calculated as follows: 100-([Mean FL1 of cells treated with GFP siRNA/polymer]/[Mean FL1 of cells treated with scrambled siRNA/polymer] x %). Percent decrease in GFP-positive cells was calculated as follows: [% of GFP-negative cells of cells treated with GFP siRNA/polymer] - [% of GFP-negative cells of cells treated with scrambled siRNA/polymer].

A comparison of the transfection efficiency between lipid-modified polymers and commercial transfection reagents (PEI25, Lipofectamine™ 2000, Lipofectamine™ RNAiMax, DOTAP, Metafectene, Interferin, Ibofect and Fugene HD) was also performed. Twenty-four hours prior to transfection, 15,000 cells were seeded in 100 µL of complete medium in 96-well plates. On the day of the transfection, complexes were prepared following the manufacturer's instructions as closely as possible using GFP-siRNA and scrambled siRNA as it follows: first, the siRNA and the carriers were diluted in 75 µL Opti-MEM separately to make carrier:siRNA weight ratios of 2:1, 4:1 and 8:1 and a final siRNA concentration of 24 nM. After a 5-min incubation of the reagents in OptiMEM, siRNA and reagents solutions were mixed by pipetting few times, except Interferin that was vortexed. PEI25 complexes were incubated for 30 min, Lipofectamine® 2000, Lipofectamine® RNAiMax, DOTAP, Metafectene, Interferin were incubated for 20 min and Fugene HD was incubated for 15 min prior to drop-wise addition (50 µL) of complex solution to cells. The differences in complexation protocols (as well as relative ratios of carrier:siRNA) were due to differences in recommendations of each manufacturer, which was adopted as closely as possible rather than re-optimizing the formulations to match

the polymer formulations developed in this study. GFP silencing was assessed by flow cytometry 72h post-transfection as described above.

2.2.7 Apoptosis Analysis

For the apoptosis assay, K562 cells were seeded 24 hours prior to transfection on 24-well plates at a 1 x 10^5 cells/mL. On the day of the transfection, complexes were prepared with control siRNA and a mixture of three BCR-ABL siRNAs at 50 and 100nM (one third of each siRNA) and PEI1.2-PA (1.98 PA per PEI) with polymer:siRNA ratio of 4:1. After 1, 2 and 3 days after transfection, apoptosis was assessed by Annexin kit following the manufacturer's protocol. For this, cells were collected in tubes, washed twice with cold HBSS and, aliquots of 1 x 10^5 cells diluted in 100 µL of 1X were incubated in dark for 15 min at room temperature with 5 µL of FITC-Annexin V and 5 µL of Propidium Iodide (PI). This solution was dissolved in 400 µL of 1X Binding Buffer and analyzed with a BD LSR Fortessa flow cytometer (Franklin Lakes, NJ) (Flow cytometry facility, University of Alberta).

2.2.8 BCR-ABL Silencing in K562 cells

K562 cells seeded on 6-well plates in 2.5 mL of complete medium were treated with complexes prepared with control and BCR-ABL siRNA (catalog number: ABP-Ri-VAsi-D09 from Allele Biotechnology) at a polymer:siRNA weight ratio of 4:1 and a 100 nM siRNA concentration. Twenty-four and 48 hours after transfection, levels of *BCR-ABL* were assessed at the mRNA level. First, treated cells were transferred to tubes, washed twice with HBSS and the total RNA was extracted using the RNAeasy Mini Kit following manufacturer's instructions except that 2-mercaptoethanol was omitted from the extraction. The integrity of the RNA extracted was then checked by spectrophotometry (GE Nanovue). For each sample, 500 ng of RNA were then reverse-transcribed with M-MLV reverse transcriptase, following the manufacturer's instructions. Oligo (dT) as well as random primers were used for the cRNA synthesis [23], [24]. Finally, for real-time

PCR analysis, 2X SYBR green master mix with ROX (MAF Center, University of Alberta) was used to follow the fluorescence intensity. Specific forward and reverse primers used to detect expression levels are the following: beta-actin (housekeeping endogenous gene): 5'-CCA CCC CAC TTC TCT CTA AGG A-3' and 5'-AAT TTA CAC GAA AGC AAT GCT ATC A- 3'[23], BCR-ABL: 5'-CAT TCC GCT GAC CAT CAA TAA G-3'; 5'-GAT GCT ACT GGC CGC TGA AG-3' [22]. A 10 µL volume containing 5 µL of 2X master mix SYBR Green, 2.5 µL of 3.2 µM primer and 2.5 µL of cDNA (10 ng/µL) template for each sample in triplicate were transferred to a Fast Optical 96-well plate. Using an Applied Biosystems StepOnePlus instrument, reaction mixtures were heated for 2 min at 95 °C before going through 40 cycles of a denaturation step (15 sec at 95 °C) and an annealing/elongation step (1 min at 60 °C). Analysis to determine differences in gene expression was performed by $2^{-\Delta\Delta CT}$ method using the no-treatment groups as the calibrator. *BCR-ABL* C_T was normalized against Beta-actin C_T and the results are expressed as relative quantity of the targeted mRNA.

2.3 RESULTS AND DISCUSSION

A library of PEI2 polymers modified with CA, MA, PA, SA, OA, and LA at different lipid substitutions was previously described in Reference [18] and **Table 2.S1**. During the synthesis, the lipid:PEI mole ratio was controlled so as to control the extent of lipid substitutions (determined by ^{1}H-NMR), which was generally increased with increasing lipid:PEI ratio. Similarly, a library of PA-substituted PEIs was previously described before [18]-[20] where the PA substitution was again controlled by the lipid:PEI ratios during the synthesis. The resultant polymers were readily dissolved in water, which made them suitable for siRNA complexation under aqueous conditions. A variability in siRNA delivery efficiency of lipid-modified polymers was previously reported among anchorage-

dependent cell lines in References [19], [25]-[27], letting us to believe that the most effective lipopolymer needed to be tailored for an individual cell type. In the absence of previous experience with CML cells, we first set out to determine the delivery and silencing efficiency of lipid-substituted PEI2 polymers.

2.3.1 Comparison of siRNA Delivery to K562 and MDA-MB-231 using Lipid-Substituted PEI2s

Since lipid-modified PEI2 mediated effective delivery of siRNA to attachment-dependent MDA-MB-231 breast cancer cells [28], we first compared siRNA delivery efficiency to K562 and MDA-MB-231 cells head-to-head in this study. The siRNA delivery with the lipid-modified polymers was substantially higher in MDA-MB-231 cells: siRNA delivery was >45-fold greater than the no carrier (siRNA alone) group with most of the polymers (**Figure 2.1Ai**). As in previous results in Reference [28], PEI2LA again gave the highest delivery in MDA-MB-231 cells (mean delivery ~400-fold greater than no carrier group). Although siRNA delivery to K562 cells was lower, PEI2LA again had the highest delivery efficiency in these cells (mean delivery >29-fold greater than no carrier group; **Figure 2.1Ai**). Almost all MDA-MB-231 cells were positive for the siRNA delivered with lipid-substituted PEIs (>85% with most effective polymers), while the highest levels of siRNA-positive K562 cells were obtained only with PEI25 and PEI2LA polymers (~76% and 67%, respectively) (**Figure 2.1Aii**). A strong correlation in siRNA cellular delivery was evident between MDA-MB-231 and K562 cells (**Figure 2.1B**; $r = 0.843$, based on mean fluorescence values from **Figure 2.1Ai**). Although this is suggestive of the similar performance of each polymer in both cell types, the difference was more evident in the absolute quantities of siRNA delivered (**Figure 2.1Ai**).

We next explored the influence of cell density on siRNA delivery to K562 cells. Cells were seeded at initial densities of 0.4, 0.7, 1, 1.5 and 2 x 10^5 cells/mL and treated with siRNA complexes for 24 hours (**Figure 2.1C**). Although there was a trend of decreasing

siRNA delivery with increasing cell concentration for two of the polymers (PEI25 and PEI2LA), these trends were not strong enough to lead to a significant change in siRNA delivery. It was conceivable that delivery of siRNA to K562 cells was limited due to their higher cell density in these cells (since they undergo rapid proliferation) as compared to MDA-MB-231 cells, but this did not appear to be the reason for the reduced level of siRNA delivery to K562 cells.

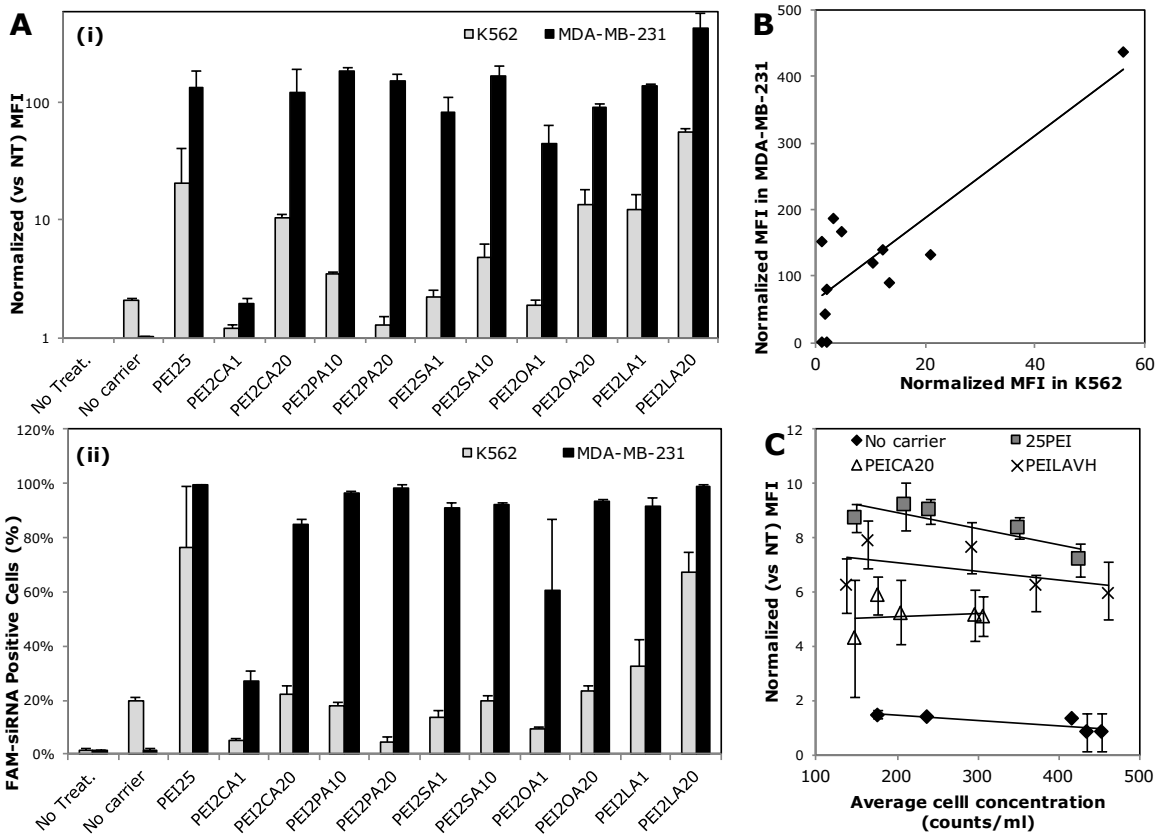

Figure 2.1. Cellular uptake of polymer/siRNA complexes in K562 and MDA-MB-231 cells. (**i**) Mean fluorescence intensity (MFI) of treated cells normalized against MFI of non-treated (NT) cells after 24 h exposure to complexes prepared with FAM-labeled siRNA and the indicated polymers. Two levels of substitutions were used for each lipid, and complexes were prepared at a polymer:siRNA ratio of 8:1 and added to cells at siRNA concentration of 36 nM. (**ii**) Percentage of FAM-siRNA positive K562 and MDA-MB-231 cells after 24 h exposure to complexes. Non-treated cells were set at 1% positive cell as a background. **B**) Correlation of siRNA cellular delivery between K562 and MDA-MB-231 cells with a correlation coefficient of 0.843 (based on MFI values from

(**Ai**); p = 0.001). **C**) Effect of cell seeding density on MFI of K562 cells exposed to FAM-siRNA complexes for 24 h (polymer:siRNA ratio of 8:1 and 36 nM siRNA concentration). Average of final cell densities counted by flow cytometer were plotted against the normalized MFI.

The difficulty of delivering nucleic acids to suspension growing cells is generally appreciated in the field [29]-[32]. It is likely that the interaction of the complexes with the adherent cells is facilitated by the monolayer formation by these cells; once the complexes settle to the bottom of tissue culture plates, they have a larger cell membrane area to which they can interact with. In contrast, the interaction of complexes with suspension cells could be reduced since both components are in suspension and electrostatic charges between the complexes and cells might not be strong enough for tight binding and subsequent endocytosis [29]. The increased gene delivery efficiency in adherent cells in comparison with suspension cells was also explained by the interaction of cationic particles with the components of the cell membrane involved in cell anchoring to the extracellular matrix, which are absent in suspension cells [29], [33]. For this reason, suspension cells such as hematopoietic and T lymphocytes cells have been made to adhere to a monolayer of cells to improve the liposomal-mediated transfection efficiency [29].

Previous studies in Reference [19], [23] indicated that LA-substitution sustained most silencing among the lipid-substituted PEI2s for down-regulation of GAPDH and P-glycoprotein expression in MDA-MB-435 multidrug resistant cells [19], as well as GFP and CXCR4 in acute myeloid leukemia cells (THP-1) [23]. Combined with the fact that LA substitution again gave the most siRNA delivery to K562 cells, this polymer was further investigated for silencing efficiency in K562 cells.

2.3.2 GFP silencing with PEI2 polymers and Lipofectamine® 2000 in K562 cells

The silencing efficiency of siRNA delivered with PEI2LA was assessed in GFP-K562 cells (**Figure 2.2**). The GFP silencing was evaluated after 48 and 72 h post-transfection by comparing complexes prepared in either Opti-MEM or 150 mM NaCl at a carrier:siRNA ratio of 8:1 and at 36 nM siRNA concentration. The carriers additionally used were the unmodified PEI2, PEI25 and Lipofectamine® 2000. The latter was chosen as it was found to be among the most effective commercial reagents tested for GFP-silencing in GFP-K562 cells at a range of carrier:siRNA ratios (2:1, 4:1 and 8:1) and using a final siRNA concentration of 24 nM (**Figure 2.S1**). The range of carrier:siRNA ratios used in this initial screening fell into the manufacturer's suggestion for optimization of the reagents. Lipofectamine® 2000 was chosen since, in addition to being most effective in this study (along with PEI25), it is widely used in the field and it has a relatively lower cost in comparison to another highly efficient carrier, Lipofectamine® RNAiMAX.

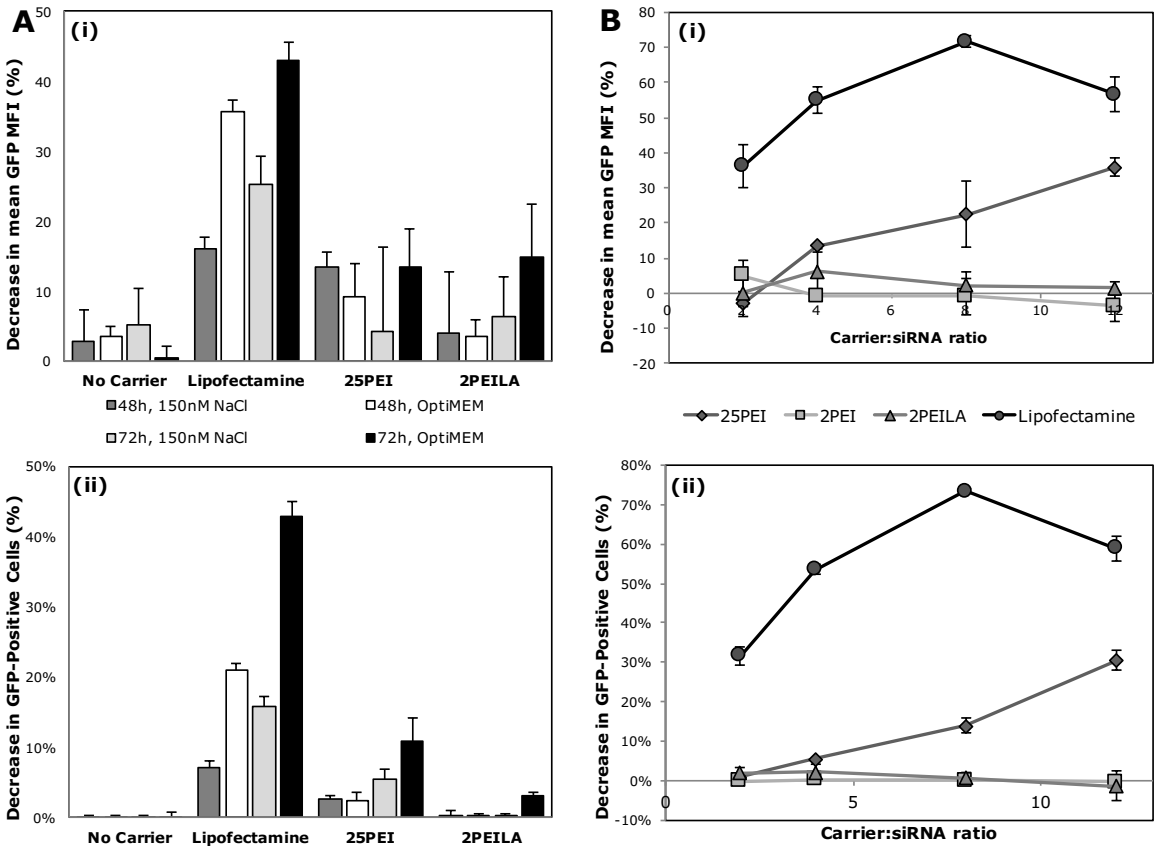

Figure 2.2. GFP silencing in GFP-K562 cells after 48 and 72 hours of siRNA treatment. A) GFP silencing of complexes prepared in 150 mM NaCl and Opti-MEM and presented as (**i**) decrease in mean GFP fluorescence and (**ii**) decrease in GFP-positive cell population. The carriers used were Lipofectamine® 2000, PEI25 and PEI2LA (2.1 LA/PEI) at a carrier:siRNA ratio of 8:1 (36 nM siRNA concentration). **B**) GFP silencing in GFP-K562 cells treated with PEI25, PEI2, PEI2LA and Lipofectamine® 2000 at carrier:siRNA ratios of 2:1, 4:1, 8:1 and 12:1 (36 nM siRNA concentration). GFP silencing was analyzed by flow cytometry 72 hours after siRNA treatment and summarized as decrease in mean GFP fluorescence (**i**) and decrease in GFP-positive cell population (**ii**).

The GFP silencing was summarized both as a percent decrease in mean GFP fluorescence (**Figure 2.2Ai**) and the decrease in proportion of GFP-positive cells (**Figure 2.2Aii**). Since control (scrambled) siRNA generated a minor (insignificant) change in the GFP fluorescence distinct for each carrier (see **Figure 2.S2** for this data from day 3 assessment), silencing in cells treated with GFP-specific siRNA complexes was normalized against the control siRNA complexes. PEI2LA was found to have a small effect in decreasing the mean GFP fluorescence (<10% at both time points), while PEI25-delivered siRNA reduced the GFP expression by ~10% after three days of transfection. PEI25 appeared to perform better than PEI-LA based on the reduction of GFP-positive cells (**Figure 2.2Aii**). No significant changes were found in GFP silencing with complexes prepared with 150 mM NaCl and Opti-MEM (for PEI25 and PEI2LA) after two days of transfection (**Figure 2.2Ai**). Since the silencing with complexes prepared in Opti-MEM was only increased by a minor amount (both for PEI25 and PEI2LA at 72 h post-transfection; **Figure 2.2Aii**), we decided to prepare the complexes with 150 mM NaCl in subsequent studies. On the other hand, Lipofectamine® 2000 gave a significant reduction in GFP (as much as 43% in mean GFP fluorescence), which was higher for the complexes prepared in Opti-MEM. The GFP silencing was also increased after 72 h post-transfection (from 16% to 25% for 150 mM NaCl vs. from 36% to 43% for Opti-MEM; **Figure 2.2Ai**). The stronger silencing effect for Lipofectamine® 2000 was also evident in the changes in the percentage of GFP-positive cells after 48 and 72 h post-transfection (from 21% to 43% for Opti-MEM **Figure 2.2Aii**).

To evaluate whether the silencing efficiency of PEI2LA could be increased, the carrier:siRNA ratio was next varied since this ratio influenced the silencing efficiency of the complexes in other cell types [34], [35]. While keeping siRNA concentration constant (36 nM), the varying of carrier:siRNA ratio was found to affect the efficiency of PEI25 and Lipofectamine® 2000 (**Figure 2.2B**). Complexes prepared with PEI25 led to increasing GFP silencing at increasing carrier:siRNA ratio, based on both mean decrease in GFP fluorescence and GFP-positive cell population (**Figure 2.2Bi** and **2.2Bii**), ultimately reaching to a ~30% silencing (based on mean GFP Fluorescence) with ratio 12:1 (**Figure 2.2Bi**). However, the concentration of PEI25-treated cells was reduced at this ratio to ~78% in comparison with no-treated cells (from flow cytometry; not shown), indicating the cytotoxicity of this carrier. With Lipofectamine® 2000, GFP silencing reached its peak level at the carrier:siRNA ratio of 8:1 (~70% silencing) with no apparent changes in cell concentration (not shown). PEI2 and PEI2LA polymers gave insignificant silencing at the ratios evaluated (**Figure 2.2Bi** and **2.2Bii**).

The less than optimal performance of LA-substituted PEI2 was surprising since this polymer previously showed effective silencing in several attachment-dependent cells in previous studies [19], [28], [36], [37] as well as in the acute myeloid leukemia THP-1 cells [23]. The low levels of siRNA delivery observed to K562 cells with respect to attachment dependent cells might partly explain this result. The possibility of K562 cells not being conducive to siRNA-mediated silencing was also considered (e.g., due to low RISC complex), but the fact that Lipofectamine® 2000 provided effective silencing argues against this possibility. To better explore the relationship between siRNA delivery and silencing efficiency, a more detailed comparison of PEI2LA against Lipofectamine® 2000 was next conducted.

2.3.3 Correlating siRNA Delivery to GFP Silencing

The comparison of siRNA delivery and GFP silencing efficiencies was performed

with wild-type K562 and GFP-K562 cells (**Figure 2.3**). PEI2LA, PEI25 and Lipofectamine® 2000 carriers were used for this purpose to prepare the complexes at an increasing range of siRNA concentrations (36, 72 and 140 nM). A general increase in siRNA delivery was evident for all carriers at increasing siRNA concentration, with Lipofectamine® 2000 showing the highest siRNA delivery (**Figure 2.3Ai** and **2.3Aii**). A positive correlation between the siRNA delivery and GFP silencing was found for PEI25 and Lipofectamine® 2000: i.e., increasing delivery resulted in increasing GFP silencing (**Figure 2.3A** and **2.3B**). Although PEI25 was effective in silencing (~40% decrease in mean GFP fluorescence and ~25% in GFP-positive cell population at 140 nM siRNA; **Figure 2.3Bi** and **2.3Bii**), a significant (~50%) decrease in viable cell concentration was evident in both delivery (**Figure 2.3Aiii**) and silencing studies (**Figure 2.3Biii**), again indicating the high cytotoxicity induced by this polymer. The cytotoxicity of Lipofectamine® 2000 was less than the one of PEI25 (**Figure 2.3Aiii** and **2.3Biii**). Although the siRNA delivery efficiency of PEI2LA was nearly as high as the PEI25 (**Figure 2.3Ai** and **2.3Aii**), the GFP silencing efficiency was considerably lower (**Figure 2.3Bi** and **2.3Bii**). No major changes in cell concentration were found with PEI2LA (**Figure 2.3Aiii** and **2.3Biii**), consistent with the previously published compatibility of this polymer with different cell types [19], [23].

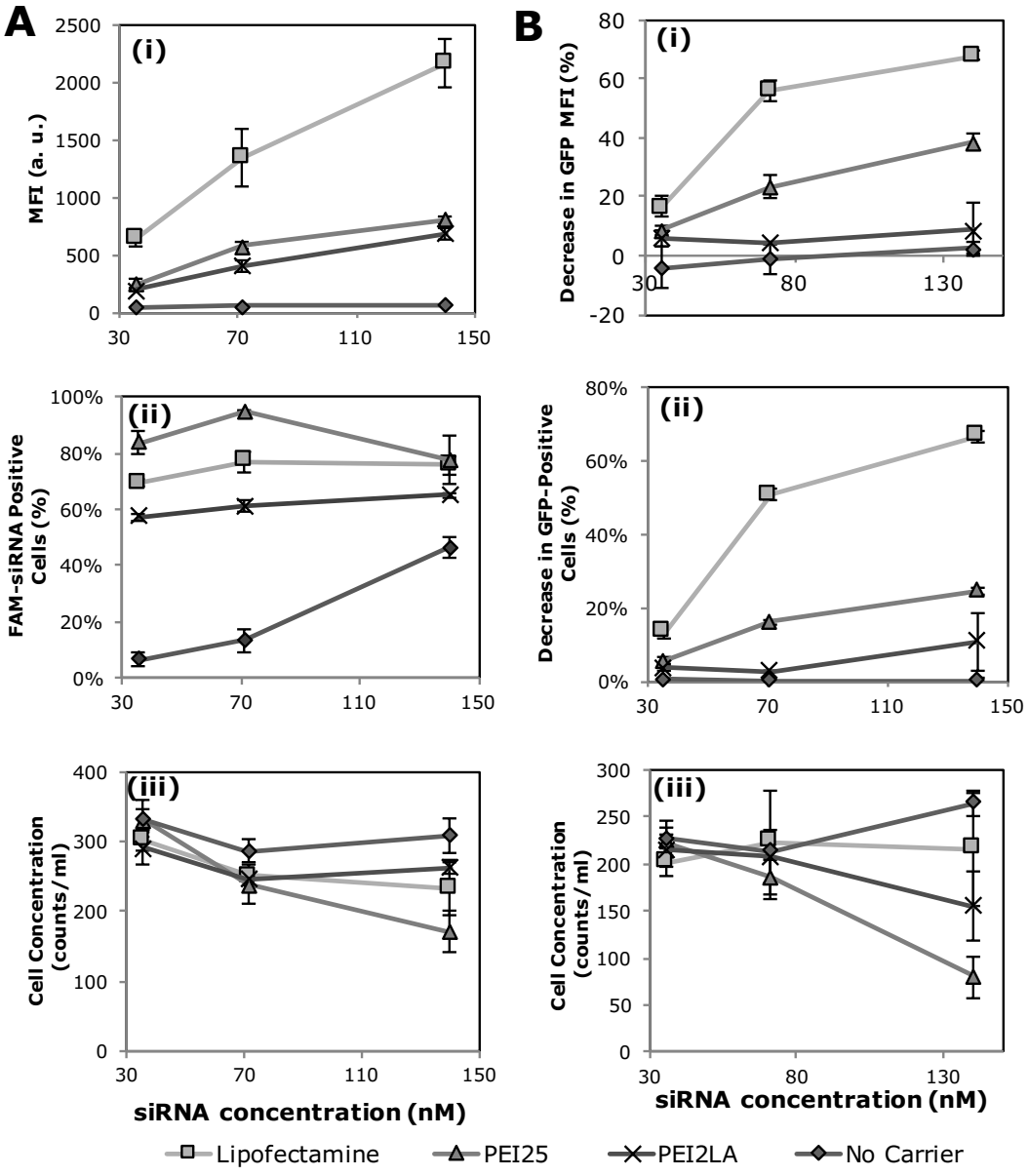

Figure 2.3. FAM-siRNA uptake in K562 cells and GFP silencing in GFP-K562 cells. The cells were exposed to siRNA complexes for 24 h (for uptake studies in **A**) or 72 h (for GFP silencing in **B**). The complexes were prepared with FAM-siRNA (**A**) and GFP-siRNA (**B**), and exposed to cells at siRNA concentrations of 36, 72 and 140 nM. A carrier:siRNA ratio of 4:1 was used for 25PEI and PEI2LA (2.1 LA/PEI) complexes, and 2:1 for Lipofectamine® 2000 complexes. Cell concentrations obtained after the siRNA treatment periods, as determined by the counts from flow cytometry, are summarized in (**iii**) in **A** and **B**.

These results indicate that increasing siRNA delivery to K562 cells with PEI2LA did not necessarily lead to GFP silencing. This was unlike Lipofectamine® 2000, which gave the most significant siRNA delivery as well as the silencing efficiency. It is likely that other barriers exist to the successful silencing in the case of PEI2LA. Among the likely reasons are a poor binding of the complexes to the cell membrane that does not promote endocytosis [38], endosomal entrapment followed by lysosomal degradation in cytoplasm [17], and/or a lack of dissociation of the siRNA from the complex due to tight binding of the siRNA molecules to the polymer backbone [34], [39]. Since Lipofectamine® 2000 is a small cationic lipid (exact structure not disclosed by the manufacturer) that forms relatively large and less tight complexes with nucleic acids [26] for a better siRNA release, we next explored a readily available library of PA-modified PEI polymers of lower MW to evaluate whether the lipid modification on 0.6 and 1.2 kDa PEI can delivery siRNA more efficiently in K562 cells than the 2 kDa counterpart.

2.3.4 GFP Silencing with PA-modified PEIs in K562 cells

A library of PA-substituted PEIs was next explored, which was constructed by using PEIs of different backbones (0.6 vs. 1.2 vs. 2.0 kDa; [20], **Table 2.S2**). The lower MW PEIs were particularly appealing to us since the small molecular lipid Lipofectamine® 2000 (structure and molecular weight not known to us) was effective in GFP silencing in K562 cells, as shown in **Figure 2.3**. Previous studies with this library indicated that plasmid DNA delivery to immortal 293T cells was achievable upon PA substitution on the ineffective native polymers as described in Reference [20]. The higher MW PEIs (1.2 and 2.0 kDa) performed generally better in plasmid DNA delivery and transgene expression, since they afforded a higher degree of PA substitution (up to 2 for 1.2 kDa PEI and up to 3 for 2 kDa PEI) as compared to 0.6 kDa PEI (<0.5 PA per PEI). The ζ-potentials of complexes prepared with these PA-modified polymers were found to be strongly positive

and higher than those of unmodified PEI25 complexes [20]. However, these polymers were not previously evaluated for siRNA delivery.

For GFP silencing efficacies of PA-modified PEIs in GFP-K562 cells, the complexes were formed at a polymer:siRNA ratio of 8:1 and the cells were treated at a 72 nM siRNA concentration for 3 days. As expected, PEI25 showed ~54% decrease in mean GFP fluorescence (**Figure 2.4Ai**) and ~40% in percentage of GFP-positive cells (**Figure 2.4Aii**). However, this was accompanied by a severe reduction in cell concentration (~22% of non-treated cells, **Figure 2.4Aiii**). Among the PA-substituted PEIs, the PEI1.2PAIII (with a lipid substitution of 1.98 PEI/PA) stood out in GFP silencing, in that it showed a ~63% decrease in the mean GFP fluorescence (**Figure 2.4Ai**) and a ~55% decrease in GFP-positive cells (**Figure 2.4Aii**), while giving a milder effect on cell concentration in comparison with PEI25 (~62% of non-treated cells, **Figure 2.4Aiii**). The rest of the polymers had <30% decrease in mean GFP fluorescence and <20% decrease in GFP-positive cells (**Figure 2.4Ai** and **2.4Aii**). The extent of GFP silencing (based on mean GFP values) was correlated to the extent of lipid substitution for each type of PEI; a positive correlation was evident between the extent of PA substitution and GFP silencing (**Figure 2.4B**). Among the three different MW PEIs evaluated, the GFP silencing was more sensitive to PA substitution for smaller PEIs, as compared to the 2 kDa PEI, which silencing efficiency was not as strong as the lower MW PEI at equivalent PA substitution.

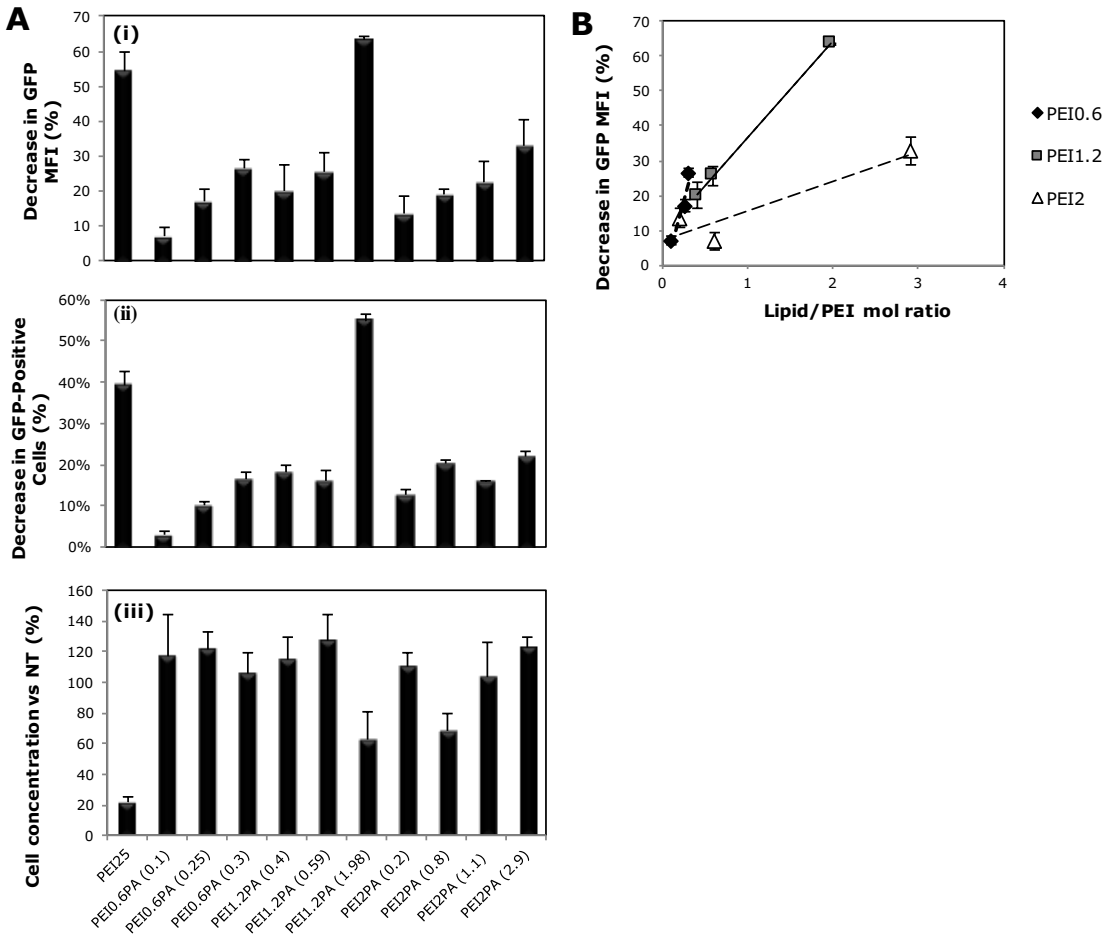

Figure 2.4. GFP silencing in GFP-K562 cells with PA-substituted polymers. Reduction in GFP MFI (**Ai**), percent decrease in the GFP-positive population (**Aii**) and cells concentration/mL (as a percentage of non-treated cells (NT); **Aiii**) were assessed by flow cytometry 3 days after siRNA treatment. The complexes were prepared at a polymer:siRNA ratio of 8:1 and used at 72 nM GFP-siRNA concentration. **B**) Correlation between the percent decrease in GFP MFI (data from **Ai**) and the extent of lipid substitution based on the number of lipids per PEI (shown in parenthesis in the polymer labels of **Aiii**). Correlation for each MW PEI is shown separately.

A more detailed comparison of the GFP silencing of PEI1.2PAIII with PEI25 and Lipofectamine® 2000 was further evaluated (**Figure 2.5**). The siRNA concentrations of 50 (**Figure 2.5A**) and 100 nM (**Figure 2.5B**) were tested using low and high carrier:siRNA ratios of 4:1 and 8:1 for the polymers and 2:1 and 4:1 for Lipofectamine® 2000. At 50 nM siRNA and high ratio (8:1 for PEI1.2PAIII with PEI25 and 4:1 for

Lipofectamine® 2000) (**Figure 2.5A**), PEI25 showed a ~34% decrease in the mean GFP fluorescence and ~16% decrease in GFP-positive cells; in contrast, PEI1.2PAIII and Lipofectamine® 2000 had similar performances, giving 61% and 60% decrease in the mean GFP fluorescence (**Figure 2.5Ai**), and 52% and 58% decrease in GFP-positive cells (**Figure 2.5Aii**), respectively. The cell concentration under the same conditions was decreased to ~55% in comparison to non-treated group for the three carriers (**Figure 2.5Aiii**). At the low ratio (4:1 for PEI1.2PAIII with PEI25 and 2:1 for Lipofectamine® 2000), the decrease in mean GFP fluorescence at 50 nM siRNA was increased when a 100 nM concentration was used: going from 8.3% to 41.4% with PEI1.2PAIII, from ~21.3% to ~34% with PEI25 and from ~38% to ~61% with Lipofectamine® 2000 (**Figure 2.5Ai** and **2.Bi**). As expected, as the GFP silencing levels increased from 50 to 100 nM at the low ratio, an opposite effect was found in the cell concentration: going from a cell concentration of ~90% in comparison to no-treated cells at 50 nM for the three carriers (**Figure 2.5Aiii**) to cell concentrations of ~67% for PEI1.2PAIII, ~53% for PEI25 and ~73% for Lipofectamine® 2000 when 100 nM of siRNA were used (**Figure 2.5Biii**). Finally, when the high ratios (8:1 for polymers and 1:4 for Lipofectamine® 2000) were used at 100 nM siRNA, the decrease in mean GFP increased to ~75% with the three carriers (**Figure 2.5Bi**) Again, a decrease in cell concentration associated with high GFP silencing effect was seen: the cell concentration after Lipofectamine® 2000 treatment was decreased to ~50%; however, the cell concentration with PEI1.2PAIII and PEI25 polymers was decreased severely (to ~1% for both polymers, **Figure 2.5Biii**) in comparison with non-treated cells. These results show that siRNA concentration as well as carrier:siRNA ratio are key factors for optimization of the transfection in order to find a balance between silencing efficacy and toxicity for each of the carriers.

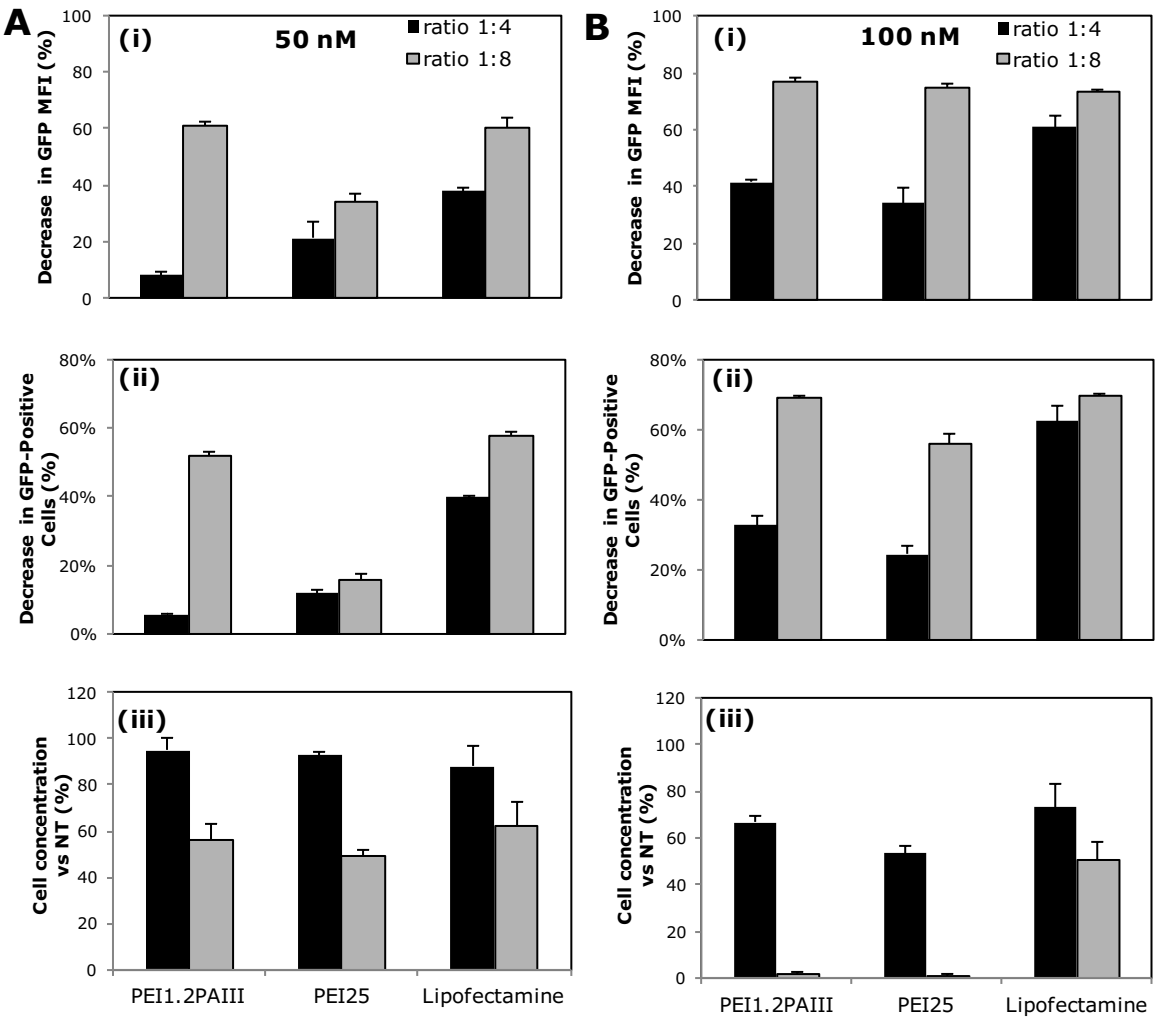

Figure 2.5. GFP silencing in GFP-K562 cells transfected with PEI1.2PAIII and the commercial reagents. GFP silencing in GFP-K562 cells transfected with PEI1.2PAIII (1.98 PA/PEI) and the commercial reagents PEI25 and Lipofectamine® 2000 at siRNA concentrations of 50 nM (**A**) and 100 nM (**B**). Low carrier:siRNA ratios corresponds to 4:1 for PEI1.2PAIII and PEI25, and to 2:1 for Lipofectamine® 2000. High carrier:siRNA ratios corresponds to 8:1 for PEI1.2PAIII and PEI25, and to 4:1 for Lipofectamine® 2000. Percent decrease in mean GFP MFI (Ai and Bi), percent decrease in GFP-positive population (**Aii** and **Bii**) and cell concentration/mL (as percentage of non-treated cells (NT; **Aiii** and **Biii**) were assessed by flow cytomentry 3 days after transfection.

The general relationship between the extent of lipid substitution and delivery efficiency for nucleic acids has been reported for a number of polymers [18]-[20], [23], [40]. This relationship has been particularly well studied in our hands with PEI2 polymers

for siRNA delivery in attachment dependent cells [19], [23]. The role of lipid substituent was previously explored by using different lipids, where we noted longer lipids (e.g., LA) to be more effective for silencing certain targets/cells (e.g., P-gp in MDA-MB-435 cells) as described in Reference [19], whereas shorter lipids (e.g., CA) were more effective for other targets/cells (e.g., survivin in MDA-MB-231 cells) as described in Reference [28]. We now report that lower MW backbone will be more beneficial for suspension-growing cells such as K562 cells. This outcome might be related to dissociation of complexes; as the MW of PEI backbone is decreased, we expect a better dissociation of complexes inside the cells. Another reason might be related to the strongly positive ζ-potentials of these complexes as it has been shown before with DNA [20] given that this could improve the cell membrane/complexes affinity and consequently allow the endocytosis. Further studies to better reveal the role of dissociation are currently underway. It was also interesting to note that when the current PA-substituted PEI library was used in plasmid DNA delivery, the most effective polymer identified here for siRNA delivery was also equally effective in sustaining transgene expression [20]). Modification of 0.6 and 1.2 kDa PEIs with other lipids such as LA, OA and CA could be also effective in K562 cells, but these polymers have not been synthesized/explored yet.

2.3.5 BCR-ABL Silencing in K562 cells and Apoptotic Response

Finally, we investigated the ability of PA-substituted polymers to induce apoptosis and to silence the endogenous target *BCR-ABL* product. For this, the cells were treated with control (cr-siRNA) and BCR-ABL specific siRNAs at 50 and 100 nM at a polymer:siRNA weight ratio of 4:1, and assessed the extent of apoptosis after 1, 2 and 3 days post-transfection (**Figure 2.6A**). A ratio of 4:1 was used for these experiments with the purpose of decreasing the cell toxicity due to the polymer at a ratio of 8:1 (as shown above in **Figure 2.4** and **2.5**). Based on Annexin-positive cells (**Figure 2.6Ai**), a significant elevation of early-apoptotic cell population was evident with 50 nM BCR-

ABL siRNA on day 2, and with 100 nM BCR-ABL siRNA on day 2 and day 3. Based on PI-positive cells (**Figure 2.6Aii**), a significant elevation of late-apoptotic cell population was evident with 100 nM BCR-ABL siRNA on day 2 and day 3. The silencing kinetics was investigated in a parallel study by using q-PCR (**Figure 2.6B**); compared to no treatment samples, treatment with control siRNA did not give any changes in the BCR-ABL mRNA levels on day 1 and day 2. However, BCR-ABL siRNA resulted in significant reduction of the corresponding mRNA on day 1 (~20%), after which the relative quantity of the specific mRNA became equivalent to other groups.

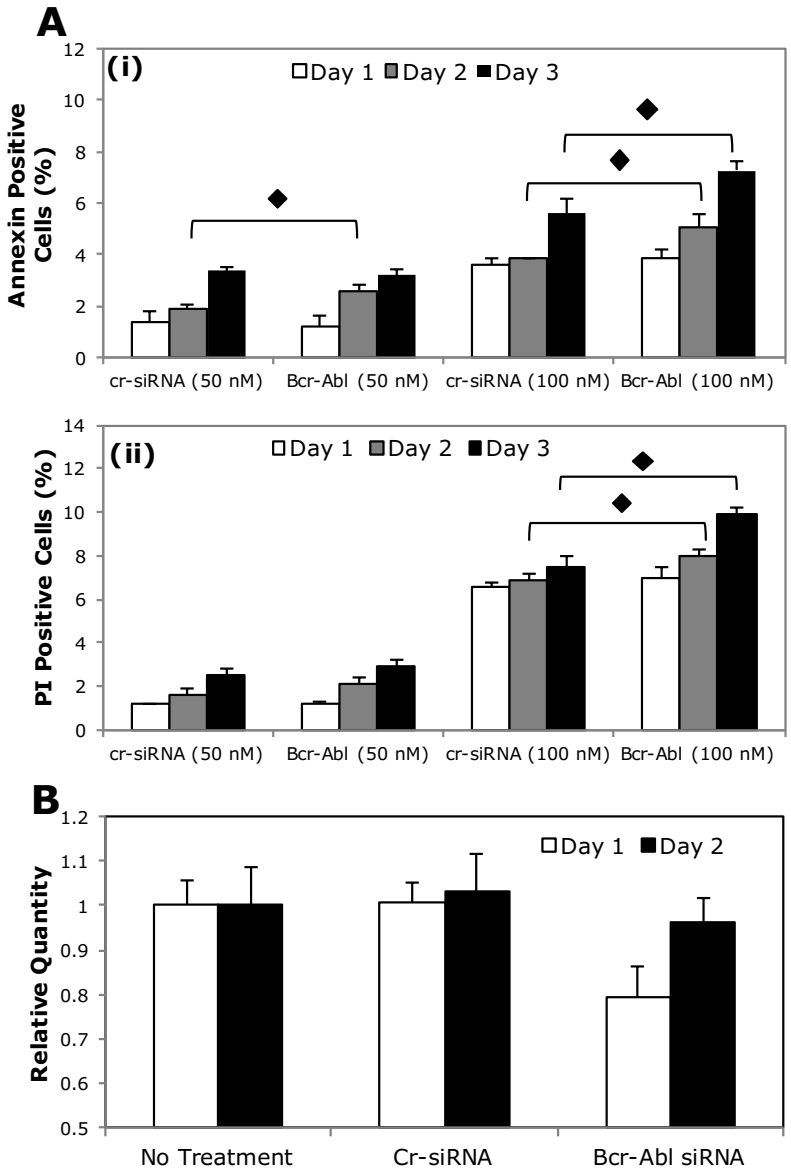

Figure 2.6. BCR-ABL-siRNA induced apoptosis and changes in BCR-ABL mRNA expression. A) FITC-Annexin V/PI staining of K562 cells exposed to PEI1.2PA (1.98 PA/PEI) complexes for 24, 48 and 72 hours. The siRNAs used were either control-siRNA (cr-siRNA) or a mixture of 3 BCR-ABL-siRNAs, with a polymer:siRNA ratio of 4:1 and final siRNA concentration of 50 and 100 nM. Percentage of Annexin-positive cells (i.e., early apoptotic population) was the sum of the percentage of Annexin+/PI- and Annexin+/PI+ populations (**Ai**). Percentage of PI-positive cells was the sum of percentage of the Annexin-/PI+ and Annexin+/PI+ populations (**Aii**). Diamond represents significant increase on early (**Ai**) or late (**Aii**) apoptosis ($p<0.05$). **B**) Quantitative PCR results showing BCR-ABL mRNA levels in K562 cells with no-treatment and cells treated with PEI1.2PA complexes of control siRNA (cr-siRNA) or BCR-ABL siRNA at a polymer:siRNA concentration of 4:1 and a final siRNA concentration of 100 nM. mRNA levels were quantitated 1 and 2 days after siRNA treatment.

A transient reduction of BCR-ABL mRNA levels was expected by siRNA delivery given that once these molecules bind and activate the RNA-induced silencing complex (RISC) in cytoplasm, its targeting and cleavage effect last only for a few days [11], [41], possibly due to siRNA degradation within the cell [17]. Several other studies have also targeted *BCR-ABL* in K562 cells mediating non-viral delivery, for example: (i) Whithey *et al.* achieved a 90% transfection efficiency and a 84% protein suppression 48 hours after having transfected the cells twice (with a 24 hours interval) using Oligofectamine™ (siRNA concentration not specified) [42]; (ii) Arthanari *et al.* used the Tat-LK15 peptide to deliver siRNA (71 to 2142 nM concentration by our calculation) and found a minimum of 70% reduction in $p210^{BCR-ABL}$ 48 h post-transfection for all concentrations, but no cell viability decrease was detected after 48 hours in comparison with control siRNA group [15]; (iii) Wilda *et al.* found that BCR-ABL mRNA levels of Oligofectanime-transfected cells were reduced to ~33% after 48 hours post-transfection, and that number of histone-associated DNA fragments (apoptosis induction) was at the same level in K562 cells treated either by RNAi or with Imatinib (1 μM) [10], and; (iv) Zhelev *et al.* used Lipofectamine-mediated transfection to deliver 3 different siRNAs (3 x 60 nM) every 2 days for a 6 day period and observed a reduction in BCR-ABL mRNA levels, $p210^{BCR-ABL}$ oncoprotein and proliferation capacity by 82%, 64% and 50% by the end of the treatment, respectively [43]. Our PCR studies showed an early decrease of BCR-ABL mRNA levels (24 hours post-transfection) and a biological response (apoptosis) to this silencing in K562 cells, albeit apparently at a lower dose and/or frequency of treatment. Although several carriers seem to be functional *in vitro*, it remains to be seen if they are all suitable for use in animal models. The polymeric carriers developed here, being fully described, could act as leads in this respect and offer possibilities for further optimization for animal use.

Finally, we must note that K562 cells used in this study served as a cell model to

characterize, understand and improve the siRNA delivery system with lipid-modified polymers in suspension cells representing CML. Studies involving the use of these delivery systems in patient cells to target other over-expressed proteins involved in CML as well as animal studies to test the efficacy of delivery system *in vivo* will need to be carried out to further access the potential of the described polymeric delivery system.

2.4 ACKNOWLEDGEMENTS

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3. SIRNA LIPID-POLYMERIC NANOPARTICLES TO ARREST CELL AND TUMOR GROWTH IN CHRONIC MYELOID LEUKEMIA CELLS

3.1 INTRODUCTION

Synthetic siRNA molecules can be designed to specifically bind mRNAs of interest with the aim of preventing specific protein expression. Different proteins that play key roles in cancer development, such as the mediators responsible for over-proliferation, aberrant cell death/survival mechanisms, and resistance against drugs, can be controlled by using synthetic siRNAs [1]. The benefits of this therapeutic approach have been proved successful in preclinical models and nanoparticle-delivered siRNA technology has entered clinical testing in certain diseases, such as the solid tumors in lung, prostate, breast, liver, melanoma, pancreas, respiratory diseases, hepatitis B virus infection (liver), hypercholesterolemia (liver), and transthyretin-mediated amyloidosis (liver) [1]-[3]. However, delivery of siRNA in cells that are difficult to transfect, such as stem/bone marrow cells and attachment-independent leukemia cells, have yielded limited success *in vitro* due to non-effective functional delivery systems, so that only a couple of cases have been reported in preclinical animal models [4].

The main barrier for the siRNA technology to be fully developed for attachment-independent cells is the lack of a proper delivery agent that interacts efficiently with the cells of interest. In the search for improved siRNA delivery agents for leukemia specifically, we have been working on siRNA delivery systems that: 1) have high efficiency *in vitro* to enable sufficient down-regulation of target mRNA to control leukemic cell behavior, 2) induce minimal toxic effect after siRNA delivery, and most importantly, 3) have the capability to be translated to a clinical setting- unlike delivery systems such as electroporation and viruses that cannot be applied *in vivo* and/or represent an unacceptable risk for the patient. Leukemic cells, due to their nature to grow as anchorage-independent, cannot be generally localized at one site and a lack of cell-surface adhesion molecules and smaller cell surface area make them difficult targets to reach [5], [6]. Delivery systems based on lipid grafting on low molecular weight (MW)

polyethylenimine (PEI) are being used for efficient siRNA delivery and transfection in leukemia cells *in vitro*. Grafting of linoleic acid or caprylic acid lipids onto low MW PEI have yielded carriers with high siRNA delivery efficiency and induced therapeutic effects *in vitro* in acute myeloid leukemia (AML) cells, leukemic stem/progenitor AML cells, and cutaneous T-cell lymphomas (CTCLs) [7]-[9]. Likewise, for siRNA delivery against the fusion gene BCR-ABL by using a lower MW PEI (1.2 kDa) grafted with a palmitic acid (PA) (PEI1.2-PA) decreased BCR-ABL mRNA levels in chronic myeloid leukemia (CML) cells, which caused cell growth inhibition and increased the percentage of apoptotic cells [5]. PEI1.2-PA was also found to be less cytotoxic than high MW PEI (25 kDa) and effective to the similar extent as liposomal reagents [5].

To investigate the potential effect of lipid-modified polymers for CML therapy, we present in the current study a new formulation of lipid-conjugation on 1.2 kDa PEI as siRNA carrier in CML cells with lower cell toxicity and similar transfection efficiency to its previous ancestor (1.2PEI-PA) [5]. This new polymer, based on alpha-linoleic acid (αLA) conjugation on 1.2 kDa PEI (PEI1.2-αLA), was explored in human-derived K562 CML cells. The PEI1.2-αLA polymer was evaluated in Green Fluorescent Protein (GFP)-positive K562 cells after delivery of siRNA against *GFP* gene to quantify the extent of silencing and duration of its silencing effect. Moreover, PEI1.2-αLA polymer was used *in vitro* to deliver siRNA against the *BCR-ABL* fusion gene present in CML to quantify its effect on *BCR-ABL* mRNA levels and cell proliferation of CML cells. Finally, efficacy of BCR-ABL siRNA transfection with PEI1.2-αLA was evaluated in a CML *in vivo* model.

3.2 METHODS

3.2.1 Materials.

Branched PEI with MW of 1.2 kDa (PEI1.2) was purchased from Polysciences, Inc. (Warrington, PA). PEI with MW of 25 kDa (PEI25), anhydrous dimethylsulfoxide (DMSO), α-linoleyl chloride (αLA), MTT and trypsin/EDTA were obtained from Sigma-Aldrich Corporation (St. Louis. MO). Unlabeled scrambled siRNA and Lipofectamine® 2000 reagent were purchased from Invitrogen (Burlington, ON). GFP siRNA (GFP-22) was from Qiagen (Toronto, ON). A custom-synthesized BCR-ABL siRNA (5-GCAGAGUUCAAAAGCCCTT-3' and 3-TTCGUCUCAAGUUUUCGGG-5') was obtained from Integrated DNA Technologies, Inc. (IDT) (Coralville, IA), while another BCR-ABL siRNA was obtained from Allele Biotechnology (San Diego, CA; catalog numbers: ABP-Ri-VAsi-D09). The RPMI Medium 1640 medium with L-glutamine, low-glucose DMEM, Opti-MEM® I reduced serum medium, penicillin (10,000 U/mL), and streptomycin (10 mg/mL) were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from PAA Laboratories Inc. (Etobicoke, ON). Turbofect^{TM} was from Thermo Scientific.

3.2.2 Synthesis and characterization of lipid-substituted polymers

The synthesis procedure for the PEI1.2-PA polymer has been described previously [5], [10]. For the synthesis of PEI1.2-αLA, 200 mg of PEI was dissolved in 10 mL of dichloromethane (DCM), under nitrogen while being stirred. After 30 min, 80 µL of 1.15 mmol of triethylamine was added to the PEI solution and the solution was stirred for an additional 30 min. Sufficient linoleic acid chloride to get a PEI1.2: αLA mol:mol ratio of 1:4 was dissolved in 10 mL of DCM and added drop wise to the PEI solution while being stirred for 20 min. After incubation for a 24-hour period under constant stirring, the product was precipitated twice in 500 mL of anhydrous diethyl ether. The polymer was dissolved in ~5 mL distilled/deionized water and freeze dried to obtain the water-soluble

fraction. The polymers were analyzed by ^{1}H NMR (Bruker 600 MHz; Billerica, MA) in D_2O using the characteristic proton shifts of lipids (δ ~ 0.8 ppm; −CH3) and PEI (δ ~ 2.5–2.8 ppm; NH–CH2–CH2–NH–) to calculate the extent of lipid on the synthesized polymer. The calculated substitution level of PEI1.2-αLA polymer is 0.94 lipid per PEI.

3.2.3 Cell culture

The K562 cells, a CML cell line that expresses the BCR-ABL fusion protein, and GFP-expressing K562 (GFP-K562 [11]) cells were propagated in RPMI medium containing 10% heat-deactivated fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO_2. For cell expansion and subculture, spent medium was discarded by centrifugation (600 rpm, 5 min) and 1 x 10^6 cells were diluted in 20 mL of fresh medium (~1:20 dilution) typically once a week.

3.2.4 Preparation of siRNA/polymer complexes

The desired siRNAs and polymers were separately dissolved in nuclease free water at 0.14 and 1 µg/µL, respectively. For preparation of siRNA/polymer complexes, siRNA solutions were first diluted in RPMI and the desired polymer solutions (PEI1.2, PEI1.2-PA and PEI1.2-αLA) were added to the diluted siRNA solutions. Solutions were mixed briefly with vortex and incubated for 30 min at room temperature (RT) to allow complex formation. RPMI medium alone (no complexes) was used for no treated groups (NT), while scrambled-siRNA/polymer complexes (prepared similarly) were used for negative controls. The siRNA:polymer (weight:weight) ratios in complexes were controlled and kept either at 1:8 or 1:12 as specified in each Figure's legend.

Commercial reagents Turbofect™ and Lipofectamine® 2000 were used for comparison of transfection efficiency with lipid-modified polymers. For the preparation of siRNA/carrier complexes, a siRNA:carrier ratio of 1:4 was used as per manufacturer's suggestion and after in-house optimization. With Lipofectamine® 2000, siRNA and

carrier were diluted separately in OptiMEM® and, after a 5-min incubation, siRNA solution was mixed with the carrier solution by pipetting. The final solution was incubated for 20 min at room temperature. Complexes prepared with TurboFect™ were prepared in the same way as polymeric complexes with the exception that TurboFect™ complexes were incubated for 20 min at room temperature.

3.2.5 *In vitro* transfection experiments

For assessment of GFP silencing, 100 µL solutions containing GFP-siRNA/carrier complexes at the indicated siRNA:polymer ratios and siRNA concentrations were added to empty wells in 48-well plates. GFP-K562 cells suspended in 300 µL of supplemented RPMI medium were then added to complex solutions at ~1.2 x 10^5 cells/mL per well. Cells were incubated at 37°C in a CO_2 for three or more days. For cells cultured for more than four days, cells were diluted six times in fresh medium and re-seed in new wells. At indicated time points, cells were harvested and washed twice with HBSS by centrifugation (1400 rpm, 5 min) and, fixed and suspended in 3.7% formaldehyde solution. Mean fluorescence and percentage of GFP-negative of cell populations were quantified using a Beckman Coulter QUANTA SC flow cytometer using the FL-1 channel. No treated (NT) GFP-K562 cells were gated to ~2% of GFP-negative cells. Percent decrease in mean fluorescence was calculated as follows: 100-([Mean FL1 of cells treated with GFP siRNA/polymer] / [Mean FL1 of cells treated with scrambled siRNA/ polymer] × %). Percent decrease in GFP-positive cells was calculated as follows: [% of GFP-negative cells of cells treated with GFP siRNA/ polymer] − [% of GFP-negative cells of cells treated with scrambled siRNA/polymer].

MTT assay was performed to assess the viability and growth of K562 cells *in vitro* after treatment with BCR-ABL siRNA. Complex solutions were prepared with polymer and BCR-ABL siRNA in RPMI at a final volume of 150 µL (triplicate). Fifty microliters were added to empty wells of a 96-well plate and 50 µL of cell suspension at 1 x 10^5 cells/mL

per well was added to the complexes. At the indicated time points after transfection, 25 µL of MTT (5 mg/mL in HBB) was added to each well. One hour after the cells were incubated with MTT at 37 °C and 5% CO_2, medium and MTT were removed. Hundred microliters of DMSO were added to wells to dissolve the formazan crystals. Absorbance was acquired at a wavelength of 570 nm. Background, considered as absorbance values from cells without MTT, was subtracted from absorbance readings to give final values. Results from treated groups were normalized against no treatment groups (100%) to obtain the percentage of viable cells.

3.2.6 CML *in vivo* animal studies

All experiments were performed in accordance with the University of Alberta guidelines for the care and use of laboratory animals. Ethical approval for animal studies were obtained before the start of the experiments. Four to six-week old male NCR nu/nu nude mice were purchased from Taconic Farms (Albany, NY) and kept in a bio-containment facility. For tumor formation, 10-20 x 10^6 GFP-K562 cells were injected into the right flank of the mice. For studies that used Matrigel® (Corning) for cell injection (second and third studies), 100 µL of RPMI media containing the cells were mixed with 100 µL of Matrigel® right before injections into mice. Tumor growth was monitored every 2-3 days tumor volume was measured with a digital caliper (tumor volume = length x width^2 x 0.4). After ~2 weeks, tumors that reached a size of more than 60 mm^3 were used for injections. Only those tumors that were in the exponential growth phase were included in the study. Three *in vivo* studies were performed: tumors were injected 1) subcutaneously (vicinity of the tumor) with three injections every 72h with RPMI alone, GFP (control)-siRNA//PEI1.2-αLA complexes or BCR-ABL-siRNA/PEI1.2-αLA complexes; 10 µg siRNA, ratio 1:12 in 20 µL RPMI per injection; 2) intraperitoneally with three injections every 72 h with 10 µg siRNA, ratio 1:8 in 300 µL RPMI per injection, and 3) intraperitoneally with four injections every 72 h with 10 µg at ratio 1:12 (1st injection)

and 15 µg siRNA at ratio 1:12 (2^{nd} to 4^{th} injections) in 300 µL RPMI per injection. Tumor volumes from each mouse were measured at the indicated time points and were divided by the tumor volume at the onset of the study to obtain a relative tumor volume. Three days after the last injection, mice were euthanized by CO_2 asphyxiation and tumors were harvested for ddPCR analysis.

3.2.7 Quantification of mRNA by droplet digital PCR (ddPCR)

Tumors were surgically excised, homogenized using a Pyrex™ Tissue Grinder (Sigma-Aldrich), filtered through a 40-µm mesh (Fischer Scientific) and collected. Total RNA was extracted using TRIzol® (Life Technologies). For this, cells were lysed with 1 mL of Trizol reagent and incubated for 5 min. Chloroform (0.2 mL per mL of Trizol) were added to lysed samples. After vigorous shaking, and centrifugation, the aqueous phase was extracted. RNA was then precipitated with isopropanol and centrifugation and finally washed with 75% ethanol. RNA was dissolved with RNAse free water and the integrity and concentration of total RNA was determined by a spectrophotometer (NanoDrop LITE; Thermo Scientific). For each sample, 2 µg of RNA were reverse-transcribed using oligo (dT), random primers and M-MLV reverse transcriptase (Invitrogen). Copy number of BCR-ABL mRNA was determined by droplet digital PCR (QX100, Bio-Rad) using 3 ng of cDNA from each tumor sample, ddPCR Probe Supermix (Bio-Rad), PrimeQuest qPCR (IDT) assay for BCR-ABL (forward 5'-CAT TCC GCT GAC CAT CAA TAA G-3'; reverse 5'-GAT GCT ACT GGC CGC TGA AG-3'), and TaqMan® gene expression assay for B-actin as reference gene (Hs01060665_g1, Life Technologies). For droplet generation, the 20 µL PCR reaction mixture was loaded on wells of droplet generation DG8 cartridge. A volume of 70 µL of droplet generation oil was added to each reaction mixture. Cartridge was then plated into a QX100 Droplet generator (Bio-rad). The generated droplets were then aspirated and dispensed into a 96-well PCR plate, which was heat-sealed using foil sheets. cDNA amplification was performed into thermal cycler using the following

program: one cycle of initial denaturation at 95 °C for 10 min; followed by 45 cycles of denaturation at 94 °C for 30 s and annealing at 60 °C for 60 s, and one cycle of extension at 98 °C for 10 min. After PCR, 96-well PCR plate was loaded into a QX100 Droplet Reader (Bio-Rad) to measure the fluorescence of each droplet from each well. Results were analyzed using the QuantaSoft Software (Bio-Rad).

3.2.8 Statistical Analysis

All results were expressed as mean ± standard deviation of indicated number of replicates. One-way ANOVA followed by the Tukey test were used to analyze the significance of any difference among the study groups (GraphPad Prism v6 software). Where stated, the data between controls and treatment groups were analyzed for statistical difference by Student's t-test (two-tailed distribution and unequal variance). The level of significance was set at $p < 0.05$ and NQ (not quite) significant, where $0.05 > p < 0.10$.

3.3 RESULTS

3.3.1 Comparison of GFP silencing with lipid-polymers and commercial reagents

We first compared the GFP silencing with the newly-prepared PEI1.2-αLA to the previously effective PEI1.2-PA, the parent polymer PEI1.2, and the commercial carriers PEI25, Turbofect™ and Lipofectamine® 2000. The GFP-K562 cells were transfected with 75 nM of GFP siRNA at a siRNA:polymer ratio of 1:8, except for Lipofectamine® 2000 and Turbofect™, where a ratio of 1:4 was used (to reduce toxicity). GFP fluorescence levels and resultant concentration of GFP-K562 cells were assessed by flow cytometry 3 days after transfection (**Figure 3.1**). The PEI1.2 with no lipid modification did not decrease the GFP fluorescence (as expected), while PEI25 gave a decrease of

81.5±0.3% in mean GFP fluorescence (**Figure 3.1A**). However, only 11.6±0.9% of the cells could be recovered (compared to non-treated cells) after treatment with PEI25 complexes (**Figure 3.1B**). The extent of silencing with PEI1.2-PA polymer was 61.9±5.8% with 28.4±8.1% of the cells remaining, while PEI1.2-αLA gave 54.3±4.9% decrease in mean GFP fluorescence but with a greater recovery of cells (~41%). Turbofect™ and Lipofectamine® 2000 gave 77.6±1.4% and 86.5±0.3% decrease in mean GFP fluorescence with the cell recoveries of 22.0±4.0% and 70.0±4.8%, respectively.

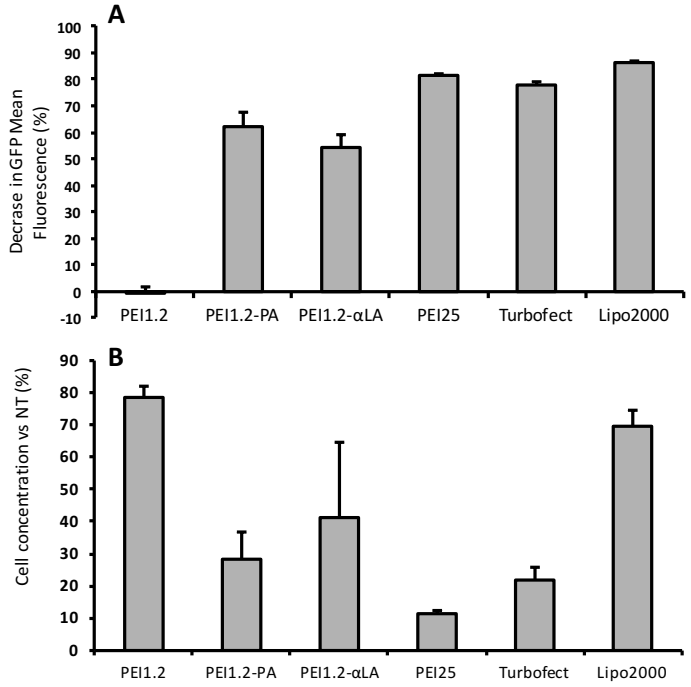


Figure 3.1. GFP siRNA-mediated silencing in GFP-K562 cells with lipid-modified polymers and commercial transfection reagents. GFP fluorescence of K562 cells was measured by flow cytometry 3 days after transfection. **A**) Decrease in mean GFP fluorescence (in comparison with cells treated with control siRNA with the indicated carrier). **B**) Cell concentration/mL (as a percentage of non-treated (NT) cells). Complexes were prepared at a siRNA:polymer ratio of 1:8 with a final siRNA concentration of 75 nM and siRNA:carrier ratio of 1:4 with commercial reagents PEI25, Turbofect™ and Lipofectamine® 2000. Lipid-modified polymers PEI1.2-PA and PEI1.2-αLA gave a similar silencing effect (~55%), while commercial reagents gave a silencing of ~83% (**A**) cell recovery (vs NT) with PEI1.2-PA and PEI1.2-αLA was between Lipofectamine (no toxicity) and PEI25 and Turbofect™ (high toxicity).

We further explored the silencing effect of low molecular PEIs at a lower siRNA concentration (36 nM) with siRNA:polymer ratios of 1:8 (**Figure 3.2A**) and 1:12 (**Figure 3.2B**) in GFP-K562 cells. Flow cytometry data for the GFP fluorescence (**Figure 3.2Ai** and **Bi**), percentage of cells with GFP silencing (**Figure 3.2Aii** and **Bii**), and cell concentration (normalized against non-treated cells; **Figure 3.2Aiii** and **Biii**) are shown. Comparing PEI1.2-PA with the PEI1.2-αLA, PEI1.2-PA had a greater reduction in GFP fluorescence at the ratio of 1:8 (40.3±2.0% vs. 22.8±3.1% in mean GFP fluorescence [**Figure 3.2Ai**]; 21.4±0.7% vs. 11.6±1.0% in silenced cell population [**Figure 3.2Aii**]) but not at the ratio of 1:12 (47.2±4.0% vs. 44.6±5.8% in mean GFP fluorescence [**Figure 3.2Bi**]; 22.9±5.2% vs. 27.7±3.9% in silenced cell population [**Figure 3.2Bii**]). However, PEI1.2-PA showed a higher toxicity compared to PEI1.2-αLA: 20.6±3.4% and 9.8±4.7% cells were recovered with PEI-1.2-PA at the ratios 1:8 and 1:12 (**Figure 3.2Aiii** and **Biii**), while 33.0±4.5% and 35.0±9.4% cells were recovered with PEI-1.2-αLA at the ratios 1:8 and 1:12, respectively (**Figure 3.2Aiii** and **Biii**). αLA substitution on higher molecular weight PEI2.0 (instead of 1.2 kDa) gave low silencing effect (6.4±0.9% in mean GFP fluorescence and 2.0±1.8% in silenced cell population with 1:8 ratio [**Figure 3.2Ai** and **ii**] and 9.8±1.1% in mean GFP fluorescence and 1.9±3.9% in silenced cell population with 1:12 ratio [**Figure 3.2Bi** and **ii**]), whereas PEI2.0 without any lipid gave no silencing effect and no changes in cell recovery (**Figure 3.2A** and **B**).

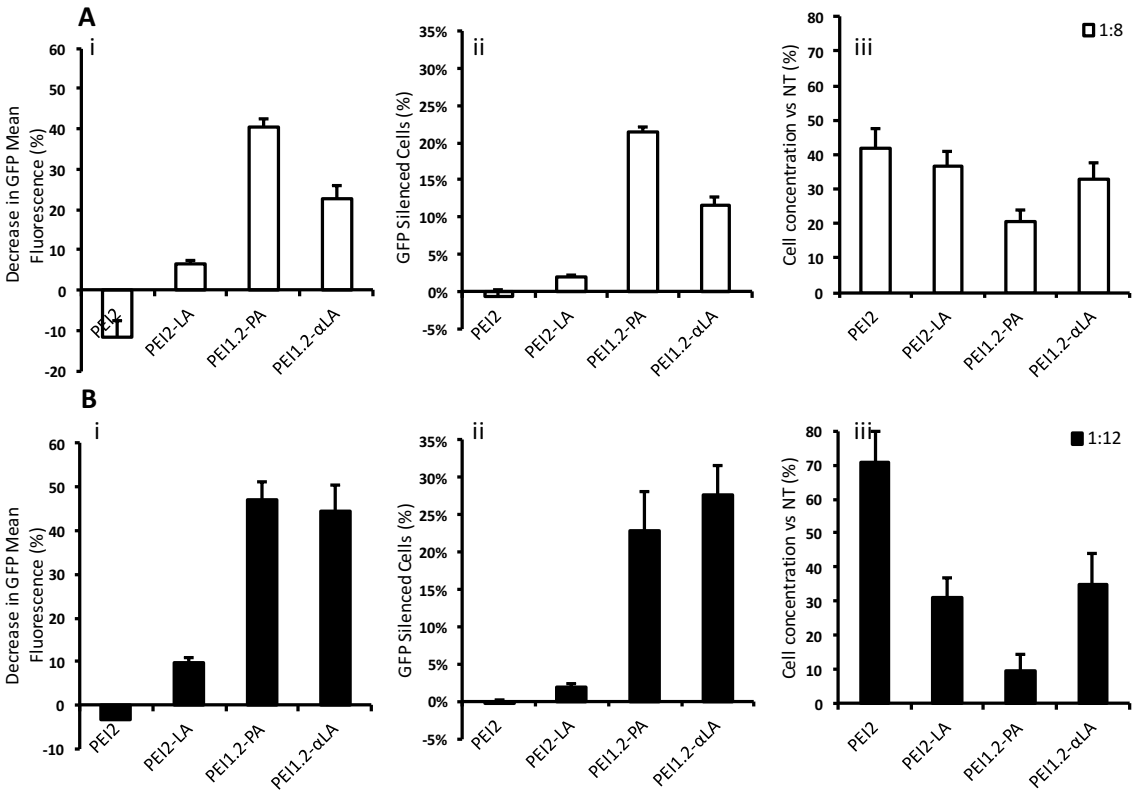


Figure 3.2. GFP silencing in GFP-K562 cells with PEIs substituted with PA, LA, and αLA lipids. A) Decrease in GFP mean fluorescence, **B**) percentage of silenced cells, and **C**) cell concentration/ml (vs NT) were assessed by flow cytometry 3 days after siRNA treatment with 36 nM at ratios 1:8 (**Ai-iii**) and 1:12 (**Bi-iii**). Lower molecular weight PEI1.2 with lipid substitution gave higher GFP silencing effect at both 1:8 and 1:12 ratios. At ratio 1:12, PEI1.2-αLA gave similar silencing effect (~50% decrease in fluorescence and 25% in silenced cell population) than PEI1.2-PA and lower negative effect on cell recovery (35% vs 10%).

To evaluate the lasting effect of silencing, GFP fluorescence and cell recovery were assessed after 2, 4, 7 and 9 days of a single siRNA treatment (20, 40 and 80 nM) with PEI1.2-αLA (best performing polymer from experiments above) at the siRNA:polymer ratios of 1:8 (**Figure 3.3Ai, ii**) and 1:12 (**Figure 3.3Bi, ii**). The 20 nM siRNA treatment at both ratios showed a decrease of ~20% or less in mean GFP fluorescence, which remained constant for up to 9 days (**Figure 3.3Ai, Bi**). At the 40 nM concentration, decrease in GFP fluorescence with 1:8 ratio was slightly higher than the 20 nM dose but

also less than 20% (**Figure 3.3Ai**). At the 1:12 ratio, the 40 nM siRNA treatment displayed a steady increase in silencing from 11.1±2.0% to 44.8±3.5% over the 9 day studied period (**Figure 3.3Bi**). For the 80 nM concentration and ratio of 1:8, there was a gradual increase on the extent of silencing (decrease GFP fluorescence) from 29.2±1.6% on day 2 to 83.1±0.4% on day 9 (**Figure 3.3Ai**). In the case of ratio 1:12, the extent of silencing gradually increased to 83.9±1.5% on day 9 (**Figure 3.3Bi**).

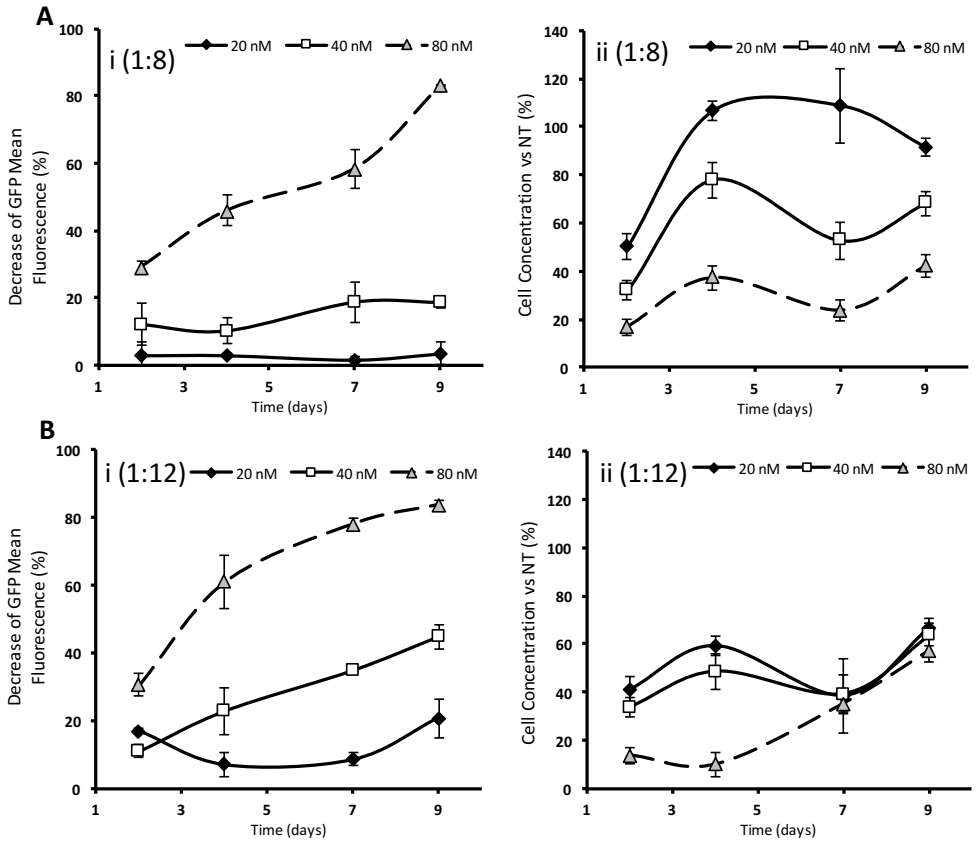


Figure 3.3. GFP silencing in GFP-K562 cells with PEI1.2-αLA as a function of time. GFP-K562 cells were treated with one siRNA dose at 20 nM, 40 nM, and 80 nM with PEI1.2-αLA at siRNA:polymer ratios 1:8 (**A**) and 1:12 (**B**). Decrease of GFP mean fluorescence (Ai and Bi) and cell concentration/ml (vs NT) was assessed by flow cytometry on day 2, 4, 7 and 9 after transfection. Cells were sub-cultured on day 7 with fresh medium. Silencing effect progressively increased from day 2 up to day 9 (from 30 to 80% decrease) with siRNA treatments at 40 nM and 80 nM. Cell recovery was affected two days after transfection; cells at 1:8 recovered by day 4, whereas cells are ratio 1:12 too longer to recover.

The recovery of the cells was most robust with the low concentration of siRNA (20 nM) at the low (1:8) ratio; at this ratio, the initial decrease in cell concentration ranged from 16.9±6.9% to 50.0±10.6%, and higher concentrations of siRNA treatments resulted in reduced recovery (**Figure 3.3Aii**). At the 1:12 ratio, a higher decrease in cell concentration was initially seen, ranging from 13.7±6.3% to 41.2±16.9% and a slower cell recovery was observed, ultimately reaching ~62% cell recovery for all siRNA concentrations on day 9 (**Figure 3.3Bii**). It is also important to note that the cells were sub-cultured (7-times dilution) on day 4 and that this is the reason for seeing lower cell concentrations at the last two time points (**Figure 3.3Aii** and **Bii**).

3.3.2 BCR-ABL siRNA treatment *in vitro*

The silencing of BCR-ABL mRNA after specific siRNA delivery with PEI1.2-αLA at the siRNA:polymer ratios 1:8 and 1:12 and siRNA concentrations of 30 and 60 nM was quantified by ddPCR one day after transfection (**Figure 3.4**). Treatment of cells with 30 nM siRNA yielded 11.7±1.7% BCR-ABL silencing at the 1:8 ratio (in comparison with negative GFP-siRNA treatment, no significant) and 12.8±6.7% BCR-ABL silencing at the 1:12 ratio (no significant). Silencing efficiency was increased when 60 nM siRNA was used for cell treatment: 27.3±6.5% silencing at the 1:8 ratio ($p<0.001$) and 49.6±2.5% with the 1:12 ratio ($p<0.001$), indicating the significant effect of siRNA:polymer ratio on BCR-ABL down-regulation.

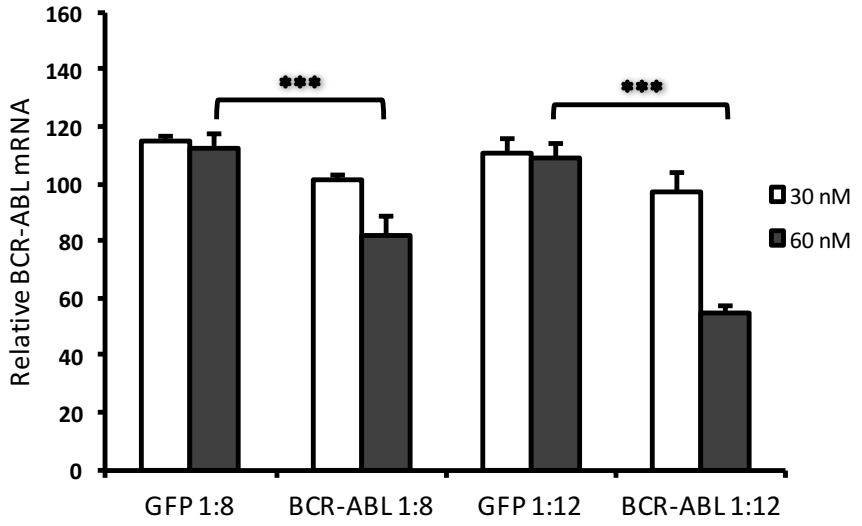


Figure 3.4. ddPCR analysis in GFP-K562 cells after BCR-ABL siRNA transfection. GFP-K562 cells were treated with GFP-siRNA (negative control) or BCR-ABL-siRNA with PEI1.2-αLA at siRNA concentrations of 30 nM and 60 nM with siRNA:polymer ratios 1:8 and 1:12. mRNA levels were quantified by ddPCR one day after siRNA treatment. The significant level *** $p<0.001$ were calculated between corresponding GFP- and BCR-ABL-siRNA groups.

To evaluate the specific effect of BCR-ABL silencing on cell viability, K562 cells were treated with BCR-ABL siRNA and GFP siRNA as a negative control using PEI1.2-αLA as the carrier (siRNA:polymer ratios of 1:8 and 1:12 and siRNA concentration of 60 nM). Cell viability was quantified by the MTT assay 1, 2 and 4 days after transfection (**Figure 3.5Ai-iii**). At the siRNA:polymer ratio of 1:8, cell viability was 93.9±2.1% on day 1 for the GFP-siRNA treated cells and 84.2±4.4% for the BCR-ABL siRNA treated cells (**Figure 3.5Ai**), decreasing to 78.1±4% and 79±4% for day 2 (**Figure 3.5Aii**) and 53.6%±2.2 and 55.5%±3 for day 4 (**Figure 3.5Ai**), for GFP-siRNA and BCR-ABL-siRNA groups respectively; hence showing a decrease of 9.6% in cell inhibition with BCR-ABL group vs GFP group on day 1 and no BCR-ABL specific effect thereafter. At the siRNA:polymer ratio of 1:12, however, cell viability on day 1 was 41.6±3% for GFP-siRNA treated cells and 35.9±5.1% for the BCR-ABL siRNA treated cells (5.7% decrease, **Figure 3.5Ai**),

48.1±15.2% and 35±9.8% for day 2 (13.1% decrease, **Figure 3.5Aii**), and 57.5±3.6% and 34.3±1.6% on day 4 (23.2% decrease, $p<0.001$, **Figure 3.5Aiii**), for GFP-siRNA and BCR-ABL-siRNA groups, respectively. At the 1:12 ratio, the GFP siRNA treated cells recover slowly from the initial toxicity (going from 42% on day 1 to 58% on day 4 vs NT), unlike the BCR-ABL siRNA treated cells where cell growth was subsequently arrested (remaining at 35% vs NT up to day 4, $p<0.001$) (**Figure 3.5B**).

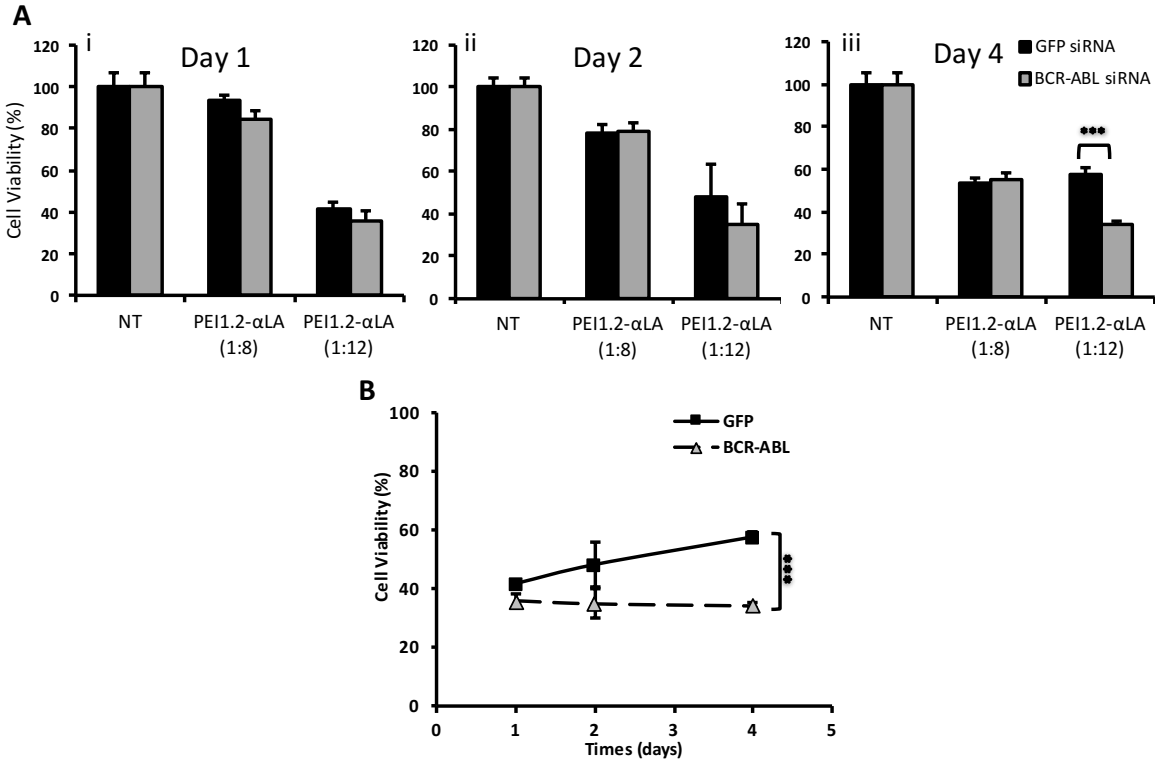


Figure 3.5. Cell viability assessment after BCR-ABL siRNA transfection. GFP-K562 cells were transfected with GFP siRNA or BCR-ABL siRNA and PEI1.2-αLA at a siRNA concentration of 60 nM and 1:8 and 1:12 ratios. **A**) Cell viability was assessed by MTT assay on days 1 (**Ai**), 2 (**Aii**) and 4 (**Aiii**) after siRNA treatment. **B**) Cell viability values of cells treated with ratio 1:12 in **A** are summarized as a function of time. Absorbance values were normalized to NT values. Cell viability on day 4 of cells treated with BCR-ABL siRNA at a 1:12 ratio was significantly different than cells treated with GFP siRNA (23.2% decrease in viability, $p< 0.001$).

3.3.3 BCR-ABL siRNA delivery in *in vivo* Animal Model

The effect of BCR-ABL siRNA delivery was investigated for the first time, to the best of our knowledge, in CML xenograft models to treat tumor growth. The xenografts derived from GFP-K562 cells were injected either 1) subcutaneously (vicinity of the tumor, SC) with 3 injections of 10 µg of siRNA each at ratio 1:12, 2) intraperitoneally (IP1) with 3 injections 10 µg of siRNA at ratio of 1:8, and 3) intraperitoneally (IP2) with four injections of 10 µg (first injection) and 15 µg of siRNA at the ratio of 1:12 for subsequent 3 injections. Tumors injected with RPMI alone served as no treatment control tumors and GFP-siRNA/PEI1.2-αLA complexes as negative treatment controls. Tumors that had achieved between 60 and 300 mm^3 of volume were introduced into the study (usually 2- 2.5 weeks after cell injections). Since not all tumors grew at the same rate, some tumors that also reached this volume were introduced into the study ~1 more week after. The average volumes of tumors on day of first injection were 133.8±71.1 mm^3 for SC study, 143.6±35.1 mm^3 for IP1 study, and 153.4±99.2 mm^3 for IP2 study. It is important to note that there was a large variation in the growth of tumors in the mice. From the SC study, where cells were injected in RPMI (no Matrigel®), 12 of 30 injected mice developed a tumor (40% success rate); whereas for the IP studies, where cells were injected with Matrigel®, also 12-13 mice out of 30 developed a tumor, indicating that Matrigel® did not improve the development of tumor [12], [13]. The rest of the mice were excluded from the study mainly because (i) they either did not develop tumors, (ii) the tumor disappeared a few days after injection of cells, or (iii) the tumors were not in the exponential growth phase remained non-growing.

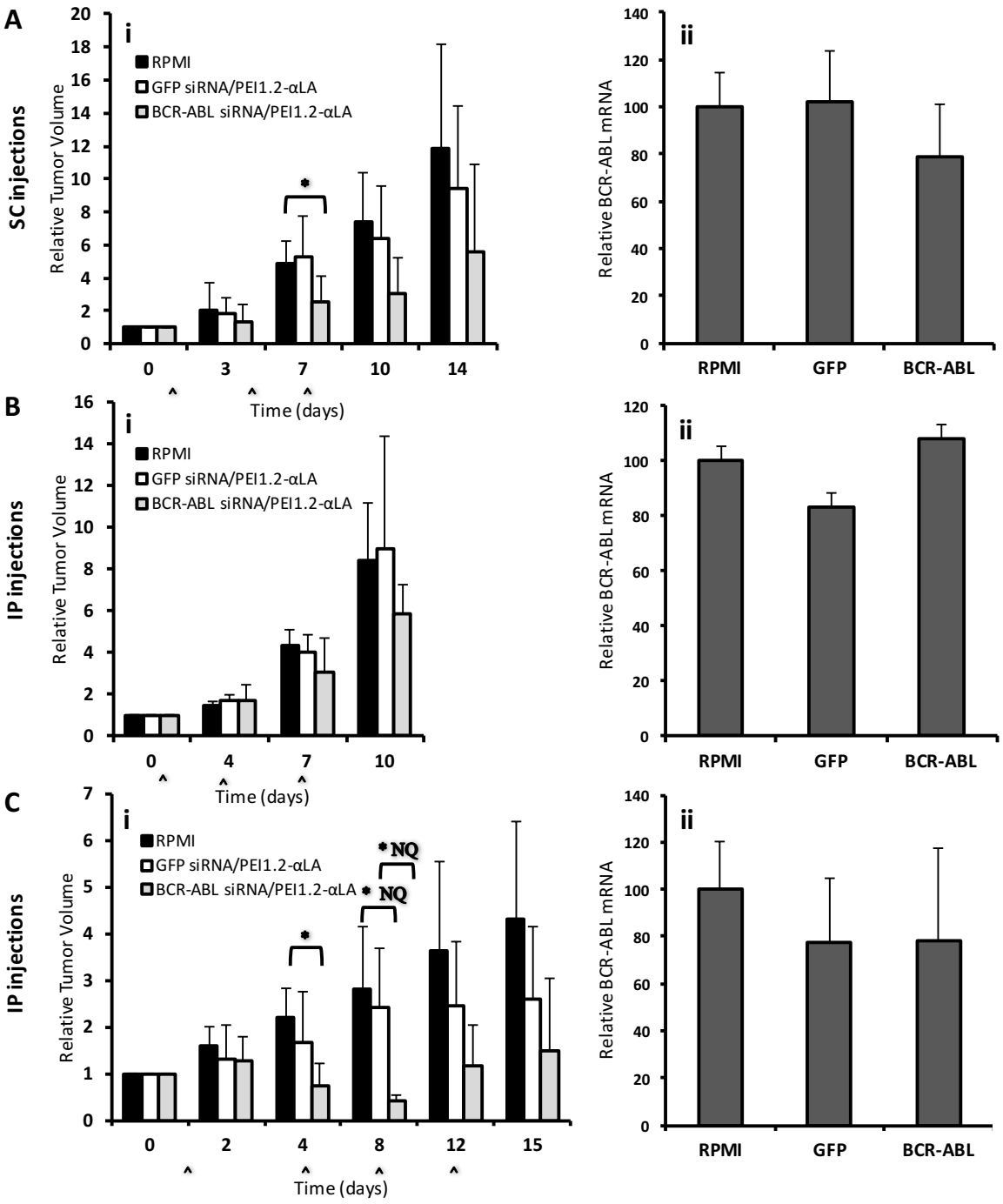
Changes in the relative tumor volumes (in comparison with tumor size on day 0) after first injection are shown as bar graphs in **Figure 3.6 A**, **B**, **C** and a table with the mean ± standard deviation values and the number of animals used per group (n) (**Figure 3.6D**). For xenografts tumors that were treated subcutaneously (10 µg siRNA, 1:12,

3X), GFP siRNA resulted in a small decrease in tumor volumes after day 10 in comparison to RPMI group (no significantly different, **Figure 3.6Ai**). Tumor growth with BCR-ABL siRNA showed a slower growth trend in comparison with RPMI and GFP siRNA groups (**Figure 3.6Ai**). This effect was more evident on days 7 and 10, where there was a significant difference on day 7 between RPMI and BCR-ABL siRNA groups ($p<0.05$ by t-test), but the rest of the groups were not significantly different from RPMI and GFP siRNA groups (BCR-ABL vs RPMI group: $p=0.1$ on day 10, and; BCR-ABL vs. GFP group, $p=0.012$ and $p=0.2$ on day 7 and day 10, respectively). A lower reduction of tumor size was still appreciated on day 14 but the difference was less evident than the previous days. The quantification of mRNA expression levels by ddPCR of the extracted tumors (**Figure 3.6Ai**) indicated a ~21% reduction of the BCR-ABL mRNA expression in tumors treated with BCR-ABL siRNA in comparison with RPMI group ($p= 0.1$), where no changes in BCR-ABL levels with the GFP siRNA were found (**Figure 3.6Aii**).

The growth rate of tumors treated intraperitoneally with 10 µg of BCR-ABL siRNA (1:8, 3X) also show a slower growth in the BCR-ABL group but the effect is less evident than the SC study (**Figure 3.6Bi**). Tumors treated with BCR-ABL siRNA showed a decreased relative tumor volume on days 7 and 10 (**Figure 3.6Bi**), but there was no significant difference from the RPMI and GFP siRNA groups (BCR-ABL vs RPMI group: $p= 0.35$ and $p=0.13$ on days 7 and 10, respectively, and; BCR-ABL vs GFP group, $p=0.25$ and $p=0.13$ on days 7 and 10, respectively). No reduction of the BCR-ABL mRNA levels was found in any of the treated groups, especially in the group treated with BCR-ABL siRNA (**Figure 3.6Bii**).

A second IP study was performed with higher siRNA dose and siRNA:polymer ratio and four injections (10µg [1X], 15 µg [3X], 1:12) (**Figure 3.6Ci** and **ii**). Growth of tumors treated with GFP siRNA showed a slower growth in comparison with RPMI group (no significant difference was found at any time point) (**Figure 3.6Ci**). Retardation of

the tumor growth of BCR-ABL siRNA group was much more evident than the SC and first IP experiments. A decrease in volume starts from day 4 and lasts up to day 15; where statistical differences were found on day 4 (between RPMI and BCR-ABL, $p<0.05$) and on day 8 (between RPMI and BCR-ABL, and GFP and BCR-ABL, both $p<0.05$ by t-test). [NOTE: One-way ANOVA statistical analysis gave not quite significant p values for day 8: $p=0.054$ for RPMI vs BCR-ABL, and $p=0.092$ for GFP vs BCR-ABL). On day 8, where the decreased volume was much more evident, the volume of tumors treated with BCR-ABL was 6 times less than the volume of tumors treated with GFP siRNA. Changes of volume of tumors treated with BCR-ABL siRNA on days 12 and 15 were no longer significantly different in comparison with RMPI ($p=0.076$) and GFP siRNA ($p= 0.097$) groups (**Figure 3.6Ci**). BCR-ABL mRNA analysis does not show a significant decrease in the BCR-ABL group in comparison with the GFP siRNA as mRNA levels in both group are reduced ~23% in comparison with RPMI group, perhaps suggesting some toxicity in the tumors treated with GFP siRNA (**Figure 3.6Cii**).


A
SC injections
i
RPMI
GFP siRNA/PEI1.2-αLA
BCR-ABL siRNA/PEI1.2-αLA
Relative Tumor Volume
Time (days)
ii
Relative BCR-ABL mRNA
RPMI
GFP
BCR-ABL
B
IP injections
C
IP injections
NQ

D

SC	Day 0		Day 3		Day 7		Day 10		Day 14	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
RPMI	1.00 ± 1.00	4	2.23 ± 1.43	4	4.88 ± 1.37	4	7.42 ± 2.96	3	11.87 ± 6.29	2
GFP	1.00 ± 1.00	4	2.14 ± 1.00	4	5.27 ± 2.49	4	6.39 ± 3.13	3	9.47± 5.01	2
BCR-ABL	1.00 ± 1.00	5	1.35 ± 1.00	5	2.54 ± 1.55	5	3.02 ± 2.19	5	5.62 ± 5.3	5

IP1	Day 0		Day 4		Day 7		Day 10	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
RPMI	1.00 ± 1.00	3	1.42 ± 0.24	3	4.29 ± 0.82	3	8.37 ± 2.75	3
GFP	1.00 ± 1.00	5	1.71 ± 0.27	5	3.98 ± 0.86	5	8.95 ± 5.41	5
BCR-ABL	1.00 ± 1.00	5	1.71 ± 0.75	5	3.01 ± 1.69	5	5.86 ± 1.40	5

IP2	Day 0		Day 2		Day 4		Day 8		Day 12		Day 15	
	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
RPMI	1.00 ± 1.00	4	1.58 ± 0.40	4	2.18 ± 0.65	4	2.81 ± 1.34	4	3.63 ± 1.90	4	4.30 ± 2.09	4
GFP	1.00 ± 1.00	5	1.31 ± 0.73	5	1.68 ± 1.07	5	2.42 ± 1.27	5	2.44 ± 1.38	5	2.57 ± 1.56	5
BCR-ABL	1.00 ± 1.00	3	1.26 ± 0.52	3	0.74 ± 0.46	3	0.41 ± 0.13	3	1.16 ± 0.88	3	1.50 ± 1.55	3

Figure 3.6. Effect of BCR-ABL siRNA treatment in GFP-K562 in vivo models. Tumors were treated **A**) subcutaneously (SC) (10 µg siRNA at ratio 1:12, 3X), **B**) intraperitoneally (IP) (10 µg siRNA at ratio 1:8, 3X), and intraperitoneally (10 µg siRNA at ratio 1:12, 1X; and 15 µg siRNA at ratio 1:12, 3X). **Ai**), **Bi**), and **Ci**) Changes in the relative tumor volume (tumor volume at indicated day vs tumor volume at day 0) after injections with RPMI alone (no treatment), GFP (control) siRNA/PEI1.2-αLA and BCR-ABL siRNA/ PEI1.2-αLA complexes. **Aii**), **Bii**), and **Cii**) Harvested tumors (excised 3 days after last injection) were analyzed for BCR-ABL mRNA levels by ddPCR. * was significantly different ($p > 0.05$) by T-test and NQ was not quite significant ($0.05 > p < 0.10$) by one-way ANOVA. ^ indicates day of injections. **D**) Values shown of the mean ± standard deviation and the number of animals used per group (n) from **A**), **B**), and **C**).

3.4 DISCUSSION

3.4.1 BCR-ABL siRNA treatment *in vitro*

In search of a polymeric delivery vehicle to be used in clinical CML therapy, we explored PEI-based polymers in this study. As expected, unmodified low MW PEI polymers were ineffective in siRNA delivery, possibly because of a lower degree of interaction with the cell membrane due to its lower cationic charge in complex with siRNA, as compared to siRNA complexes prepared with high MW PEI25 [1], [14], [15]. It is also possible that complexes from low molecular weight PEI and siRNA form a loose

and not stable enough structure that adversely affect the internalization of the complexes through the cell membrane [1]-[3], [16]. The effectiveness of PEI25 was attributed to its high MW and high cationic charge that forms a strong binding with siRNA and strongly interacts with cell membrane [4], [10]; however, this efficiency is inversely related to cell viability, as the substantial decrease in cell numbers was evident with the latter PEI (**Figure 3.1B**). With the aim of balancing toxicity with efficiency, we have grafted lipid moieties onto PEI1.2 to increase its ability to deliver siRNA due to increased lipophilicity of the polymer/siRNA complexes [5], [6], [10]) across cell membranes, especially in suspension growing cells. Our first attempt to successfully delivery siRNA in K562 cells with lipid-modified polymers was explored with palmitic acid substituted PEIs that conferred higher GFP silencing and milder cytotoxicity in comparison to PEI25. Moreover, BCR-ABL siRNA delivery in K562 cells yielded a 20% decrease at the BCR-ABL mRNA level and increased levels of apoptosis in comparison with cells treated with control siRNA in previous studies [5], [7]-[9]. This level of performance, although promising, was not considered very significant in going forward towards clinical utility. A new lipid substitution on PEI1.2 was used in the current study with the purpose of having improved silencing and less cytotoxic features. This reduced toxicity was seen with PEI1.2-αLA when the siRNA concentration of 36 nM was used at the polymer:siRNA ratio of 1:12 showed a similar reduction in GFP fluorescence for both polymers, but with PEI1.2-αLA giving a higher percentage of recovered cells (**Figure 3.2Biii**). These results suggest that PEI1.2-PA and PEI1.2-αLA can achieve similar levels of silencing but with PEI1.2-αLA polymer displaying lower cytotoxicity. In comparison to PEI1.2-αLA, commercially available transfection reagents PEI25, Turbofect™ and Lipofectamine® 2000 gave more robust silencing efficiencies (based on GFP as the reporter gene. **Figure 3.1**), but again the polymeric carrier PEI1.2-αLA gave better cell recoveries after treatment (except for Lipofectamine® 2000), suggesting that the lipid-modified polymer induced less toxicity on the cells. A mechanism behind the effectiveness of PEI1.2-αLA

is that the cationic nature in combination with lipidic part, allows better interaction with the cell membrane and promotes the entrance into the cells. Once inside the cell, the H^+-sponge effect properties of the PEI (i.e., ability to absorb free H^+ in endosome), together with the likelihood of the loosely bound carrier/siRNA complexes (decreased siRNA binding capacity due to consumption of primary amines and steric hindrance from the aliphatic chain [5], [17]-[19]), will allow escape of siRNA cargo from the endosome (with a lesser toxic effect than high MW PEI) and consequent release of siRNA into the cytoplasm.

When the GFP siRNA was delivered to K562 cells at a range of concentrations between 20 to 80 nM and the silencing was assessed over 9 days, results showed a proportional relationship between the siRNA concentration and the silencing effect (**Figure 3.3Ai, Bi**), as well as an inverse relationship between siRNA (and polymer) concentration and cell recovery (**Figure 3.3Aii, Bii**). Furthermore, it was surprising to find that the use of effective siRNA concentrations (40 nM or more) resulted in increasing silencing up to 9 days after one dose of GFP siRNA and, that the extent of silencing reached similar levels (83%) at both 1:8 and 1:12 ratios at day 9 (siRNA dose of 80 nM). Although a cytotoxic effect was evident due to transfection at both ratios, cells slowly recovered from this effect after day 2. Cell recovery tended to be faster for the 20 and 40 nM doses at 1:8 ratio than for the 20 and 40 nM doses at 1:12 ratio.

The effect of BCR-ABL siRNA on cell growth was tested first *in vitro*. Cell growth was arrested for at least 4 days when cells were treated with BCR-ABL siRNA (**Figure 3.5**), which was significantly different from the group treated with GFP siRNA. In agreement with these results, a 50% decrease of BCR-ABL mRNA level was confirmed by ddPCR (**Figure 3.4**). Although there was an initial toxicity (~60%) due to transfection with GFP siRNA complexes on day 2 (**Figure 3.5**), cell recovery was seen over time and these cells reached 57.4% of cell mass (compared to NT controls) 4 days after transfection (in

comparison to 34.4% of cell treated with BCR-ABL siRNA). Progressive cell recovery was expected to continue in culture. Various BCR-ABL siRNAs have shown a high potency to arrest growth in CML cells in other studies using various delivery techniques. Scherr et al. employed electroporation for the siRNA delivery (estimated siRNA concentration of 357 nM) and stated that cell viability declined by 75% after 4 days (effect on cell viability with control siRNA was not presented) [5], [20]; Zhelev et al. delivered the siRNA with Lipofectamine® 2000 transfection reagent (3 doses of 60 nM every 2 days, 180 nM total) and reduced the cell viability by 50% after 6 days. Delivery of control siRNA with same transfection reagent did not affect cell viability (approx. 2%)[5], [21]. Elmaagacli et al. decreased cell viability by 22% 24 hours after BCR-ABL siRNA transfection with DOTAP reagent (estimated siRNA concentration of 286 nM) [5], [10], [22]. Rangatia et al., used electroporation (1 µg siRNA per 5 x 10^5 cells) and showed proliferation arrest for at least 2 weeks after silencing [11], [23]. Collectively, these studies report a cell growth retardation at high siRNA concentrations and using delivery methods that are not applicable *in vivo*, so that the use in the current study of a practical delivery vehicles that can be used *in vivo*, is a significant leap forward for the translation of siRNA therapy.

3.4.2 BCR-ABL siRNA treatment *in vivo*

With the aim of evaluating the effect of BCR-ABL siRNA treatment on tumor growth *in vivo*, this study used the lipophilic polymer PEI1.2-αLA for delivery in a CML animal model. In three independent studies, the overall growth tendency of the tumors treated with BCR-ABL siRNA/PEI1.2-αLA complexes was slower in comparison to tumors treated with GFP siRNA/PEI1.2-αLA complexes and untreated tumors. The SC siRNA injection study (10 µg siRNA, 1:12, 3X), showed an evident decrease of tumor growth from day 7 to day 14, with a significant decrease of tumors treated with BCR-ABL siRNA/PEI1.2-αLA complexes in comparison with the untreated tumors. Two IP siRNA treatment studies were also performed: in the first IP study (10 µg, 1:8, 3X), BCR-ABL

siRNA/PEI1.2-αLA treated tumors showed a small retardation on tumor growth in comparison with GFP-siRNA/PEI1.2-αLA treated and untreated tumors, but there was no significant difference. The systemic injection (with lower siRNA exposure to tumors) and a lower siRNA:polymer ratio used (1:8 instead of 1:12) were probably the likely reasons. In the second IP study, a more significant tumor growth retardation was seen after BCR-ABL siRNA/PEI1.2-αLA treatment: decrease in tumor volume was shown from day 4 to day 15, being significantly different on days 4 and day 8 from at least one of the control groups. This increased effect may be due to increased siRNA:polymer ratio (from 1:8 to 1:12), siRNA dose (from 10 to 15 μg) and number of siRNA injections (from 3 to 4), all indicating a dose-dependent effect in the inhibition of tumor growth. Moreover, a decrease in the tumor growth was seen with GFP siRNA/PEI1.2-αLA on day 12 and 15 in comparison with untreated tumors, which may indicate some toxicity caused by the siRNA/polymer treatment.

With regards to ddPCR analysis, tumor cells treated with SC BCR-ABL siRNA/PEI1.2-αLA showed a ~21% BCR-ABL mRNA silencing, indicating that the silencing effect was detected 7 days after the last injection, which may indicate a long-lasting silencing effect after siRNA injections. In the first IP injection study, however, given the small decrease in tumor volume on day 7, no silencing effect at the mRNA level was found on day 10. For the second IP study, where the strongest efficacy was found, we were expecting to observe the strongest BCR-ABL mRNA silencing; however, this was not the case: although there was a ~20% decrease of mRNA in BCR-ABL group, this decrease was also found with the GFP group, suggesting a siRNA/polymer-related toxicity that may have masked the BCR-ABL siRNA silencing effect. Specific toxicity studies might be needed to confirm whether this is the case (i.e., apoptosis levels in extracted tumor). Longer *in vivo* studies under similar conditions may be useful to evaluate whether this reduction of the tumor volume is transient and whether the cells recover from this

toxicity (as found in *in vitro* studies).

A wide variation was observed in the growth of tumors in mice; while some tumors were highly vascularized and surrounded by host cells, other tumors remained intact and almost free of host cells for reasons not clear to us. The infiltration of host's cells into the tumors may be a sign of innate immune activation. Tumors that produce abundance of pro-inflammatory cytokines induce inflammatory responses that potentiate the vascular formation and therefore the tumor growth, whereas those tumors that do not produce enough cytokines are less likely to form vasculature and therefore impede the tumor growth [12], [13], [24]. Most of the tumors were increasing in size soon after cell inoculation but one week after, some tumors were no longer palpable and disappeared, suggesting a local reaction (inflammation) rather than actual tumor formation. Other tumors did not enter the exponential growth phase at all. To improve K562 cell survival, success rate of tumor grafting and heterogeneity in tumor formation, cells were injected with Matrigel® in the following two IP studies; however, this strategy was not successful since this inoculation also led to inconsistent tumor growth, which was similar to when cells were injected without it (implantation success rate of 40% in all studies). Moreover, not all tumors reached a volume between 60 and 100 mm^3 after ~2 weeks of cell injection, so that different set of tumors had to be put on study at different times (over a period of 7 day window). An implantation success rate of ~40% together with the different growth rates among tumors may explain the large error bars for the study groups, which may have impeded a cleaner assessment of the BCR-ABL siRNA effect on tumor growth. Regardless of this heterogeneity, a retardation of tumor growth was seen with BCR-ABL siRNA, indicating the potential of our treatment to effectively deliver siRNA to tumors in the chosen animal model.

Drug efficacy studies in 5-10 mice per treatment group are ideal, so that larger study groups will be needed if we were to use the same CML animal model. However, the low

success of grafting, and the variation of tumor growth make the use of the xenograft model suboptimal. There are several reasons for the low grafting rate: Athymic nude mouse have a greatly reduced number of T-cells but they do have B-cells and natural killer cells. Moreover, the natural killer cells can recognize tumor cells and induce cell death [25]. Also, as the mice age, the rudimentary thymus can still produce a small population of T-cells that might create a xenograft rejection [26]. In this study, the use of GFP-positive K562 cells may have represented a good model for *in vitro* assays, they may not be optimal for tumor formation in mice (tumor formation capacity may be lost after modification for GFP expression [11]), and therefore wild type K562 cells was considered to be able to recapitulate the disease better (and tumors) in future studies. Other mouse models, such as the NOD/SCID or NOD/SCID/γc mice, may represent a better alternative for the CML xenograft formation [11], [27]. Given that these mice are T-cell and B-cell deficient, as well as NK-impaired, less rejection will allow tumor formation which may translate into higher implantation success rate. For future studies to evaluate the effect of siRNA delivery systemically, CML can be induced systemically by transplanting retroviral-transduced mice bone marrow cells [28] or CML bone marrow patient cells [29] using NOD SCID mouse models (i.e., patient-derived xenograft models).

We are aware of only one other *in vivo* study that focused on nucleic acid delivery in a CML model using non-viral vehicles. To downregulate the expression of Bcl-2 that confers drug resistance in leukemias, Zhang et al. [30] delivered an antisense oligonucleotide (G3139) with transferrin receptor (TfR) targeted lipopolyplexes (LPs) to K562 (that highly express Tf-R) xenografts in athymic mice. As expected, *in vitro* Bcl-2 downregulation by Tf-LP G3139 was ~60% while downregulation with LP-G3139 and free G3139 was ~53% and ~58%, respectively. For *in vivo* studies, although C3139 was delivered into tumors more efficiently in Tf-LP than in its free form (2.4 folds) and, Tf-

LP G3139 delivery suppressed tumor growth that resulted in increased survival, the tumors treated with free G3139 resulted in lower Bcl-2 protein expression as compared to the Tf-LP G3139 group [30]. The reason attributed to this ambiguity was first, that the G3139 oligonucleotide contains 2 CpG motifs that have been shown to trigger TLR9 activation. For this, the authors confirmed this activation by finding increased levels of IL-12 in serum and enlargement of the spleen in tumor bearing mice with the Tf-LP G3139 treatment group; while these increments were not found in the treatments with free G3139 or empty Tf-LP. The authors then suggested that the antitumor activity of Tf-LPs G3139 may be associated with the capacity to induce cytokine production (due to nanoparticle uptake of Tf-rich macrophages and immunoactivation) rather than the specific action of the oligonucleotide in downregulating Bcl-2 [30]. This suggests that not only an effective delivery vehicle is needed but also a careful design of the silencing agent is needed so that the secondary effects are optimized and the intended effect takes place. Innate immune activity will be an important area to monitor in siRNA delivery with lipopolymer delivery systems in *in vivo* models in the future. The fact that no published work was found that delivered siRNA into *in vivo* CML models, may reflect the limited successful work of siRNA delivery with the currently used siRNA delivery systems. The encouraging results from the current study laid the groundwork for the evaluation of siRNA delivery systems in CML in an *in vivo* mouse model.

3.5 ACKNOWLEDGMENTS

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4. FIBRONECTIN-MODIFIED SURFACES FOR EVALUATING THE INFLUENCE OF CELL ADHESION ON SENSITIVITY OF LEUKEMIC CELLS TO SIRNA NANOPARTICLES[4]

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4.1 INTRODUCTION

Chronic Myeloid Leukemia (CML) is initiated once the chromosomal translocation t(9;22) takes place in hematopoietic stem cells, giving rise to the constitutively active BCR-ABL oncogene. This oncogene is a tyrosine kinase that aberrantly triggers the activation of multiple proliferation and cell survival pathways, and promotes the progression of the disease. Current drug therapies are based on the use of tyrosine kinase inhibitors (TKI), which target CML cells that remain trapped in the cycling proliferative state. However, a proportion of the transformed cells could become insensitive to the effect of these drugs mainly because they divide infrequently and remain in a quiescent state for weeks or even months [1], [2]. The bone marrow microenvironment provides a niche that promotes cell-to-cell and cell-to-matrix interactions that are responsible for maintaining hematopoietic and progenitor cells at quiescence and regulates their cellular renewal, differentiation and maintenance [3], [4], so that molecular interactions in this niche can also dictate the fate of cells embedded in this niche [5]. Among the factors that contribute to the engraftment of leukemic cells in the bone marrow are extracellular matrix components, including fibronectin and collagen, soluble factors, such as SDF-1, and stromal cells [3], [4].

When leukemia develops, components of this environment can reduce the therapeutic effects of anti-cancer drugs and protect the cells against the drugs [1]. This is the case as well in CML, where the bone marrow anchored leukemic cells are known to display resistance to TKIs (such as imatinib mesylate) and other chemotherapeutic drugs. This could explain, at least partially, why the full eradication of CML is not achieved in many cases [4]. To understand the means by which leukemic cells can bind to extracellular matrix components, Rainaldi *et al.* using flow cytometry and antibody staining, found that the cellular receptors for vitronectin, collagen and hyaluronan were not present on the cell surface of the widely used CML-model K562 cells, while the

fibronectin (FN) receptor (VLA-5; $\alpha_5\beta_1$) was the only cell membrane receptor expressed on these cells [6]. This suggests that the adhesion of this type of leukemic cells to extracellular matrix occurs preferentially through the integrin-FN interactions. There is, therefore, a need to better understand the effects of this particular interaction on the response of CML cells to current therapies.

Delivery of short interfering RNA (siRNA) against oncogenic targets is an emerging and promising therapy for the management of CML. The siRNA therapy has the potential to silence the expression of virtually any gene of interest in leukemia and to curb the uncontrolled proliferation of leukemic cells [1], [7], [8]. We have developed non-viral siRNA nanoparticles that effectively deliver nucleic acids (i.e., siRNA) to suspension-growing leukemic cells and induce silencing of the expression of particular genes, such as the *BCR-ABL* oncogene [3], [4], [9]. (This work is summarized in **Chapter 2** and **Chapter 3**)**.** As in other studies in the literature, our studies evaluated the efficacy of siRNA nanoparticles in suspension growing cells but no information exists on the response of adhered leukemic cells to siRNA therapy. Mimicking the bone marrow environment could be a valuable strategy for evaluating therapies that can promote the efficacy of emerging drugs in CML.

Towards this end, this study explored the modification of polytetrafluoroethylene (PTFE) films to make them more conducive for attachment of CML cells. We took advantage of the known role of FN in mediating attachment of CML cells to extracellular matrix, and created surface-aminated and covalent-grafted FN and FN-derived RGDS peptide on PTFE films. These types of modifications have been investigated previously in vascular grafts applications to promote endothelialisation [5], [10]-[12], but not to explore the attachment of leukemia cells and to evaluate the response to therapy under these conditions. In this study, the adhesion and growth of the CML model (K562 cells) on FN-grafted PTFE films was first explored. Next, the sensitivity to siRNA delivery was

evaluated on the adherent leukemic cells and compared with the effect on the suspension-growing cells.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The PTFE with a 250 µm of thickness was purchased from Goodfellow Corp. (Berwin, PA, USA) and sulfo-succinimidyl-4-(p-maleimidophenyl)butyrate (S-SMPB) from Life Technologies (Burlington, ON, Canada). 5-Bromosalicylaldehyde, 1-ethyl-3-(3-dimethyl-amonipropyl) carbodiimide hydrochloride (EDC), MES buffer, glutaric anhydride (GA), bovine serum albumin (BSA), trypsin/EDTA, formaldehyde solution, hexamethyldisilazane (HMDS), paraformaldehyde, spectroscopic grade dimethysulfoxide (DMSO) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). FN was acquired from ERRM (Université de Cergy-Pontoise, France). The CRGDS peptide (~600 g/mol) was purchased from Celtek Peptides (Franklin, TN, USA). The rabbit primary polyclonal antibody anti-FN (F3648) was purchased from Sigma-Aldrich and the anti-rabbit IgG horseradish peroxidase-linked secondary (HRP-IgG) antibody was from Amersham/GE Healthcare BioSciences (Piscataway, NJ, USA). The Amplex® Red reagent was from Life Technologies (Burlington, ON, Canada). The RPMI Medium 1620 with L-glutamine, Dulbecco's Modified Eagle Medium (DMEM) F12, Hank's Balanced Salt Solution (HBSS; without phenol red), penicillin (10,000 U/mL solution), streptomycin (10,000 µg/mL) and heat-inactivated fetal bovine serum (FBS) were purchased from Invitrogen (Gran Island, NY, USA). Recombinant human basic fibroblast growth factor (bFGF) was obtained form the Biological Resource Branch of NCL-Frederickton (Bethesda, MD, USA). Coomassie brilliant blue was purchased from Bio-Rad (Mississauga, Ontario, Canada). The control

(scrambled) siRNA was purchased from Invitrogen (Burlington, ON, Canada). The GFP siRNA (GFP-22) was from Qiagen (Toronto, ON, Canada).

4.2.2 Polymeric siRNA Carrier

A lipid-modified polymer, which consists of α-linoleoyl chloride (αLA) grafted on low molecular weight (MW = 2.0 kDa) polyethyleneimine (PEI2) by N-acetylation (PEI2-αLA), was used as the siRNA carrier and was prepared in house as follows: αLA (2 mM) and PEI2 (1 mM) were dissolved separately in anhydrous chloroform and kept in ice for 30 min. Hundred µL of triethylamine were added to the PEI2 solution before it was mixed with the αLA solution under stirring in ice. This mixture was then left stirring over night at room temperature. The crude product of PEI2-αLA was precipitated three times in ice-cold diethyl ether and dried under vacuum for 2 days. The structural composition of PEI2-αLA was analyzed by ^{1}H-NMR spectroscopy (Bruker 300MHz, Billerica, MA) using TMS as an internal standard in D_2O to calculate the lipid substitution levels. A substitution level of 2.72 lipids per PEI in PEI2-αLA was calculated based on this method.

4.2.3 siRNA/Polymer complexes: Preparation and Characterization

The siRNA binding capacity of PEI2-αLA and native PEI2 polymers was elucidated by agarose gel retardation assay as described previously [13]. Briefly, 4 µL of scrambled control siRNA (0.3 µg/µL) was diluted in NaCl (150 mM). Then, polymer solution (0.38 µg/µL) was added to the diluted siRNA solution to get final siRNA/polymer complexes ratios ranging from 0 to 1:1. After a 30-min incubation at room temperature, complexes were mixed with 4 µl of loading dye (30% glycerol) and loaded onto agarose gel (0.8%, EtBr at 2 µg/mL) prepared in TAE buffer (1X). The gel was electrophoresed at 120 mV for 30 min. siRNA bands were visualized under UV (Alpha Imager EC).

The stability of the siRNA in polymer/siRNA complexes was explored by serum digestion and quantified through gel retardation assay. Briefly, siRNA/polymer

complexes of ratio 1:5 (w/w) were prepared as described above. Then the complexes were incubated with fetal bovine serum (final concentration 50% v/v) at 37 °C for 24 h. The nuclease activity in serum was inactivated with 25 mM EDTA at 90 °C for 15 min, and then the complexes were dissembled with heparin (final concentration 50 U/mL) treatment for 1 h at room temperature. The crosslinked serum components were removed by centrifugation (2000 rpm, 5 min). Finally, the complexes were electrophoresed as described above and the siRNA recovery was quantified.

Hydrodynamic size (Z-average) and surface charge (ζ-potential) of the siRNA/polymer complexes (1:12 w/w) was assayed in ddH2O using dynamic light scattering (DLS) and electrophoretic light scattering (ELS) using Zetasizer Nano-ZS (Malvern, UK) equipped with He-Ne laser and operated at 10 mW.

4.2.4 Preparation of PTFE Films and Surface Functionalization with Plasma Treatment

The PTFE films (3 X 3 cm) were washed successively with acetone, water and methanol in an ultrasonic bath for 10 min and air-wiped between steps. Clean PTFE films were stored under vacuum until use. XPS analysis confirmed the efficiency of the cleaning procedure as the survey spectra revealed the presence of only carbon and fluorine in the appropriate stoichiometric ratio. For the surface modification with amine groups, PTFE films were placed on the grounded electrode of a parallel-plate dielectric barrier discharge (DBD) reactor and treated in a gas environment containing 5% of H_2 and 95% of N_2. The gas flow was introduced directly between the electrodes through a diffuser and was kept constant at 10 L/min. The mixture flowed through an oxygen trap (Restek, Bellefonte, PA, USA) to nominally reduce the O_2 content of the gases to ppb levels. The frequency, gas, gap between electrodes and time were kept constant (3 kHz, 2 mm, 45 s), while the applied voltage was varied to ensure that the same discharge power (or energy) was dissipated on the samples in the different atmospheric conditions

(P=0.25 W/cm^2, E= 15 J/cm^2).

The amine (NH_2) surface concentration of plasma-treated PTFE films was quantified by vapor-phase chemical derivatization using 5-bromosalicylaldehyde followed by XPS analysis (below) to record survey spectra as described previously [3], [4], [14]. Taking into account the nine newly bounded atoms upon reaction of 5-bromosalicylaldehyde with the amino groups of the surface, the relative amine surface concentration was be determined using the following equation: $\%NH_2 = [\%Br/(100 - 9 \times \%Br)] \times 100$.

4.2.5 Gluteraldehyde (GA) Grafting on PTFE Films

Plasma-treated PTFE films were immersed in a GA solution at 85 mg/mL under agitation. Fresh GA solution was added two more times 20 and 40 min after the beginning of the reaction. Sixty minutes after, the reaction was stopped and the films were successively washed and vortexed three times with acetone and once with deionized water. The films were then air-wiped and kept under vacuum. Prior to FN grafting, carboxylic acid functionalities of GA-grafted PTFE films were activated with excess of EDCA in MES buffer (0.1 M, pH 4.75) under agitation and at room temperature. To avoid water-induced hydrolysis, EDCA was added three times every 10 min. After 30 min of the reaction, the films were washed with MES buffer and used immediately for FN grafting [1], [15].

4.2.6 S-SMPB Grafting on PTFE Films

Plasma-treated PTFE films were individually covered with 600 µL of 3 mg/mL S-SMPB (referred as SMPB from now on) in phosphate buffered saline (PBS) and incubated for 2 h at room temperature, under agitation and protected from light. The SMPB-grafted PTFE films were then rinsed in PBS, air-dried and used immediately for FN grafting (Protocol modified from [4], [11]).

4.2.7 FN and RGD Peptide Grafting on PTFE Films

FN was purified from human plasma by a three-step combination of gelatin and heparin-cellufine affinity chromatography as described previously [6], [16]. This protocol yielded a highly purified FN (>99.5%) devoid of blood plasma contaminants. The purified FN was filtered through a 0.2 mm filter and stored at 8 ºC in 10 mM Tris buffer, pH 7.4. GA and SMPB modified PTFE films were reacted with 3 µg/mL of FN in PBS 7.4 solution for 3 h under agitation at room temperature. After the reaction, the films were washed and vortexed five times with PBS. For peptide grafting, PTFE films previously modified with SMPB were individually covered with 600 µL of 20 µM solution of CRGDS peptide in PBS and incubated for 3 h at room temperature (protocol adapted from [11]). Peptide-grafted films were then rinsed in PBS, air dried and stored under vacuum. A schematic representation of the chemical modifications performed on plasma-treated PTFE films is shown in **Figure 4.1**.

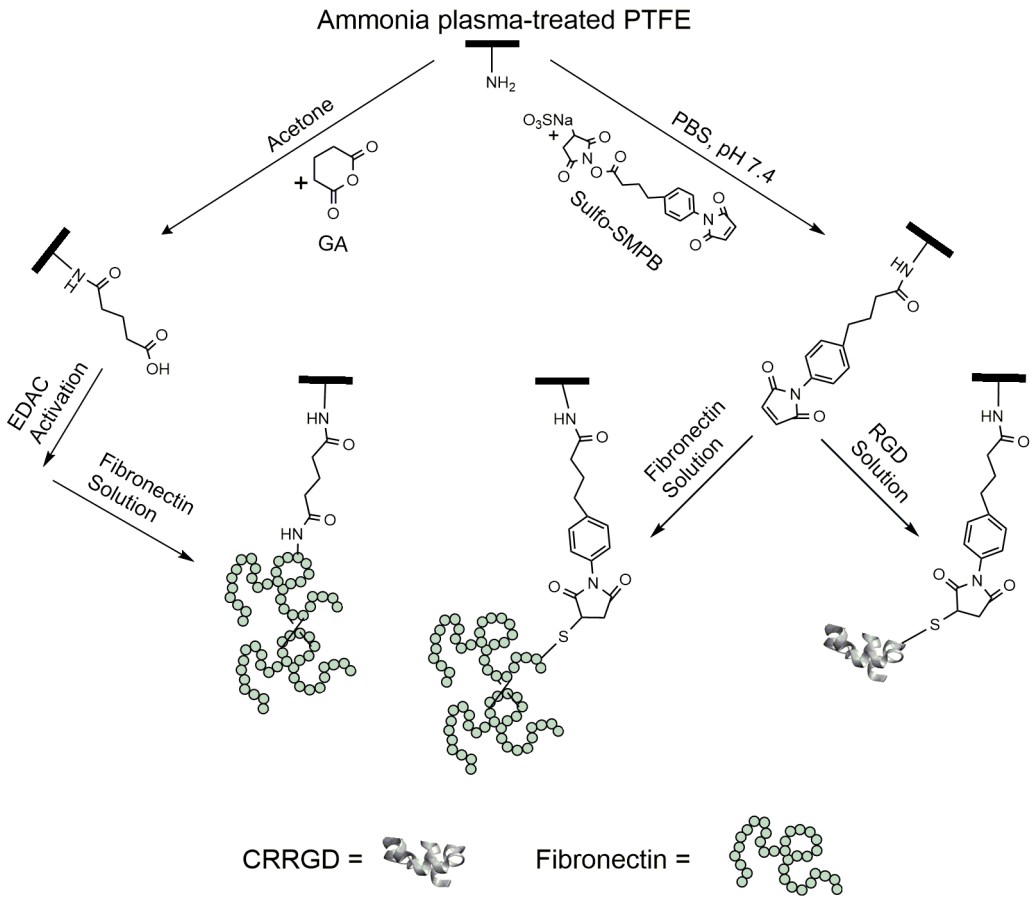

Figure 4.1. Schematic representation of conjugation methods to covalently graft fibronectin or CRGDS molecules on plasma-treated modified PTFE films. Two chemical crosslinkers were used for fibronectin grafting: GA (left) and sulfo-SMPB (right).

4.2.8 XPS Analysis

XPS analysis was performed at each step of the surface modification process. A PHU 5600-ci spectrometer (Physical Electronics, Eden Prairie, MN) was used to record the spectra. A monochromatic aluminum X-ray source (1486.6 eV) at 300 W was used along with a charge neutralizer to record survey spectra while a monochromatic magnesium X-ray source (1253,6 eV) at 300 W was used to record high-resolution spectra. The detection angle was set at 45° with respect to the normal of the surface. Three survey and high-resolution spectra were obtained for each sample.

4.2.9 ELISA Assay for Bound FN

The untreated PTFE and treated PTFE films were subjected to ELISA analysis for FN quantitation. The films were placed in 96-well plates and blocked with 1% BSA in PBS for 1 h at room temperature and rinsed once with PBS. The rabbit primary polyclonal antibody against FN (1:2500 dilution) was added on the films and incubated for 2 h at room temperature. After primary antibody was rinsed three times with PBS, the anti-rabbit secondary antibody HRP-IgG was added at a dilution of 1:3000 and incubated for 2 h at room temperature. Films were rinsed three times with Tris buffer and then transferred to new wells. Finally, 100 µL of Amplex red solution (50µM containing 1 mM H_2O_2) was added to each film and incubated for 30 min in the dark at room temperature. A fixed amount of volume was transferred from each well to new wells and fluorescence intensity was read at 530/590 using a BioTek FL600 reader.

4.2.10 Water Contact Angle

Static contact angle measurements were performed on the samples using a VCA 2500 XE system (AST, Billerica, MA). Drops of deionized water (1 µl) were deposited on surfaces. At least three drops per samples and three samples per modification step were analyzed.

4.2.11 Cell Culture

K562 cells, which are derived from a *BCR-ABL* positive CML patient in blast crisis [17], were used as the CML model. A GFP-expressing K562 cell line (GFP-K562) was generated by transduction of K562 cells with a retroviral vector containing the green fluorescent protein (GFP) gene [18]. GFP-K562 cells were kindly provided by Dr. Xiaoyan Jiang (Terry Fox Laboratory, British Columbia Cancer Agency, Canada). GFP-K562 cells were used as the silencing model due to convenience of assessing GFP silencing. GFP-K562 cells were maintained in RPMI medium containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin under incubation at 37 °C and 95% air/5% CO_2. Twice per

week, cells were diluted 10 times (1 x 10^6 cells) in 20 mL of fresh medium for cell expansion or as noted for cell seeding.

Rat bone marrow stem cells (rBMSC) were isolated from both femurs of the female Sprague-Dawley rats (rats obtained from Biosciences; U. of Alberta). The isolation protocol was described in detail previously [19]. rBMSC were maintained in culture with DMEM/F12 containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and supplemented with 5 ng/mL bFGF under incubation at 37 °C and 95% air/5% CO_2. On confluence, the cells were trypsinized with 0.08% trypsin/0.04% EDTA, typically diluted four times with the same medium and sub-cultured on 25 cm^2 flasks with 10 mL of fresh medium.

4.2.12 Cell Adhesion Assay

The PTFE films were cut with a hole puncher (~6.4 mm diameter cylinders) and washed with sterile HBSS. In 96-well plates, 100,000 rBMSC or 15,000 K562 cells suspended in 100 µL of respective media were seeded on PTFE films. After one day, unattached or loosely bound cells were removed with a HBSS wash, and attached cells were fixed with 3.7% (v/v) formaldehyde solution and then stained with 0.1% of coomassie brilliant blue. Staining solution was washed out with HBSS, and films were then transferred to new plates and imaged using a scanner Epson Perfection V550 Photo. As controls, unmodified PTFE, adsorbed FN (ads FN) on plasma-treated PTFE films (no crosslinker) and plasma-treated PTFE films with grafted GA (no FN) were used.

4.2.13 Scanning Electron Microscopy (SEM) Analysis

GFP-K562 cells suspended in 300 µL of culture media (~2 x 10^5 cells/mL) were seeded on PTFE films (11 mm of diameter) placed in 48-well plates. One day after cell adhesion, films were fixed overnight at 4 °C with 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer and washed with 0.1 M phosphate buffer

for 10 min (3x). For dehydration, films were immersed in aqueous solutions of increasing alcohol concentrations for 10 min, followed by immersion in aqueous solutions of decreasing alcohol and increasing HMDS concentrations for 10 min. Dry specimens were glued on SEM stubs and sputter coated with gold/palladium. SEM images were acquired using a Philips XL30 SEM (Microscopy Facility of the Department of Biological Sciences at the University of Alberta).

4.2.14 MTT Assay for Cell Growth

In 96-well plates, 10,000 K562 cells in 100 µL of culture medium were seeded on the desired PTFE films. After 24 hours of incubation, unattached cells were washed with HBSS and films were transferred to new wells. At desired time points (1, 5 and 7 days after cell seeding) MTT solution (5 mg/mL) was added to the wells to give a final concentration of 1 mg/mL and the cells were incubated for 1 hour further, after which the medium was removed and the formed formazan crystals were dissolved with 100 µL of DMSO. The PTFE films were removed from wells and the absorbance of the wells was measured with an ELx800 Universal Microplate Reader (BioTek Instruments, Vermont, USA) at 570 nm.

4.2.15 Transfection of K562 Cells with siRNA/lipid-polymer Complexes

The desired siRNAs and polymers (PEI2-αLA) were dissolved in nuclease-free water at 0.14 and 1 µg/µL, respectively. For preparation of siRNA/polymer complexes, siRNA solutions were first diluted in RPMI medium and the polymer solution were added to the diluted siRNA solutions. Solutions were mixed briefly with vortex and incubated for 30 min at room temperature to allow complex formation. RPMI medium alone (no complexes) was used for no treatment (NT) control groups, while Control (scrambled)-siRNA/polymer complexes were used as negative controls. The siRNA:polymer (weight:weight) ratio was 1:12 and siRNA concentration in medium was 60 nM during cell treatments. The 1:12 siRNA:polymer ratio used corresponded to 48.6:1 N:P ratio

(assuming 43.1 Da for single unit of PEI, 22 bp for siRNA with 2 phosphates per base pair). The effective polymeric carrier, optimal siRNA:polymer ratio and siRNA concentration were chosen based on previous studies using the same CML model [9].

For assessment of GFP silencing, GFP-K562 cells suspended in 300 µL of culture media (1 x 10^5 cells/mL) were seeded on PTFE films (11 mm of diameter) in 48-well plates. Twenty-four hours after cell seeding (unattached cells were not removed), 100 µL solutions containing GFP-siRNA/lipid-polymer complexes were added to cells and incubated at 37°C. To take account for unspecific interactions of siRNA particles with films and siRNA effect on cells grown in suspension (positive control), cells growing in suspension in 48-well plates were similarly transfected with GFP-siRNA/lipid-polymer complexes. Silencing effect of complexes was evaluated by quantifying the reduction in GFP fluorescence in GFP-K562 cells of unattached (suspension growing) and attached cell fractions. For this, four days after transfection, cells growing in suspension were harvested and washed twice with HBSS by centrifugation (1400 rpm, 5 min) and fixed in 3.7% formaldehyde solution. Cells attached to films were trypsinized, fixed and suspended in 3.7% formaldehyde solution. GFP silencing was assessed by flow cytometry (Cell Lab Quanta Sc., Beckman Coulter) using the FL-1 channel. Percent decrease in mean fluorescence was calculated as follows: 100-([Mean FL1 of cells treated with GFP siRNA/polymer] / [Mean FL1 of cells treated with scrambled siRNA/ polymer] × %).

For assessment of long-term GFP silencing in attached cells, GFP-K562 cells suspended in 300 µL of culture media (1 x 10^5 cells/mL) were seeded on PTFE films (11 mm of diameter) in 48-well plates. Twenty-four hours after cell seeding, unattached cells were removed with HBSS and the films were transferred to new wells. On day five, fresh new media was added to cells. On day seven, spent media was removed, films were washed with HBSS, and 300 µL of fresh media was added before 100 µL solution

containing GFP-siRNA/lipid-polymer complexes were added to cells. For the cells grown in suspension, 300 µL of 1 x 10^5 cells/mL were transfected with 100 µL solution containing GFP-siRNA/lipid-polymer complexes. The GFP fluorescence in this assay was quantified by a plate reader (Assent, Thermo Scientific) at excitation and emission wavelengths of 485 nm and 527 nm, respectively. To quantify the GFP silencing, fluorescence values were normalized to the NT samples and the percent decrease in mean fluorescence was calculated as follows: 100-([Mean FL1 of cells treated with GFP siRNA/polymer] / [Mean FL1 of cells treated with scrambled siRNA/ polymer] × %).

4.2.16 Statistical Analysis

The data was summarized as the mean of the measured variables with the error bars representing one standard deviation. Where stated, the data between controls and treatment groups were analyzed for statistical difference by Student's t-test (two-tailed distribution and unequal variance). The level of significance was set at $p < 0.05$.

4.3 RESULTS and DISCUSSION

4.3.1 siRNA/polymer complexes characterization.

Fraction of unbounded siRNA was quantified to determine the binding capacity, which was based on BC_{50}; i.e., siRNA/polymer ratio required for 50% siRNA binding. As expected, binding capacity of PEI2-αLA was less (BC_{50} =0.25 ± 0.01) than native PEI2 (BC_{50} =0.16 ± 0.02) (**Figure 4.2A**). This might be the consequence of primary amine consumption and steric hindrance generated by aliphatic chain. We have been observing this phenomena in our studies [13]. In the serum digestion studies, it was found that siRNA incubation in serum completely degrades the siRNA (as expected), and that the polymers (PEI2 and PEI2-αLA) were able to protect the encapsulated siRNA from the endonuclease activity of the serum (**Figure 4.2B**). Therefore, the polymer increased

the stability of the siRNA under mimicked physiological conditions. Then hydrodynamic sizes of siRNA/polymer complexes were assessed. As usual the size of siRNA/PEI2-αLA complexes was significantly smaller (118.5 ± 13.0 nm) than the size of siRNA/PEI2 complexes (712.4 ± 17.9 nm). It is believed that the lipid grafting enabled the shrinkage of the complexes due to lipophilicity of the polymers (**Figure 4.2C**). Lipid grafting generally increases the surface charge of siRNA/polymer complexes as the higher the grafting the higher the cationic charge density. The higher cationic charge density of siRNA/PEI2-αLA complexes (38.8 ± 2.6 mV) compared to (31.9 ± 0.7 mV) might be a consequence of smaller size, which helps to concentrate the surface charge into a smaller surface area. These results are in agreement with our previous reports [13].

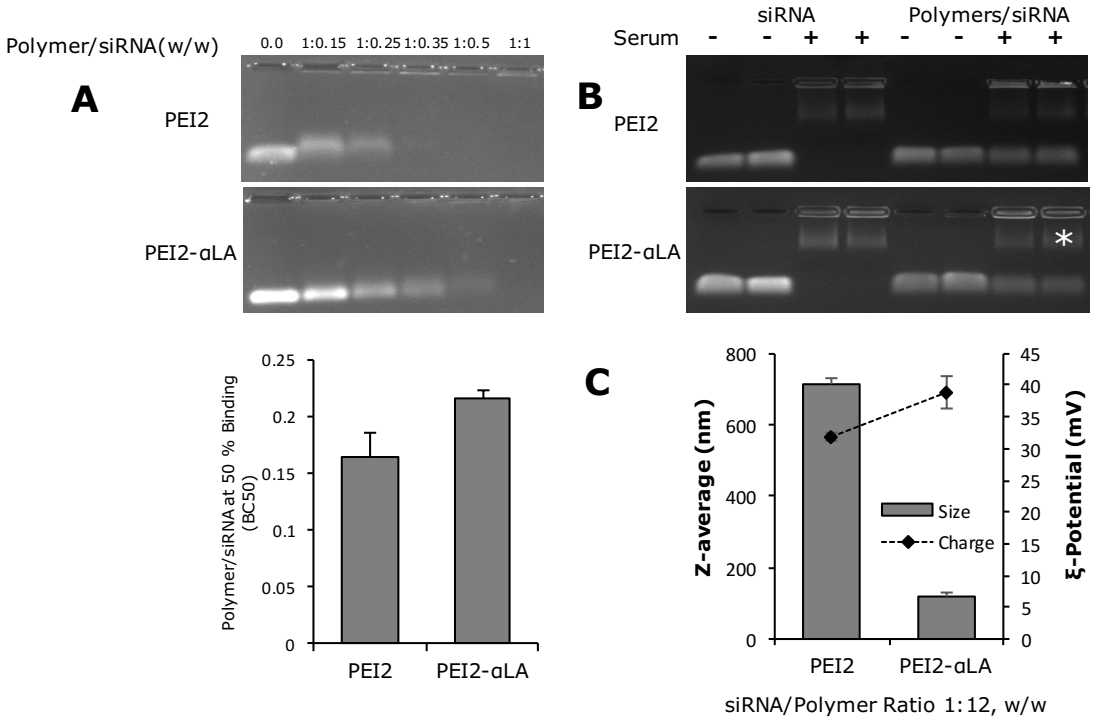

Figure 4.2. Characterization of siRNA/polymer complexes. Characterization of siRNA/polymer complexes. **A**) siRNA binding capacity of PEI2 and PEI2-αLA as a function of siRNA/polymer weight ratio (top). BC50: siRNA/polymer weight ratio at 50% binding of PEI2 and PEI2-αLA (bottom). **B**) Serum stability of siRNA/polymer complexes of native PEI2 and PEI2-αLA. Recovery of intact siRNA from complexes after 24h exposure to 50% FBS at 37 °C (by agarose gel electrophoresis). * Serum background (not shown). **C**) Size (nm) and surface charge of complexes (siRNA/polymer= 1:12 w/w) prepared with PEI2 and PEI2-αLA.

4.3.2 FN Grafting on Aminated-PTFE Films

For FN conjugation, PTFE films were first treated with N_2/H_2 plasma to allow the addition of amine groups on their surface so that further chemical groups can be grafted. The covalent binding of FN on amine groups of PTFE films was realized by the GA and SMPB crosslinkers. These two crosslinkers were used as each one reacts with different sites of the FN molecule: the aldehyde groups of GA react with primary amines of FN, whereas the maleimide of SMPB can react with the thiol groups of FN. These two crosslinkers will therefore lead to different FN conformations and orientations on the surface, which may alter FN interactions with the cells [20]. To ensure that each chemical reaction took place, surface composition was assessed with XPS at each modification step (**Table 4.1**). Starting with the unmodified (clean) PTFE films, where the F/C ratio of 2 was expected, the films showed the addition of N and O components with a decrease in the percentage of fluorine after amination, indicating the proper fluorine etching and introduction of amines to PTFE surfaces. Moreover, the calculations derived from the chemical derivatization showed that plasma-treated PTFE films had relative amine surface percentages ranging from 3.4 to 6.5%. These amine percentages are in agreement with previous studies [15]. After grafting of plasma-treated PTFE films with GA or SMPB, an increase in the percentage of O was seen due to the expected contributions of Os from the crosslinkers GA or SMPB.

Element (%) / Surface	C	F	N	O
Clean PTFE	31.8 ± 1.8	68.2 ± 1.8	-	-
Plasma-treated PTFE	54.0 ± 1.0	37.6 ± 0.9	6.1 ± 0.3	2.0 ± 0.4
PTFE + GA (with pt)	57.6 ± 1.4	27.3 ± 5.3	3.4 ± 0.3	11.2 ± 4.0
PTFE + SMPB (with pt)	64.2 ± 1.1	17.1 ± 1.9	6.1 ± 0.5	12.3 ± 1.9
PTFE + Ads FN (no pt)	55.6 ± 5.8	25 ± 3.6	6.3 ± 1.3	12.8 ± 0.9
PTFE + GA + FN	62.0 ± 1.8	18.6 ± 1.2	5.1 ± 0.6	14.1 ± 0.2
PTFE + SMPB + FN	62.7 ± 1.8	18.3 ± 1.7	6.5 ± 0.9	11.85 ± 1.8
PTFE + SMPB + RGD	63.6 ± 2.1	18.4 ± 2.9	5.9 ± 0.6	11.5 ± 0.4

Table 4.1. Surface chemical composition assessed by XPS survey spectra for PTFE before and after each modification step. Plasma treatment (pt).

Since no major changes were seen in the XPS-based atomic percentages when the FN was grafted to the films, an ELISA assay was subsequently performed to detect FN grafting, using PTFE (clean, no plasma) and plasma-treated PTFE films with adsorbed FN, and PTFE films where the FN was covalently bound with GA or SMPB (**Figure 4.3**).

The negative controls, clean PTFE and PTFE+GA films, gave no detectable FN (background fluorescence), and a reasonable amount of FN was evident on FN-adsorbed clean PTFE films (no plasma treatment). The plasma treated PTFE films gave higher FN detection (compared to non-treated films), where the values in FN adsorbed and, GA and SMPB grafted FN surfaces were equivalent. These grafting efficiencies were consistent with previous studies [15].

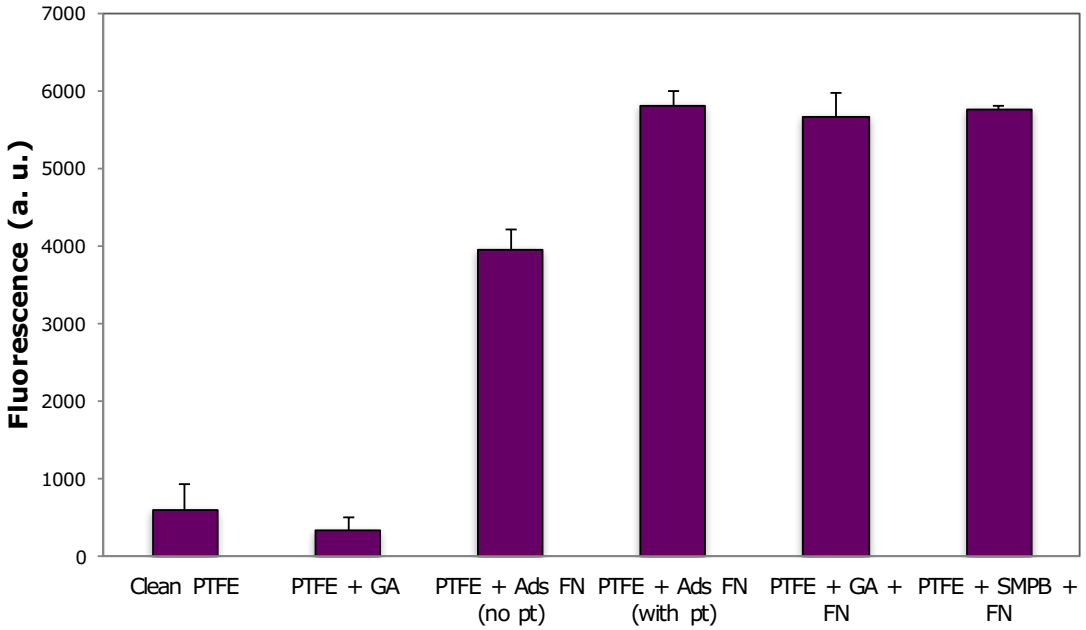

Figure 4.3. FN quantification after FN was adsorbed on films untreated or treated with plasma (pt), or grafted on GA and SMPB modified PTFE films. Negative controls were unmodified (Clean) PTFE films or GA-modified PTFE films. The FN was detected by ELISA assay using a polyclonal antibody against FN.

Samples were tested by contact angle measurements to assess the hydrophobic/hydrophilic nature of different conditions. As shown in **Table 4.2**, the wettability for Clean PTFE surface was as expected 127.9 ± 2.1° due to its hydrophobic nature, while the wettability of the plasma-treated PTFE with adsorbed FN (PTFE+Ads FN) was 115.8 ± 3.4° and, of the plasma-treated PTFE with FN grafted with SMPB (PTFE+SMPB+FN) was 75.7 ± 2.3°. Plasma treatment and FN adsorption increased slightly the hydrophilicity of the PTFE surfaces, while a more significant effect in hydrophilicity was recorded when the FN was grafted with SMPB crosslinker. These results indicated that modifying PTFE surfaces with plasma treated and grafting with FN increased substantially the hydrophilicity to make them more suitable for downstream evaluation.

Surface	Clean PTFE	PTFE+ Ads FN	PTFE + SMPB + FN
Contact angle	127.9 ± 2.1	115.8 ± 3.4	75.7 ± 2.3

Table 4.2. Contact angle measurements. Mean ± standard deviation (n=3).

It is known that FN is a highly flexible molecule, which undergoes conformational changes upon interaction with a surface. The conformational changes are dependent on the surface details and affect its biological activity [20], [21]. PTFE (untreated) is a highly hydrophobic polymer and is often regarded as chemically inert, however its surface is not inert to protein adsorption under *in vitro* and *in vivo* conditions [22]. While non-specific FN adsorption can be significant on hydrophobic PTFE surfaces, as shown in **Figure 4.3**, other studies have shown that cell adhesive and growth abilities of adsorbed FN on hydrophobic surfaces was lower than that of the FN adsorbed on hydrophilic surfaces [22]-[24]. Upon contact with different surfaces, FN acquires a conformation that is dependent on the properties of the surface and, in the case of FN adsorbed on the hydrophobic PTFE, the FN conformation is thought to be in an inactive state due to inaccessibility of cell-binding regions to cellular adhesion receptors [22], [23].

Amination of the PTFE surfaces with N_2/H_2 plasma, on the other hand, reduces the surface hydrophobicity by introducing polar amine groups on the surface that will allow FN adsorption or chemical immobilization. Increased FN adsorption on plasma-treated PTFE films most likely took place through the electrostatic interactions between the introduced amino groups (cationic) with the negatively charged amino acids of FN (in addition to the hydrophobic interactions inherently existent with the PTFE surface). This type of binding is unspecific with random interaction along the protein chain and the protein should be in different conformations on the treated surface. FN grafting by

covalent conjugation through either GA or SMPB is expected to be more stable and less prone to detachment [25]. Moreover, since the crosslinkers will bind to FN at specific sites, the grafted FN conformation and orientation on the surface is expected to be more uniform throughout the surface. Such differences in FN conformations upon FN adsorption or covalent grafting are bound to affect the biological activity of FN ultimately [26].

4.3.3 Adhesion and Growth of CML cells on FN-grafted PTFE Films

FN-grafted PTFE films were tested for cell attachment with K562 cells as well as rBMSCs. The latter cells strongly depend on attachment for growth and were used as positive control for this cell adhesion experiment. Based on coomassie-blue stained cell mass in **Figure 4.4**, K562 and rBMSC cells appeared to show a similar adhesion pattern on the tested surfaces. The extent of cell attachment on Clean PTFE was almost negligible for both cell types. Cell adhesion on the films with adsorbed FN (PTFE+Ads FN) was sporadic and un-uniform with some variations in the attached cell mass among the replicates. The PTFE+GA films showed qualitatively similar cell attachment in comparison with adsorbed FN, while GA-grafted FN-PTFE (PTFE+GA+FN) films showed an increased cell density in comparison with PTFE+GA films, although not uniform among replicates. Grafting of FN with SMPB (PTFE+SMPB+FN films) allowed higher cell attachment of both rBMSC and K562 cells than the films where the grafting of FN was with GA (PTFE+GA+FN films). Moreover, grafting of RGD peptide with SMPB (PTFE+SMPB+RGDS films) also showed high cell attachment and it was comparable with that of cell attachment to PTFE+SMPB+FN films.

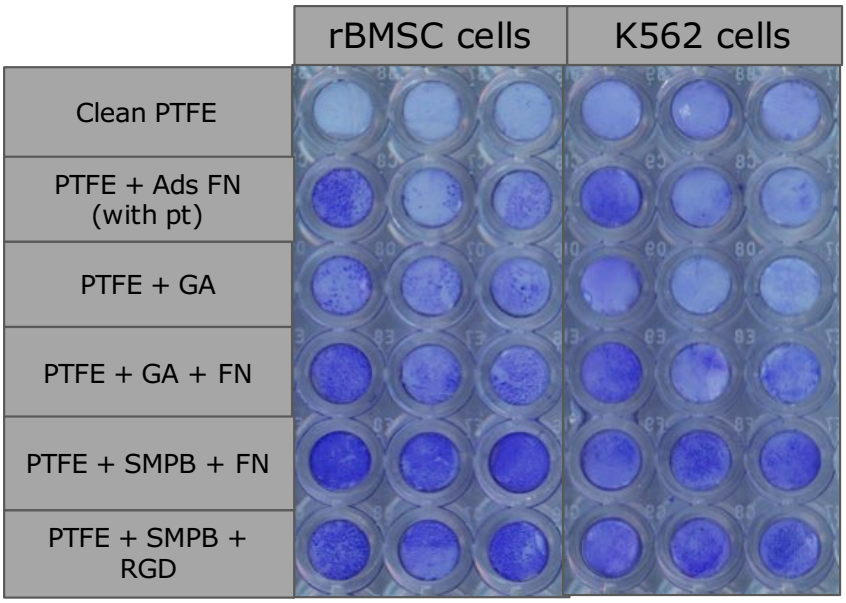

Figure 4.4. Adhesion of anchorage-dependent rat Bone Marrow Stem Cells (rBMSC) and non-anchorage dependent CML K562 cells on FN-modified PTFE films. The mass of attached cells was visualized after Coomassie Blue staining.

Further analysis of K562 adhesion on modified PTFE films was performed by SEM (**Figure 4.5**). In accordance with the results above, there was no cell attachment on the Clean PTFE surfaces (**Figure 4.5A**), while cell densities on the PTFE+GA+FN, PTFE+SMPB+FN and PTFE+SMPB+RGDS films were similar (**Figure 4.5A**). In the FN- or RGD-grafted cases, the cells appeared to interact with the films as it was evidenced by the presence of cytoplasmic projections coming out from the cells and microvilli that formed focal adhesions with the surface (**Figure 4.5B**). Moreover, some differences in the cell morphology were noted among the films. For the case of the PTFE+GA+FN and PTFE+SMPB+RGDS films (**Figure 4.5B**), cells were more cuboidal (3-dimensional) as projections from the cell bottom may be interacting with the surface, while for the PTFE+SMPB+FN films (**Figure 4.5B**), the cell morphology was flatter than the previous two surfaces, probably due to stronger interactions of the projections with the surface.

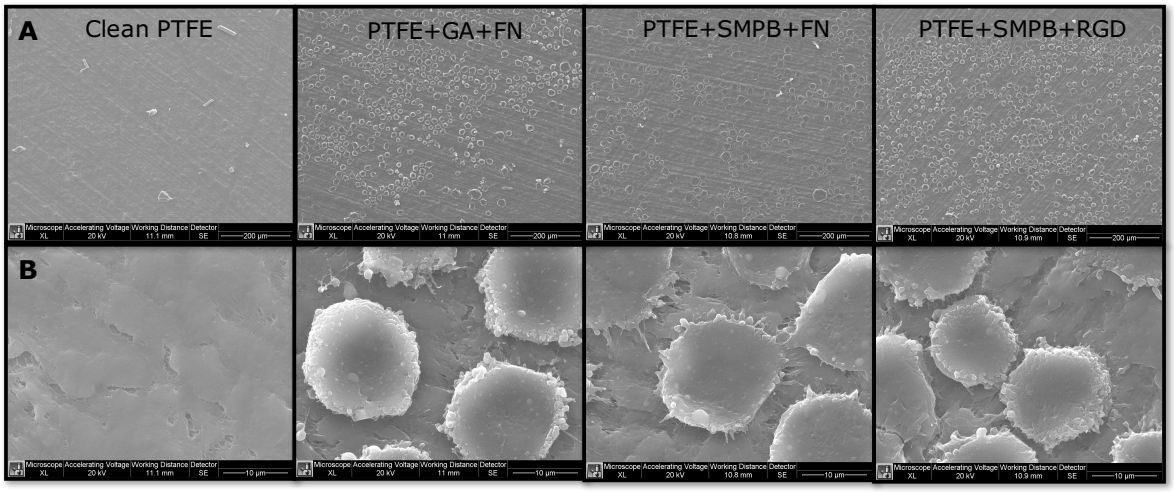

Figure 4.5. SEM images of unmodified (Clean PTFE) or FN- or RGD-grafted PTFE films. A) Wider field of view shows the cell density on the surface. B) Closer field of view shows the interaction of cells (i.e., cellular projections) with the modified PTFE films where FN or RGD were covalently grafted with SMPB.

Growth of adhered K562 cells on modified PTFE films was evaluated on days 1, 5 and 7 after cell seeding based on the MTT assay (**Figure 4.6A**). The MTT absorbance values on day 1 for the PTFE+GA, PTFE+GA+FN, PTFE+Ads FN, PTFE+SMPB+FN and PTFE+SMPB+RGDS films were higher than control films (Clean PTFE). The growth differences were noticeable among the treated films, where the growth was lower on the PTFE+GA+FN, and PTFE+Ads FN films, and higher for the last three groups, in which FN and peptides were covalently grafted with SMPB or GA (PTFE+GA+FN, PTFE+SMPB+FN, and PTFE+SMPB+RGDS). Moreover, in contrast to PTFE+GA+FN, cell growth was more consistent and greater on the PTFE+SMPB+FN and PTFE+SMPB+RGDS films, where the SMPB crosslinker was employed. This greater cell growth was evident by the significant difference ($p < 0.05$) on day 5 for PTFE+SMPB+FN, and on days 5 and 7 for PTFE+SMPB+RGDS films in comparison with Clean PTFE films. Based on the above results, PTFE+SMPB+FN films showed higher cell adhesion and growth of CML cells and were chosen for the remaining experiments of this study.

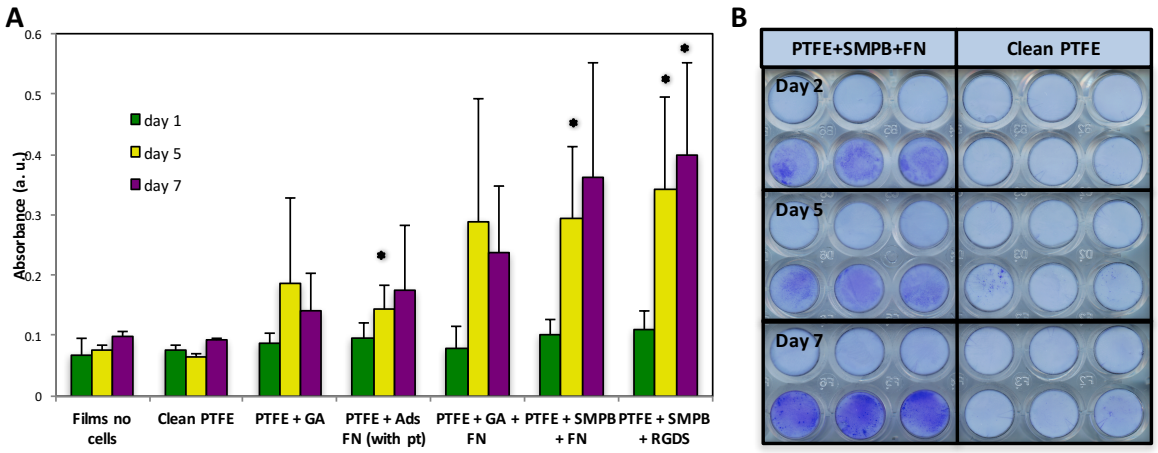

Figure 4.6. Growth of adhered K562 cells on FN-modified PTFE films. A) Growth of K562 cells measured by the MTT assay on days 1, 5 and 7 after cell seeding. Significant difference in comparison with Clean PTFE films is indicated by * ($p < 0.05$). B) Mass of adhered K562 cells on PTFE+SMPB+FN and Clean PTFE films on days 2, 5 and 7 after cell seeding. Cells were stained with Coomassie Blue.

As a validation of the MTT-based growth results, coomassie blue staining was performed on K562 cells seeded on the PTFE+SMPB+FN and Clean PTFE films on days 2, 5 and 7 after cell seeding. **Figure 4.6B** shows the stained PTFE+SMPB+FN and Clean PTFE films with no cells (upper rows) and with seeded K562 cells (lower rows). The lack of staining on the films with no cells is evident for all days (**Figure 4.6B**, upper rows), while on the Clean PTFE films where cells were seeded, there is no evidence of cell mass on day 2 and there are some sporadic traces of cell mass on days 5 and 7 (**Figure 4.6B**, lower rows). In contrast, for the case of the PTFE+SMPB+FN where K562 cells were seeded, the cell mass is clearly evident starting from day 2 and it increased over day 7 (**Figure 4.6B**, lower rows).

When Clean PTFE films are incubated with medium containing 10% serum, they may be susceptible to unspecific protein adsorption and, serum FN may adsorb to surface and promote cell adhesion [23]. Studies analyzing the protein absorption on unmodified PTFE surfaces have found that the amount and type of bound proteins is what

determines the cell attachment [22]. The Clean PTFE films did not support cell adhesion initially, and it is likely that the amount of FN adsorbed on the hydrophobic surfaces is too small since albumin, the most abundant protein present in serum, will compete with FN for adsorption due to its preferential affinity to hydrophobic surfaces [23], [27]. In PTFE+Ads FN films, there might be also a low amount of cell-binding inducive FN conformation with exposed cell binding sites [22], [27]. Therefore, the small amount of FN adsorbed or low availability of cell-binding epitopes might not be sufficient for cellular binding [22]. The PTFE+GA films support a certain degree of cell adhesion and cell growth, similar to PTFE+Ads FN films even though the PTFE+GA films do not have FN grafted. The increase of hydrophilicity after the plasma treatment may have allowed some FN adsorption from the serum that led a low degree of cell adhesion in this case. Regardless of the increased hydrophilicity after plasma treatment, uniform cell adhesion and robust cell growth was only possible to FN (and RGD) grafting. It was interesting to note that even the Clean PTFE surface had some sporadic growth on long-term studies. Such surfaces might be sufficiently 'conditioned' for cell growth with time, or 'select' specific population of cells for growth on this unique surface. Finding the reason(s) for this type of limited growth was considered beyond the scope of this study, but it will be important to pursue for better understanding of the response of sub-populations of CML cells.

4.3.4 Effect of GFP-siRNA transfection on GFP-Positive Cells Adhered to FN-grafted PTFE Films

To investigate the effect of siRNA transfection with lipid-modified polymers on cells adhered to FN-grafted films, silencing effect was assessed in GPF-K562 cells seeded on PTFE+SMPB+FN films. (PTFE+Ads FN films were not used in the transfection experiments because, as discussed above, cell adhesion on these films was not uniform among replicates, and showed slower cell growth in comparison with FN-grafted films).

Cells seeded on FN-grafted PTFE films, Clean PTFE films and in suspension (no films) were transfected with GFP-siRNA/PEI2-αLA complexes one day after seeding. Subsets of cells that did not attached to films and attached cells were analyzed four days after transfection. Specific GFP silencing is summarized as percentage of decrease in mean GFP fluorescence in **Figure 4.7A**. For the unattached cells (**Figure 4.7A**), when the cells were grown in suspension (no film), a GFP silencing of 53.9±17.0% was found, while for cells that grew on films unattached, the GFP silencing was 33.0±6.6% for Clean PTFE films and 30.5±2.5% for SMPB+FN films (**Figure 4.7A**). This was significantly lower than the case in the absence of films. This may suggest that the efficiency of the polymer/siRNA complexes to interact with the suspension growing cells decreased due to the interaction of complexes with the PTFE surfaces. It may reflect a consequence of increased complex association with surfaces. The GPF silencing for cells attached to films (**Figure 4.7B**), was 40.6±43.2% for Clean PTFE and 24.2±24.3% for PTFE+SMPB+FN films (**Figure 4.7B**), indicating a similar level of silencing efficiency in this case. However, because the numbers of cells attached to the films were much lower than the numbers of cells growing unattached (~<1/3, not shown), the number of cells was sub-optimal for flow cytometry analysis, which resulted in large error bars during the assessment of silencing for attached cells. The apparent contradiction of cells present in Clean PTFE films can be explained by the fact that few cells that were detected soon after cell seeding (**Figure 4.4**) could have increased their presence at longer incubation times (**Figure 4.6B**) due to a minimal adsorption of FN molecules of the serum-supplemented cell culture media on the Clean PTFE films. Also, since flow cytometry is more sensitive to the cell detection, these cells were more evident in this experiment than in previous assays. While a useful observation on the efficiency of siRNA complexes on suspension growing cells was made in this experiment, we considered the short experimental period and subsequently low numbers of attached cells contributing to the large standard deviation in the outcome.

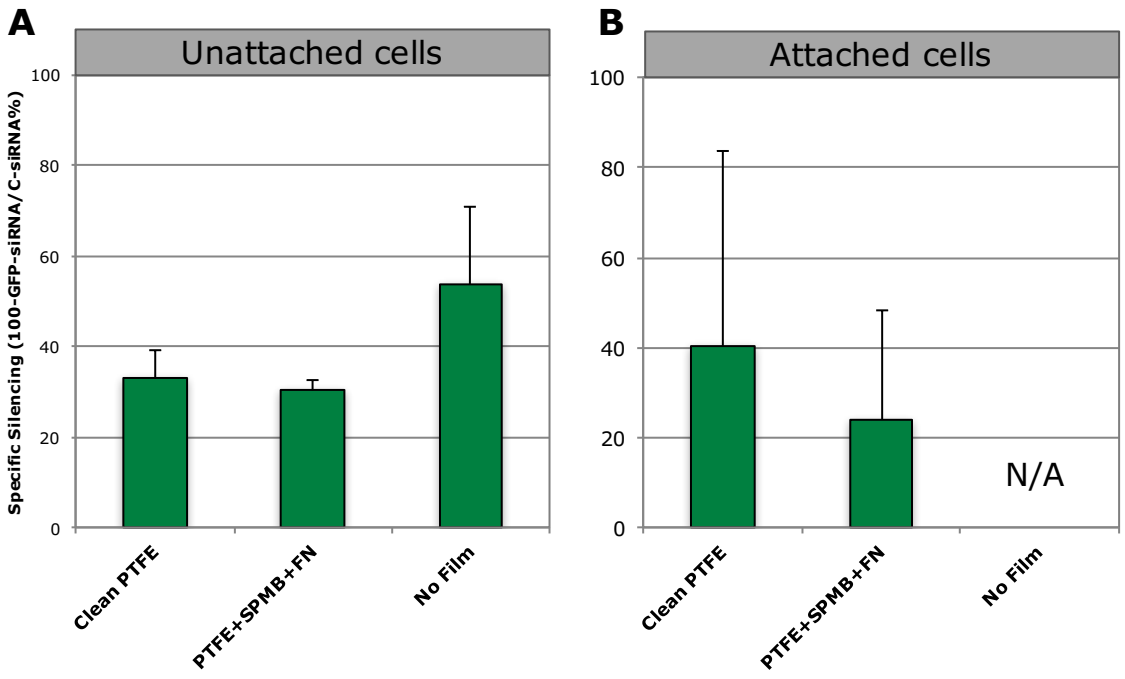

Figure 4.7. GFP silencing after GFP-siRNA nanoparticle treatment on short-term cell growth. One day after GFP-K562 cells were seeded on Clean PTFE, PTFE+SMPB+FN or in suspension (No Film), cells were treated with GFP-siRNA nanoparticles at 60 nM siRNA concentration (siRNA:polymer ratio of 1:12). Four days after transfection, GFP silencing (percent decrease in green fluorescence) was analyzed by flow cytometry of the cells unattached (A) and attached (B) to films. Large error bars in B are due to low cell numbers for optimal flow cytometry analysis. These results were from the average of 3 independent experiments.

Cells were subsequently allowed to grow for a week on films prior to transfection with GFP-siRNA/PEI2-αLA complexes. The GFP levels on the cells were assessed with a sensitive plate reader measurements four days after transfection since recovered cell numbers were again considered low for flow cytometry. The results for suspension growing cells or cells attached on films (Clean PTFE and PTFE+SMPB+FN) are summarized in **Figure 4.8**. The GFP silencing of the cells grown in suspension (No Film) was 36.4±9.0%, whereas for the cells that grew attached to films, the GFP silencing for Clean PTFE films was -7.6±3.5% whereas for PTFE+SMPB+FN films was 25.1±18.9% (**Figure 4.8**). There is no significant difference between the GFP silencing of

PTFE+SMPB+FN and No Film groups ($\rho>0.05$). Coomassie blue staining results four days after transfection confirm the presence of cells on PTFE+SPMB+FN films whereas only residual cells were present on Clean PTFE films (not shown). The lack of GFP silencing in Clean PTFE films was expected as only residual or no cells were found attached to these surfaces (**Figure 4.4** and **4.6B**). On the other hand, similar values of GFP silencing of cells attached via FN and cells in suspension may suggest that the transfection with the lipid-modified polymers is not affected by having cell attachment to surfaces.

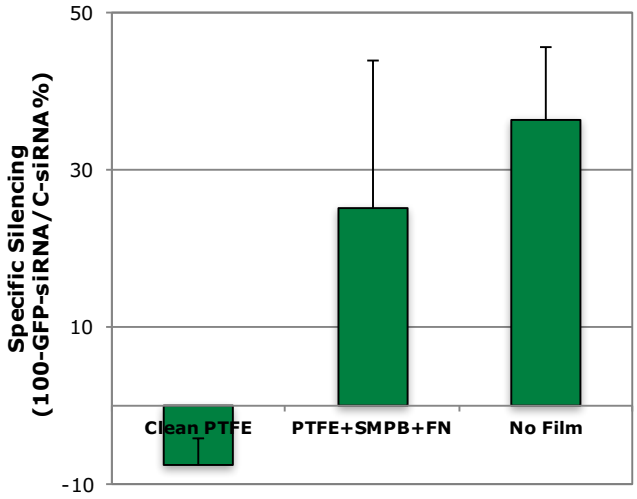

Figure 4.8. GFP silencing after GFP-siRNA nanoparticle treatment on long-term cell growth. One day after GFP-K562 cells were seeded on Clean PTFE, PTFE+SMPB+FN films and in suspension (No Film), unattached cells were removed. One week after, cells were treated with GFP-siRNA nanoparticles at 60 nM siRNA concentration (siRNA:polymer ratio of 1:12). GFP silencing (percent decrease in green fluorescence) was calculated as described in Materials and Methods using a plate reader. These results are the average of 3 independent experiments.

4.3.5 Other Studies Employing Cell Adhesive Systems

Several studies have shown that adhesion of hematopoietic cell lines to FN provides a survival advantage to cytotoxic treatments in comparison with cells grown in suspension [28]. Cells that are vulnerable to treatment with different drugs, can become resistant to drugs once grown adhered to FN [4]. In the case of K562 cells adhered to FN, a reduced cell death was seen in comparison with cells in suspension after treatment with chemotherapy drugs Imatinib and melphan, and the γ-irradiation [28], [29]. Previous studies suggested the underlying basis for the drug resistance is a crosstalk

between the β_1-integrin signaling and the BCR-ABL kinase activity. Van der Kuip *et al.* suggested the possibility of PI3K pathway activation to prevent apoptosis [28]. This adhesion-mediated resistance was found to be reversible, as once the β1 integrin receptors were blocked by antibodies, the unattached cells became sensitive to drugs again. These studies suggest that there might be ways to make FN-adhered CML cells drug sensitive again, such as by disrupting the B1-integrin mediated adhesion or downstream activators of the crosstalk with the BCR-ABL signaling. Reminiscent of siRNA-mediated silencing of BCR-ABL in K562 cells in previous studies, it might be useful to target adhesion receptors with non-viral siRNA delivery in order to reverse drug resistance. In contrast to reduced drug effects on FN-adhered CML cells, the GFP silencing by siRNA was not affected by the FN-mediated adhesion in this study, and it was comparable to GFP silencing efficiency in suspension cells.

Two other studies have delivered nucleic acids to adhered K562 cells. Yuan, *et al.* modified polycaprolactone (PCL) films via surface-initiated atom transfer radical polymerization of poly(glycidyl methacrylate) (PGMA) to covalently immobilize gelatin [30]. The authors used these scaffolds to evaluate (i) adhesion of K562 cells and (ii) transfection of cells with Lipofectamine 2000 complexes of plasmid DNA (not siRNA) [30]. K562 cells seeded on gelatin-functionalized PCL surfaces were able to adhere and proliferate while cells seeded on PCL did not proliferate. For the transfection studies, complexes were added on the scaffolds before or after cell seeding. The percentage of EGFP expression on transfected cells was slightly higher as compared to cells transfected on tissue culture plates: Percentage of EGFP-positive K562 cells was 7.4% and 4.7% on scaffolds and tissue culture plastic, respectively, while no transfection was observed on PCL [30]. The gene expression was not very efficient with this carrier, but no adverse effect of cell attachment on transfection was seen.

Another study from Hazlehurst *et al.* found that myeloma cells, which normally grow

in suspension, displayed lower sensitivity to VP-16 drug once adhered to FN (6% apoptotic cells of FN-adhered cells vs. 30% of suspension cells) and that this resistance was reversed once FN adhesion was blocked by β1 integrin antibodies (22% apoptotic cells). The authors found that the FN-adhesion mediated resistance was associated with growth and cell cycle arrest that prevented drug-induced apoptosis. To investigate this, authors targeted p27, a protein involved in impeding cell cycle progression, with antisense oligonucleotides (ASO) using a commercial transfection reagent before and after FN adhesion of myeloma cells. After the treatment, a 75% reduction of p27 protein levels was found in comparison with cells treated with mismatch ASO. More importantly, although the cell adhesion to FN was not altered, the decrease of p27 protein restored the cell drug sensitivity, and the apoptosis induced by the VP-16 drug increased from 8.8% in mismatch ASO to 22% of cells treated with p27 ASO [4]. This study showed that inhibition of the cell cycle progression induced by FN adhesion via β1 integrins cell receptors can be reverted by silencing p27 protein. This therapeutic approach could be used for myeloma cells residing in the bone marrow that interact with FN so that these myeloma cells can be sensitized to the effect of pro-apoptotic drugs.

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5. SIRNA-MEDIATED BCR-ABL SILENCING IN CHRONIC MYELOID LEUKEMIA PRIMARY CELLS WITH LIPID-MODIFIED POLYMERS

5.1 INTRODUCTION

Chronic myeloid leukemia (CML) is a malignant neoplasm characterized by the Philadelphia (Ph) chromosome at the myeloid hematopoietic stem cell level. The BCR-ABL fusion gene initiates and propagates the disease that leads to an eventual uncontrolled expansion of immature myeloid cells in the bone marrow as well as the bloodstream [1], [2]. Tyrosine kinase inhibitors (TKI) targeted against ABL tyrosine kinase (TK) have shown promise in treating CML, however subsets of patients – especially those in the accelerated and in blast crisis phases– are more likely to show early relapse and develop resistance to TKI treatment [1]-[3]. This resistance is often caused by mutations in the TK domain that impede drug binding [4]. Current strategies to treat CML use potent TKIs that target the main BCR-ABL point mutations, which include second-generation (dasatinib and nilotinib) and third-generation (ponatinib) TKIs [2], [5]. However, there is still a high risk for development of new point mutations if BCR-ABL continues to be expressed regardless of the therapy [1], [6]. Moreover, long-term toxicities particularly cardio vascular toxicity represent significant concern at the time of choosing a treatment [7]-[9]. Therefore, there is a need for novel drugs that promote the elimination of BCR-ABL$^+$ clones in order to prevent CML relapse.

Small interference RNA (siRNA) molecules are used to trigger the RNA interference mechanism to silence specific overexpressed genes. This technology has been widely explored to induce therapeutic effects in different types of leukemia in *in vitro* and *in vivo* models over the last fifteen years [10]. Among the siRNA-delivery systems used in CML studies reviewed in **Chapter 1**, all 32 studies routinely used cell line models to evaluate siRNA delivery systems to induce siRNA-mediated therapeutic effects. Moreover, 25 out of 32 (78%) studies used commercial transfection methods, including electroporation and liposomal transfection reagents. 7 out of 32 (22%) used non-commercial transfection reagents developed in investigator's labs, such as carbon

nanotubes, fusion peptides, lipid-based, and polymer-based vehicles [11]-[17]. However, among the reviewed studies only 9 out of 32 (28%) used siRNA-based silencing in CML patient cells [18]-[26]. Of the latter studies using CML primary cells, 9 of 9 used commercially available transfection methods that cannot be translated into clinics [67% studies (6 of 9) used electroporation, and 33% (3 of 9) used lipid/liposome-based transfection reagents]. These studies demonstrate that there is not yet a delivery system with potential for translation into clinics that delivers siRNA into a more relevant CML cell model such as CML patient cells.

I have been developing non-viral nucleic acid (siRNA or plasma DNA) delivery systems for the treatment of attachment-independent (suspension) cells, results which were summarized in previous **Chapters**. These delivery systems are based on the use of low molecular weight cationic polymer polyethyleneimine (PEI) as the backbone and the polymer modifications strategies to graft different lipid moieties onto amine groups of PEI that promote interaction with cell membrane and make nucleic acid delivery possible and effective [10], [27]. For siRNA delivery into suspension cells in particular, the design of delivery systems has been explored on cutaneous T-cell lymphoma (CTCL), acute myeloid leukemia (AML), and CML: Studies on CTCLs delivered siRNA with caprylic and linoleic acid substituted 2.0 kDa PEI (PEI2.0-CA and PEI2.0-LA, respectively) to Hut78 cells to induce silencing of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) or cyclin-depended kinase 18 (CDK18) genes which led to cell growth inhibition [28]. Studies on AML cells demonstrated that siRNA delivery with PEI2.0-CA, and to a slightly less extent PEI2.0-LA, sustained most silencing among the lipid-substituted PEI2.0 investigated for down-regulation of the C-X-C chemokine receptor type 4 (CXCR4) in THP-1 cells (which showed cell proliferation inhibition) [29], and in AML primary cells [30]. Furthermore, siRNA-mediated silencing of hyaluronic acid receptor CD44 was possible in CD34+ KG-1 and KG-1a cell lines and CD34+ AML primary cells using PEI2.0-

LA as the siRNA delivery system, which resulted in increased apoptosis and decreased cell adhesion [31]. These studies indicated the feasibility of silencing targets with the engineered lipid-modified polymers in patient-derived cells for the first time. For the case of CML, siRNA delivery with palmitic acid (PA)-substituted 1.2 kDa PEI [13] and α-linoleoyl chloride (αLA) substituted 2 kDa PEI [32] were used in the CML K562 cell line. Several desirable therapeutic outcomes were reported in these studies. However, previous studies did not employ patient-derived cells and whether the proposed siRNA delivery approach is functional in patient cells is not yet known.

This study investigated the use of lipid-modified polymers as siRNA delivery systems in cells isolated from patients with CML *ex vivo*. Nine different lipid-modified polymers were used in this study: the aliphatic lipids α-linoleoyl chloride (αLA) and linoleoyl chloride (LA), and the aromatic lipid cholesteryl chloroformate (Chol) were substituted on polyethyleneimine (PEI) with low molecular weights ranging from 0.6 kDa to 2kDa. The most commonly used lipid-based transfection reagent Lipofectamine® 2000 was used as a reference reagent. We performed analysis of siRNA-cell association and siRNA/polymer complex internalization, as well as the pharmacological outcomes after BCR-ABL siRNA delivery in terms of BCR-ABL silencing at the mRNA level, and growth and survival of CML primary cells.

5.2 METHODS

5.2.1 Materials

The Lymphoprep™ used in patient cell isolations was purchased from STEMCELL Technologies (Vancouver, BC). DNase I was from Sigma (D4513 Protein ≥80 %, ≥2,000 units/mg protein). Fetal Bovine Serum (FBS), Hanks' Balanced Salt solution (HBSS), RPMI Medium 1620 with L-glutamine, penicillin (10,000 U/mL solution), streptomycin

(10,000 µg/mL) from Thermo Fisher Scientific. BIT serum substitute (STEMCELL Tech.). IMDM (STEMCELL Tech.), β-mercaptoethanol (Thermo Fisher Scientific), L-glutamine (Thermo Fischer Sci.) and Flt3, IL6, IL3, and G-CSF from STEMCELL or Peprotech. Unlabeled scrambled siRNA (5'-GCGUAUUAUACGCGAUUAACG-3' and 5'-CGUUAAUCGCGUAUAAUACGC-3'), 5'-carboxyfluorescein (FAM)-labeled scrambled siRNA (5'-/56-FAM/CAGUCGCGUUUGCGACUGGUUTT-3' and 5'-AACCAGUCGCAAACGCGACUGTT-3'), and BCR-ABL siRNA (5'-GCAGAGUUCAAAAGCCCTT-3' and 3'-TTCGUCUCAAGUUUUCGGG-5') were custom synthesized from Integrated DNA Tech. (Coralville, IA). Lipofectamine® 2000 was purchased from Thermo Fischer Scientific.

5.2.2 Polymer Synthesis

Lipid modification of 0.6, 1.2 and 2.0 kDa PEIs (referred as PEI0.6, PEI1.2, and PEI2.0, respectively) was performed by N-acylation using the aliphatic lipids α-linoleoyl chloride (αLA) and linoleoyl chloride (LA), and the aromatic lipid cholesteryl chloroformate (Chol) as hydrophobic moieties as described before [32]-[34]. Briefly, each lipid (2.0 mM) and PEI (1.0 mM) solutions were dissolved separately in anhydrous dichloromethane and cooled in ice bath for 30 min. Trimethylamine (TEA, 100 µL) was added to PEI solution dropwise and homogenized. Lipid solution was then added to PEI solution under stirring in ice bath and left stirring overnight (18h) at room temperature. The lipid-modified PEI product was precipitated (3X) in cold diethyl ether and dried under vacuum for 48 h.

In addition to the modification described above, an additional carboxylic functionality was incorporated on LA through a thioester (-S-CO) linkage prior to grafting onto PEI. The thioester-containing lipids, LA and αLA were synthesized by coupling mercaptopropionic acid (MPA) with LA or αLA through thioester bonding as described previously [34]. For this, LA or αLA (332 µL, 1.0 mmol) and MPA (332 µL, 2.5 mmol)

were separately dissolved in trifluoroacetic acid (TFA, 600 µL). MPA solution was then added dropwise to LA or αLA solution and stirred for 4 h at room temperature in the dark. Thioester-modified LA (tLA) was precipitated (3X) in cold hexane and dried under vacuum for 48 h. tLA was grafted onto PEIs via N-acylation after EDC/NHS activation. Structural composition of lipid-modified PEIS was elucidated by ^{1}H-NMR spectroscopy (Bruker 300 MHz, Billerica, MA) using $CDCl_3$ and D_2O as solvents for tLA and lipid-substituted PEI, respectively. The corresponding resonance peaks in the ^{1}H-NMR spectra for lipid and PEI were used to calculate extent of lipid substitution.

5.2.3 Harvest of CML Patient Cells and Cell Culture

Peripheral blood (PB) or bone marrow (BM) from CML patient samples were obtained from newly diagnosed patients prior to TKI therapy at the University of Alberta Hospital. Clinical details of patient samples are shown in **Table 5.1**. Informed consent was obtained from patients according to the Declaration of Helsinki. The procedures were approved by the Ethics Committee of the University of Alberta Hospital (#Pro00043783). Samples were also provided from Dr. Xiaoyan Jiang's Lab at University of British Columbia, and from Dr. Michael Caligiuri's Lab at Ohio State University. Mononuclear (MN) cell fraction from fresh blood samples was immediately isolated by density gradient medium Lymphoprep™ as follows. PB or MB samples were incubated with 1.5 ml of DNase (~1mg/ml) for every 4 ml of blood and diluted with equal amount of 2% FBS in HBSS. Diluted blood was carefully layered on top of Lymphoprep™ (equal amount of undiluted blood) and centrifuged at 800g for 20 min at room temperature with the brake off. Upper plasma layer was removed and discarded. The plasma:Lymphoprep™ interface (containing MN cells) was transferred to a new tube, leaving behind the erythrocyte/granulocyte pellet. MN cells were washed with 2% FBS in HBSS and centrifuged at 300g for 10 min at room temperature. Supernatant was removed and viable cell numbers were counted by trypan blue staining exclusion and hemocytometer

before cell culture.

For the MN cells obtained from frozen samples, the cryovial was quickly thawed in a water bath at 37 °C (without dissolving ice completely), and wiped down with 70% alcohol. Thawed cells were carefully added dropwise to a 1 ml of DNase solution (for every 1 ml of cell suspension) and incubated for 2-4 min at room temperature to dissolve completely any clumps. FBS was added dropwise to the cells/DNase mixture (5 ml of FBS for every 1 ml of cell suspension). Cell suspension was distributed in 1.5 ml tubes, and spun down at 300g for 10 min at 4 °C. Supernatant was carefully removed and the tube was flicked to re-suspend cells in left over fluid. Pellets were combined and cells were counted by trypan blue staining and hemocytometer before cell culture.

For $CD34^+$ enriched populations, $CD34^+$ cells were isolated from MN cell fraction by immunomagnetic separation using the EasySep™ Human CD34 positive selection kit (STEMCELL Technologies) and EasySep™ Magnet following the manufacturer's instructions. $CD34^+$ purity was verified by staining the isolated cells with allophycocyanin (APC)-labelled antihuman CD34 mouse monoclonal antibody (1:100) (BD Biosciences, Cat. No. 17-0349-42) and quantification by flow cytometry.

The MN or $CD34^+$ enriched cells were cultured at a cell concentration between $0.5x10^5$ – $2.3x10^5$ cells/ml in IMDM (STEMCELL Technologies) serum free media supplemented with 20% BIT serum substitute (STEMCELL Technologies), 10^{-4} M β-mercaptoethanol, 1 mM L-glutamine, Flt3-ligand (100 ng/mL), IL6 (20 ng/mL), IL3 (20 ng/mL), and G-CSF (20 ng/mL) (STEMCELL Technologies or Preprotech). Cells were incubated under normal conditions (37 °C, 5% CO_2 under humidified atmosphere). Cells were incubated overnight to allow cell recovery.

K562 cells, a CML cell lines that expresses the BCR-ABL fusion protein, were maintained in RPMI medium containing 10% heat-deactivated FBS, 100U/ml penicillin

and 100 µg/ml streptomycin under incubation at 37 °C and 5% CO_2. Twice per week, 1 x 10^6 cells were diluted in 20 mL of fresh medium for cell expansion.

5.2.4 Complexes Preparation and Transfection Method

The desired siRNAs and polymers were dissolved in nuclease free water at 0.14 and 1 µg/µL, respectively. For preparation of siRNA/polymer complexes, siRNA solutions were first diluted in RPMI and the desired polymer solutions were added to the diluted siRNA solutions. Solutions were vortexed briefly and incubated for 30 min at room temperature (RT) to allow complex formation. RPMI medium alone (no complexes) was used for no treated groups (NT), while scrambled (control)-siRNA/polymer complexes were used for negative controls. FAM-labelled scrambled-siRNA/polymer complexes were used for uptake studies, whereas BCR-ABL-siRNA/polymer complexes were prepared for the treatment studies. The polymer: siRNA (weight:weight) ratio in complexes was controlled and kept either at 9:1 or 12:1, as specified in each figure legend. The commercial reagent Lipofectamine® 2000 was used for comparison of transfection efficiency with lipid-modified polymers. Lipofectamine®:siRNA ratio used was 9:1 or 12:1.

MN cells were treated using the reverse transfection method as follows: after complex preparation, 200 µL of the complex solution was added to 24-well plate, making sure the solution thoroughly covered the surface of the well, and 600 µL of cell solution from culture prepared on the day before were added on top of complexes. Likewise, for 48-well plates 100 µL of complex solution and 300 µL of cell suspension was used. The siRNA final concentration used was 60 nM and polymer:siRNA ratios were 9:1 or 12:1 as stated for each case.

5.2.5 siRNA Uptake Visualization by Confocal Microscopy

At the indicated times, after of primary cells were transfected with FAM-labelled

siRNA/polymer complexes, cells were harvested, and washed with PBS by centrifugation. Cell pellet was added inside region created with liquid repellent marker (Staining Procedures, Japan) on polylysine coated microscope slides and was allowed to attach to surface for 20 min. Microscope slide with cells was washed with HBSS three times. Cells were covered with 4% paraformaldehyde solution and incubated for 10 min. Fixative was washed with HBSS three times. Samples were mounted and with a drop of ProLong® Gold Antifade Mountant with DAPI (ThermoFisher Sci). Coverslip was put on top and sample was allowed to dry overnight. Confocal images were taken in an A1+ confocal microscope (Nikon Instruments Inc.) at the British Columbia Cancer Agency or a Zeiss LSM 710 at the Cell Imagining Facility of the Department of Oncology at the University of Alberta. Images were acquired in the FITC channel (siRNA detection), the DAPI channel (nuclei detection, and phase contrast channel (cell membrane detection). Equipped with Argo 405/488/562 nm and Helium-neon 640nm lasers.

5.2.6 siRNA Uptake Visualization by Transmission Electronic Microscopy

One day after transfection of K562 cells or primary CML cells with scrambled (control)-siRNA and the indicated carrier, cells were harvested, washed with HBSS and fixed containing 2,5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) and stored at 4 °C for 3 days. Cells were incubated for 5 min at RT in the phosphate buffer and washed by centrifugation (14000 rpm, 5 min) three times. Phosphate buffer was removed by centrifugation and cells were stained with 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hour at RT. Staining solution was removed by centrifugation and cells were washed with phosphate buffer 3 times (cells incubated in the buffer wash for 10 min at RT and then centrifuged). Cells were then dehydrated in successively increasing solutions of ethanol (50%, 70%, 90%, and 3 x 100%). Cells were incubated in each solution for 10 min at RT and then centrifuged. For the

embedding, cells were dissolved in a mix of ethanol:spurr resin mix with a 1:1 ratio and incubated for 2 h at RT. Ethanol:spurr mix was replaced with pure spurr resin and incubated for 2 h (or overnight) at RT. Cells in spurr resin were then transferred to BEEM capsules inserted in Eppendorf tubes and centrifuged to have cells collected in a pellet. Resin was cured in a 70 °C oven overnight. Samples with cells were sectioned using a Ultracut E Reichert-Jung Ultramicrotome. Sections of 70-90 nm thickness were picked up on copper grids. Samples on grids were stained with uranyl acetate for 15 min and then lead citrate stains for 5 minutes. Samples were examined by a Philips/FEI Moragagni 268 Transmission Electron Microscope operating at 80 kV with a Gatan CCD camera. Samples were prepared and images at the Advanced Microscopy Facility at the University of Alberta.

5.2.7 siRNA Uptake Quantification

The MN cells were transfected with FAM-siRNA/polymer and non-labelled-siRNA/polymer (as negative control) complexes prepared at a polymer:siRNA ratio of 9:1 and a final siRNA concentration of 60 nM in 48-well plates as described above. One day after transfection, cells were transferred to tubes, centrifuged (1400 rpm for 10 min), washed twice with HBSS and re-suspended in formalin at a final concentration of 2% in HBSS. The cell-associated FAM-siRNA was quantified in a LSR Fortessa Cell Analyzer (BD Biosciences) (Flow Cytometry Facility of the Department of Medicine and Pharmacy at the University of Alberta) using the FL1 channel and calibrating the instrument so that the negative control (i.e. no-treated cells) gave ~1% of positive cells as the background. siRNA delivery in MN cells was determined by measuring the mean fluorescence of cells and percentage of FAM-siRNA positive cells.

5.2.8 mRNA Quantification by RT-PCR

The K562 cells seeded on 6-well plates in 2.5 ml of complete medium were treated with complexes prepared with control and BCR-ABL siRNAs at a polymer:siRNA weight

ratio of 12:1 and at a 60 nM siRNA concentration. Three days after transfection, levels of BCR-ABL mRNA were assessed. First, the total RNA was extracted with TRIzol (Life Technologies) following the manufacturer's instructions. The integrity of the RNA extracted was then checked by spectrophotometry (GE Nanovue). For each sample, at least 100 ng of RNA were reverse-transcribed with VILO Superscript (Thermo Fisher), following the manufacturer's instructions except that 10µL of superscript were used per sample instead of 20 µL. Finally, real-time PCR analysis, 2X SYBR green master mix with ROX (MAF Center, University of Alberta) was used to follow the fluorescence intensity. Specific forward and reverse primers used to detect expression levels are the following: housekeeping endogenous genes beta-actin: 5'-CCA CCC CAC TTC TCT CTA AGG A-3' and 5'-AAT TTA CAC GAA AGC AAT GCT ATC A- 3' [24], GAPDH: 5'- TCA CTG TTC TCT CCC TCC GC-3' and 5'-TAC GAC CAA ATC CGT TGA CTC C -3', B2M: 5'-TAG CTG TGC TCG CGC TAC T-3' and 5'-TCT CTG CTG GAT GAC CTG AG-3', and target gene BCR-ABL: 5'-CAT TCC GCT GAC CAT CAA TAA G-3'; 5'-GAT GCT ACT GGC CGC TGA AG-3' [23]. At least two housekeeping genes were used for each analysis. A 10 µl volume containing 5 µl of 2X master mix SYBR Green, 1 µl of 10 µM forward primer, 1 µL of 10 µM reverse primer and 3 µl of cDNA template (5 ng/µl) for each sample (three independent biological replicates and two experimental replicates) were transferred to a Fast Optical 96-well plate. Using an Applied Biosystems StepOnePlus (Thermo Fisher) instrument, reaction mixtures were heated for 2 min at 95 °C before going through 40 cycles of a denaturation step (15 seconds at 95 °C) and an annealing/elongation step (1 min at 60 °C). Analysis to determine differences in gene expression was performed by $2^{\Delta\Delta CT}$ method using the no-treated cells as the calibrator. BCR-ABL CT was normalized against the geometric mean of CT values from two housekeeping genes and the results were expressed as relative quantity of the targeted mRNA.

5.2.9 Colony-Forming Cell (CFC) Assay

One day after BCR-ABL siRNA transfection (at a polymer:siRNA ratio 12:1, final siRNA concentration 60 nM), viable MN cells were counted using trypan blue exclusion staining in the hemocytometer. 200 K562 or 30,000 MN cells were mixed in 1 ml MetholCult™ (H4230 STEMCELL Technologies) containing 10% IMDM (STEMCELL Technologies) and seeded in 35 mm culture dishes. For the CFC assay of MN cells, methylcellulose-based media was supplemented with final concentrations of human EPO (3 U/mL), human IL-6 (20 ng/mL), human IL-3 (20 ng/mL), human GM-CSF (20 ng/mL) and human G-CSF (20 ng/mL) (STEMCELL Technologies or Prepotech). The colonies produced were counted 12 to 14 days based on colony numbers (K562 cells) or colony numbers/type (CML primary samples). Types of colonies identified from CML patients are: Burst-forming unit erythroid (BFU-E), Colony-forming unit granulocyte/macrophage (CFU-GM), and Colony-forming unit Granulocyte, Erythrocyte, Macrophage, Megakaryocyte (CFU-GEMM) derived colonies/burst were enumerated [35].

5.2.10 Statistical analysis

The data were summarized as the mean of the measured variables with the error bars representing one standard deviation. Where stated, the data between controls and treatment groups were analyzed for equality of variances by F-test and statistical difference by Student's t-test (two-tailed distribution). The level of significance was set at $p < 0.05$.

5.3 RESULTS

5.3.1 FAM-siRNA Delivery and Quantification in CML CD34$^+$ and MN cells by flow cytometry

The PEI0.6, PEI1.2, and PEI2.0 substituted with LA, αLA, and cholesterol (Chol), and thio-esters of LA and αLA (tLA and αLA) were chosen for this study as they have been

effective in silencing GFP and BCR-ABL genes in GFP-K562 and K562 CML cells [32]. Description of lipid-modified polymer synthesis, molecular weights of polymers used, lipid modification, and lipid/PEI feed ratios and calculated ratios are shown in **Appendix Figure 5.S2**.

All patient samples had not received TKI previously and were found to be predominantly BCR-ABL$^+$. The percentage of cell recovery from frozen samples was highly variable, and ranged from 2.3 to 96.6% (**Table 5.1**). Main reasons for low cell recoveries in first few samples tested was clump formation during the thawing process that lead to cell loss; however, implementation of a DNAse digestion step increased cell recovery in the following samples.

The siRNA delivery to CML primary cells (P1, P2, and P14) was first attempted in the CD34$^+$ cell population (**Figure 5.1**). PEI0.6-Chol, PEI1.2-Chol, and Lipofectamine® 2000 were used to deliver FAM-labelled siRNA. There was an increased uptake (mean fluorescence) at increased ratios with all carriers as expected (except with Lipofectamine® on P1, **Figure 5.1A**). PEI0.6-Chol showed higher uptake than PEI1.2-Chol in P1 and P2 (**Figure 5.1A** and **B**), and PEI0.6-Chol and PEI1.2-Chol gave similar uptake in P14 (**Figure 5.1C**). Similar uptake levels were found with lipid-modified polymers in K562 cell line and patient samples P2 and P14 (**Figure 5.1B** and **C**). Usually, percentage of CD34$^+$ (blast) cells in MN cells of CML patients in the chronic phase is between 5% and 30%, depending of the stage of CML [36], [37]. Therefore, low CD34$^+$ cell numbers are expected to be recovered from each sample, which represents a limiting factor in the evaluation and optimization assays of siRNA delivery polymers. Such was the case of CML P2 (**Figure 5.1B**), on which only one replicate was available to test each group.

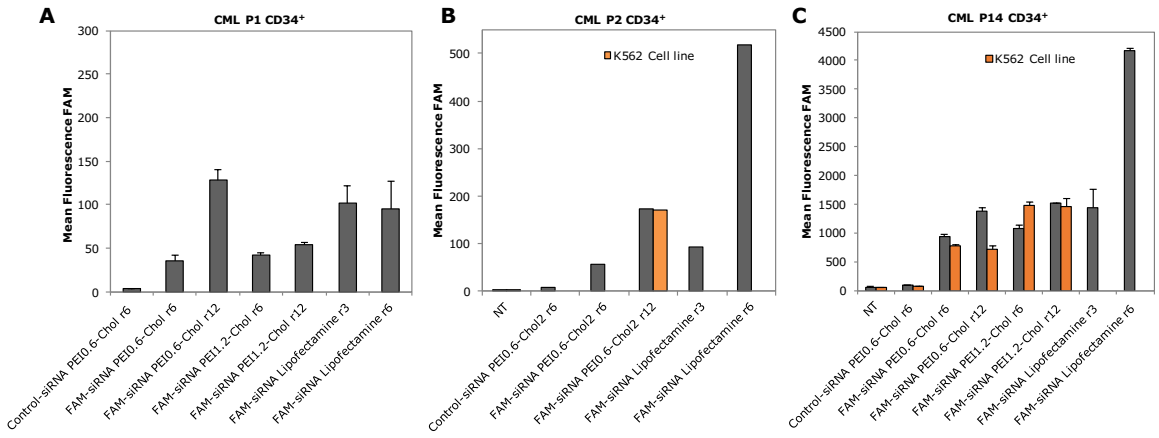

Figure 5.1. siRNA Delivery to CD34+ CML cells. siRNA delivery in $CD34^+$ isolated cells from CML patient mononuclear cells of Patients 1 (A), 2 (B), and 14 (C) on day 2 after transfection. Extent of FAM siRNA uptake in $CD34^+$ MN cells was shown as the mean fluorescence in the FL1 channel (green). K652 cells (orange bars) were transfected in parallel for comparison. Final siRNA concentration used was 60 nM and polymer:siRNA ratios 6:1 and 12:1 (r6, r12) and carrier:siRNA ratios 3:1 and 6:1 (r3, r6) for Lipofectamine® 2000. Values shown are the mean ± standard deviation where replicates were available.

Next, the MN cell fraction (with no $CD34^+$ purification) from CML primary samples was used to screen a broader array of PEI polymers and identify more effective siRNA carriers. Patient samples collected from fresh (n=3) and frozen (n=3) samples were analyzed for this purpose. **Figure 5.2** shows flow cytometry FAM-siRNA uptake results of nine lipid-modified polymers and Lipofectamine® 2 days after transfection in terms of mean fluorescence (**i-ii**) and percentage of FAM-siRNA positive-cells (**iv-vi**). Uptake patterns show some differences between fresh and frozen samples (**Figure 5.2A** and **5.2B**) as the uptake level changes but some similarities are also present. Fresh samples seemed more sensitive for uptake with PEIs polymers: PEI1.2-αLA showed the highest mean fluorescence (2/3 samples), followed by PEI1.2-LA (2/3), PEI0.6-Chol, PEI1.2-Chol (2/3), and PEI2.0-LA (2/3) (**Figure 5.2A**). Lower molecular weights PEIs (0.6 and 1.2kDa) with Chol modifications gave higher siRNA uptake (mean fluorescence and percentage positive cells) than higher molecular weight PEI2.0-Chol (**Figure 5.2A**), where PEI1.2-Chol seemed similarly effective than PEI0.6-Chol [in CML P3 (**Figure 5.2A**

i, **iv**), and in CML P13 (**Figure 5.2A iii**, **vi**)] or slightly more effective in CML P4 [(**Figure 5.2A ii**, **v**)]. A similar trend was observed with LA-modified polymers (excluding the thioester modifications on PEI1.2): substitutions on PEI1.2 had overall higher uptake and siRNA-positive cells than LA-modified PEI2.0s (**Figure 5.2A**). Mean fluorescence values were not always consistent with percentages of FAM-siRNA positive cells: PEI1.2-Chol and PEI0.6-Chol polymers showed the highest percentage levels with more than 70% of positive cells in 2 of 3 samples, which were followed by PEI1.2-LA with 59% to 74% positive cells, PEI2-Chol with 46% in 2 of 3 samples, and PEI2.0-αLA between 38% and 60% in 3 samples. For the case of the commercial Lipofectamine® 2000, it showed the highest siRNA uptake (mean fluorescence) in 1 of 3 samples and the lowest percentage in siRNA-positive cells (9% to 34%) (**Figure 5.2A**).

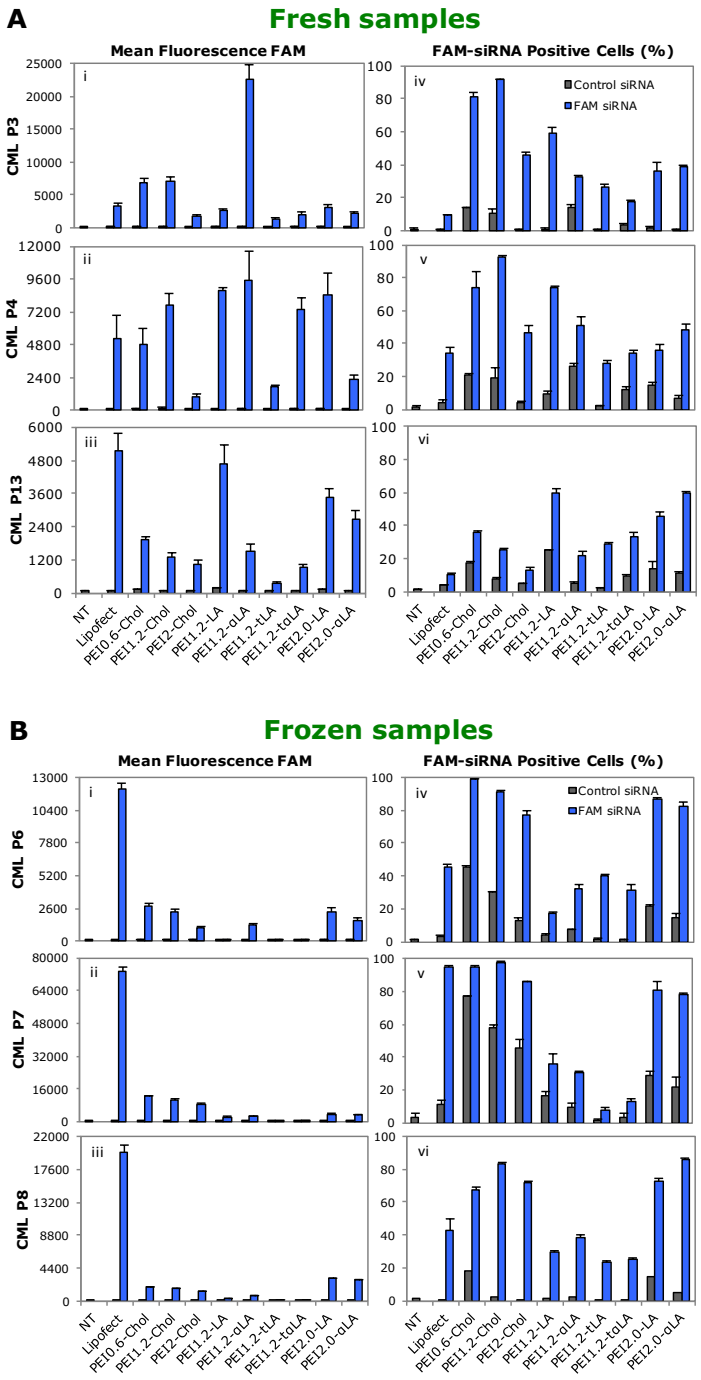

Figure 5.2. siRNA Delivery to MN CML Cells. A) Shows siRNA Delivery to fresh cells P3 (**i**, **iv**), P4 (**ii**, **v**), and P13 (**iii**, **vi**). **B**) siRNA Delivery to frozen cells P6 (**i**, **iv**), P7 (**ii**, **v**), and P8 (**iii**, **vi**). The MN cells were transfected with FAM-siRNA/carrier at a polymer:siRNA ratio of 9 and at a siRNA concentration of 60 nM. Cells were harvested and processed for flow cytometry analysis two days after transfection. Mean fluorescence (**i**, **ii**, **iii**) and percentage of siRNA-positive cells (**iv**, **v**, **vi**) is shown as the average of 3 replicates ± standard deviation.

In the frozen CML samples, PEI0.6-Chol and PEI1.2-Chol showed the highest uptake in two of three patient samples among modified PEIs (see CML P6 and P7, **Figure 5.2B**, **i-vi**). Chol polymers showed an inverse proportion between the PEI molecular weight and the uptake levels, as the mean fluorescence decreased with increased molecular weight (PEI0.6-Chol > PEI1.2-Chol > PEI2-Chol in **Figure 5.2B**, **i-iii**), where PEI0.6-Chol showed the highest uptake. In the case of LA-modified PEIs, the molecular weight vs. siRNA uptake relationship was opposite as PEI2.0s gave higher mean fluorescence than PEI1.2s (**Figure 5.2B**, **i-iii**). Percentages of positive cells patterns among frozen were similar in the three frozen samples (**Figure 5.2B**, **iv-vi**), where Chol-substituted PEIs gave the highest siRNA-positive cells (>67%), followed by LA-substituted PEI2.0s (>72%), and LA-substituted PEI1.2s (>16%). Lipofectamine® 2000 showed the highest uptake in all three frozen samples (**Figure 5.2B**, **i-iii**) and low percentage of siRNA-positive cells (approx. 43%) (**Figure 5.2B**, **iv-vi**); except in CML P7 (**Figure 5.2B**, **v**) where 95% of cells where siRNA-positive. Thioester linkage between LA lipids and PEI1.2 showed the lowest siRNA uptake of all PEIs in fresh and frozen samples (**Figure 5.2A** and **B**).

5.3.2 siRNA Delivery and internalization in MN cells by Confocal Microscopy and Transmission Electronic Microscopy

Analysis of siRNA internalization was performed by confocal microscopy. **Figure 5.3** shows localization of FAM-labelled siRNA in blue-stained nuclei of K562 cells and CML $CD34^+$ patient cells. **Figure 5.3A** shows K562 cells transfected with PEI0.6-Chol, PEI1.2-αLA and Lipofectamine® carriers at carrier:siRNA ratios 6 and 12 for polymers and 3 and 6 for the commercial reagent at 6 and 24 h after transfection. PEI0.6-Chol uptake at ratio 6 shows siRNA localized in the cell membrane, while transfection at ratio 12 shows internalization of siRNA in the cytoplasm. siRNA fluorescence was more diffused and less dense at ratio 12 than at ratio 6 (more evident at 24 h). siRNA delivered

by PEI1.2-αLA looks more compact and dense, with more aggregations at ratio 12 (6 and 24h). Similar to PEI0.6-Chol, siRNA delivery with PEI1.2-αLA at the higher ratio (12) seems more localized inside the cell (cytoplasm) in comparison with lower ratio (6); however, this cannot be concluded with clarity by this confocal microscopy technique. siRNA delivered by Lipofectamine® at the higher the ratio is denser and forms more aggregations than the PEI1.2-αLA. siRNA may be localized more towards the cell membrane as siRNA internalization was not clearly seen.

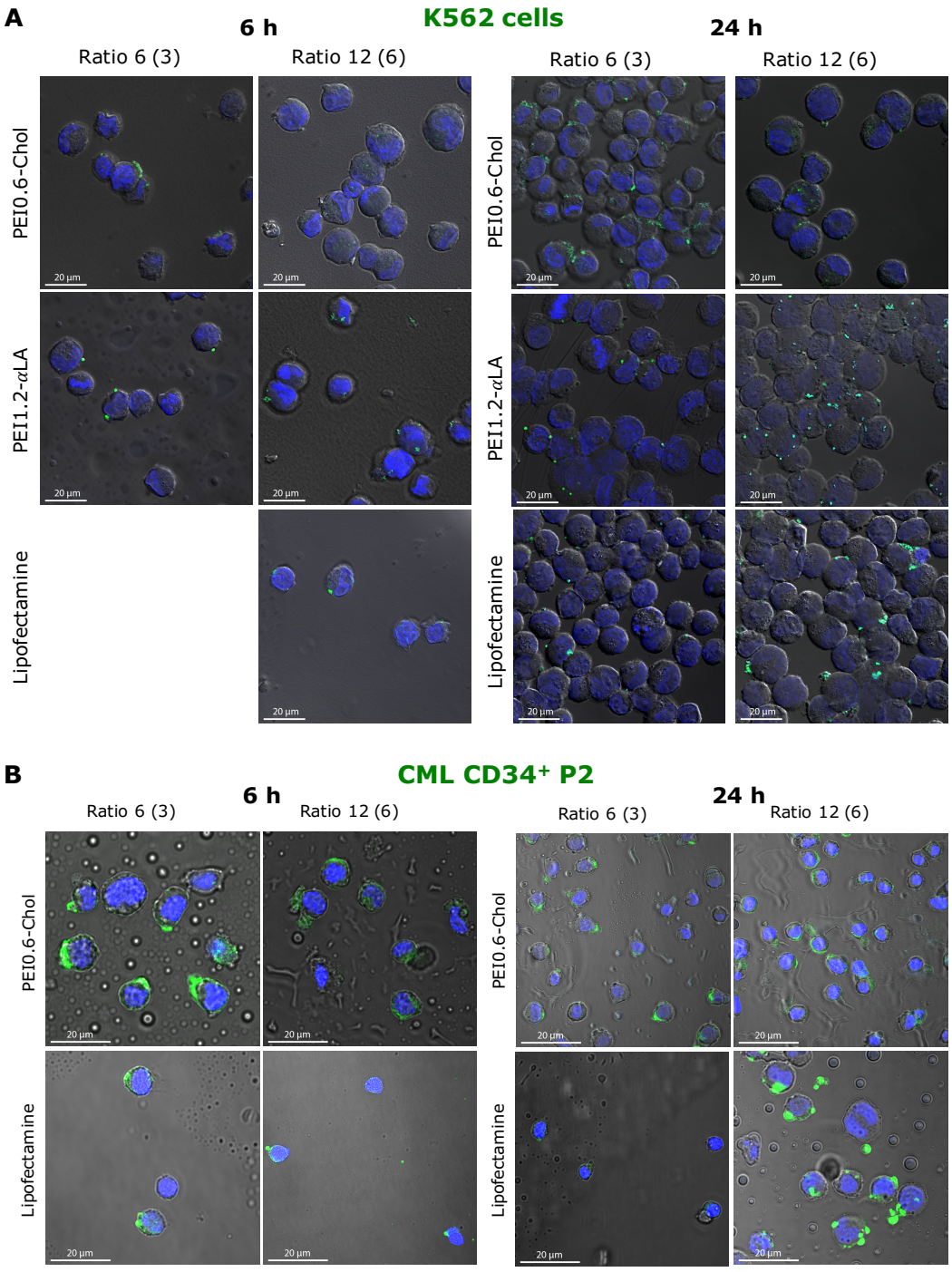

Figure 5.3. siRNA internalization after lipid-modified polymeric transfection by confocal microscopy. Cellular FAM-labelled siRNA internalization was visualized in K562 cell line (**A**) and CD34+ CML cells from patient 2 (P2) (**B**). Cells were transfected with lipid-modified polymers and polymer:siRNA ratios patient 2 indicated with an siRNA concentration of 60 nM. Rations in parenthesis are for Lipofectamine®. FAM-siRNA (green), nuclei (blue) and cell membrane (phase contrast) are shown.

Figure 5.3B shows siRNA localization in CML $CD34^+$ cells at 6 and 24 hour time points after transfection with PEI0.6-Chol (ratios 6 and 12) and Lipofectamine® (ratios 3 and 6). Similar to K562 cells, siRNA particles with PEI0.6-Chol at ratio 6 are more concentrated and denser in comparison with ratio 12, where siRNA seems more diffuse (6-hour time point); at the 24-hour time point, there was siRNA interaction with cells but no differences were found for the two ratios used. siRNA delivered by Lipofectamine® at the ratio 6 seems larger, denser and more compacted than siRNA particles at ratio 3. Given that CML primary cells have smaller cell size (5 μm - 15 μm in diameter) and have smaller cytoplasmic area in comparison with K562 cells (15 μm - 20 μm), it was not possible to assess and differentiate whether siRNA was localized in the cell membrane or cytoplasm.

To identify the siRNA/polymer particles and define exact location within the cell, TEM was performed to obtain ultrastructural information of the transfected cells. **Figure 5.4** shows the electron micrographs of K562 cells and CML primary cells untreated and treated with control-siRNA/polymer complexes. Micrographs of untreated K562 cells, siRNA/PEI0.6-Chol complexes, and K562 cells treated with PEI0.6-Chol alone, siRNA alone and siRNA/PEI0.6-Chol complexes are shown in **Figure 5.4Aa**-**h**. Structure of the siRNA complexes was a string-like amorphous network with bundles (darker areas) of presumably cholesterol moieties (**Figure 5.4Ab**). Cells treated with PEI0.6-Chol alone show polymer interaction with cell membrane (amorphous network and defined dark lines) and invagination of complex parts in vesicles inside the cell (**Figure 5.4Ac** and **d**; arrowhead and asterisk, respectively). siRNA alone-treated K562 cells do not show significant changes of morphology as expected (**Figure 5.4Ae** and **f** similar to **Figure 5.4Aa**), whereas K562 cells treated with siRNA/PEI0.6-Chol complexes confirmed the internalization of siRNA complex material in vesicles and by diffusion (**Figure 5.4Ag** and **h**; arrows and asterisk, respectively). Internalization of polymer can be evidenced by

the dark lines in the inside vesicles.

The micrographs of MN patient samples untreated and treated with siRNA/carrier complexes prepared with PEI0.6-Chol, PEI1.2-αLA (selected based on siRNA uptake studies), and Lipofectamine® are shown **Figure 5.4Bh**-**o**. NT cells show their normal state with multiple heterogeneous granules/vesicles scattered in the cytoplasm, where some granules containing small vesicles and/or a grainy substance (**Figure 5.4Bh** and **i**). The internalization of siRNA complexes is evident with all three carriers as cells display granules containing presumably complex material, as these have bigger, heterogeneous structures than differ from the vesicular and grainy material present in the NT cells (**Figure 5.4Bj**, **l**, **n**; asterisks vs. **h**). siRNA/PEI0.6-Chol and siRNA/PEI1.2-αLA complexes were captured in interaction with cell membrane (**Figure 5.4Bj**, **l**, **m**; black arrowhead). Complexes were also translocated inside cells without the aid of vesicles or granules (**Figure 5.4Bl**, **m**, **n**; black arrows). siRNA/PEI0.6-Chol complexes as found in **Figure 5.4A**, had the shape of string-like network with dark dense spots that suggest the presence of Chol (**Figure 5.4Bj**; white and black arrowheads), siRNA/PEI1.2-αLA complexes showed a paler color as they had smaller and evenly scattered dark points that suggest presence of LA (**Figure 5.4Bl**, **m**; white and black arrowheads). siRNA/Lipofectamine® complexes showed the expected multilamellar liposome vesicle dark is color due to its lipidic nature (**Figure 5.4Bn**; white arrowhead). Phagocytosis was evident in the case of primary cells treated with siRNA/PEI1.2-αLA complexes (**Figure 5.4Bl**, **m**; black arrowhead), where complexes adhered to cell membrane, which in turn the cells surrounded in preparation to engulf the material by pseudopodia present in the cell membrane.

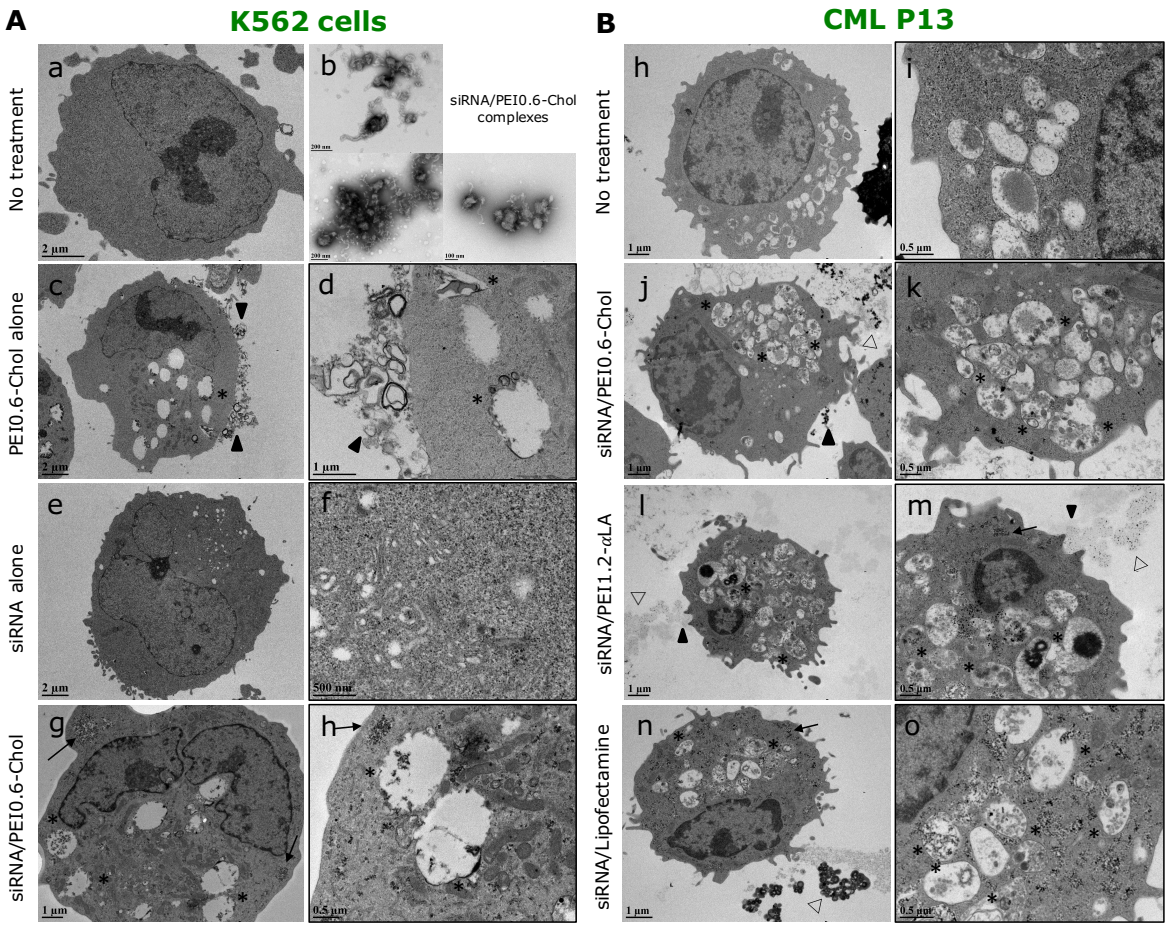

Figure 5.4. Electron micrographs of siRNA-treated K562 and CML patient cells. K562 (**A**) and CML primary cells (**B**) were transfected with scrambled (control) siRNA with the carriers indicated at a polymer:siRNA ratio 12 and at a siRNA concentration of 60 nM. Cells were prepared for TEM 24 h after transfection. Black-framed micrographs are closer looks of specific areas of micrographs on the left. Interaction of siRNA/polymer complexes with plasma membrane (black arrowheads), translocation into cells (arrows) and localization in endosomal vesicle (asterisk) or free outside of cells (white arrowheads). SiRNA and polymer is localized to a subset of granules.

5.3.3 RT-PCR and CFC Analysis of BCR-ABL siRNA silencing in MN Cells

The CML MN cells were treated with BCR-ABL siRNA/polymer complexes to evaluate whether there was a silencing effect at the mRNA level and if the ability to form colonies was altered. RT-PCR results of BCR-ABL mRNA transcript levels on fresh and frozen MN CML samples is shown in **Figure 5.2A** and **B**, respectively. Note that for the analysis of

fresh samples (P3, P4 and P13) there was enough viable cells to perform all the assays (siRNA uptake, RT-PCR and CFC), although limited polymers could be evaluated in RT-PCR and CFC assays; whereas for frozen samples there was a limited number of viable cells due a smaller initial cell concentrations and poor cell recovery rates in the thawing process (**Table 5.1**), so that for RT-PCR analysis from new patient samples (CML P9, P10) were included.

BCR-ABL mRNA reduction in CML P3 with PEI1.2-Chol and PEI1.2-αLA was not statistically significant. BCR-ABL mRNA levels in CML P4 decreased significantly by 17% and by 45% with PEI1.2-Chol and PEI1.2-αLA, respectively in comparison with the control siRNA group (**Figure 5.5A**). BCR-ABL mRNA levels in CML P13 decreased significantly by 37% with PEI1.2-LA, whereas silencing with PEI0.6-Chol, PEI1.2-Chol and PEI1.2-αLA was not evident. For frozen samples (**Figure 5.5B**), CML P9 did not show silencing with PEI0.6-Chol, whereas mRNA reduction in CML P10 with PE1.2-Chol was not significant. BCR-ABL silencing with Lipofectamine® was not evident in the samples tested despite the high siRNA uptake observed previously, especially in the frozen samples (**Figure 5.1B**).

Table 5.1. CML patient clinical details used in this study. Details provided from different sources.

Sample ID	Type	Population	BM/PB	Source	Age	Sex	Recovery (%)	Cytogenetics
CML P1	Frozen	CD34+	-	UBC	45	M	13.3*	P210 BCR-ABL
CML P2	Frozen	CD34+	-	UBC	22	M	10.4*	P210 BCR-ABL
CML P3	Fresh	Unpurified	BM	UA	67	M	-	P210 BCR-ABL
CML P4	Fresh	Unpurified	BM	UA	51	M	-	P210 BCR-ABL
CML P6	Frozen	Unpurified	PB	OSU	-	-	10.8	BCR-ABL1 positive
CML P7	Frozen	Unpurified	PB	OSU	-	-	53.1	Atypical CML
CML P8	Frozen	Unpurified	PB	OSU	-	-	7.5	BCR-ABL1 positive
CML P9	Frozen	Unpurified	PB	OSU	-	-	23.9	BCR-ABL1 positive
CML P10	Frozen	Unpurified	PB	OSU	-	-	6.9	BCR-ABL1 positive
CML P13	Fresh	Unpurified	BM	UA	59	M	-	P210 BCR-ABL

BM: Bone Marrow, PB: Peripheral Blood. * Cell recovery of unpurified cells. UBC: University of British Columbia, UA: University of Alberta, OSU: Ohio State University.

Figure 5.6 shows the CFC assay results for fresh samples CML P3, P4 and P13. The total number of colonies is shown in **Figure 5.6A**, and the colony counts according to the class of hematopoietic progenitors for CML P13 are shown in **Figure 5.6B.** CML P3 showed 35% reduction in the total number of colonies with PEI1.2-αLA in comparison with control siRNA group ($p<0.01$). CML P4 with PEI1.2-αLA showed a 32% reduction but it was not significant ($p=0.18$). CML P13 showed a 38% reduction with PEI0.6-Chol and a 26% reduction with PEI1.2-Chol in comparison to their respective control siRNA groups (both $p<0.01$). Lipofectamine® did not decrease the number of colonies of any of the samples tested (**Figure 5.6**). Colony counts according to the type of hematopoietic progenitors detected (**Figure 5.6B**), showed a significant reduction in the number of BFU-E and CFU-GM colonies: PEI1.2-Chol reduced BFU-E colonies by 24%

(p<0.01), whereas PEI0.6-Chol and PEI1.2-Chol reduced CFU-GM colonies by 41% and 24%, respectively (in comparison with control siRNA groups, p<0.01).

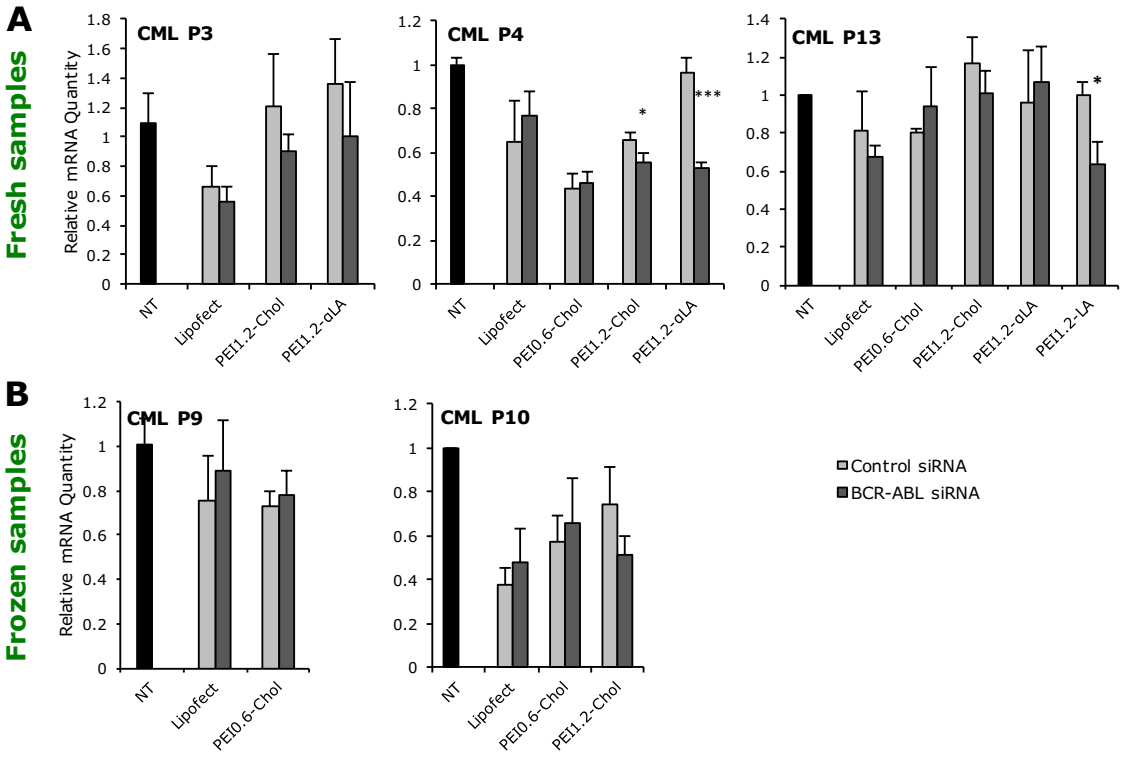

Figure 5.5. Effects of treatment with BCR-ABL siRNA/polymer complexes on BCR-ABL mRNA expression in CML MN cells. BCR-ABL mRNA levels were assessed by RT-PCR on day 3 after transfection on fresh (A) and frozen (B) MN CML patient samples at a polymer:siRNA ratio of 12 and siRNA concentration of 60 nM. Asterisks represent the level of significance, where * is p < 0.5 and ** is p < 0.01. Silencing effect at the mRNA level was evident in some samples with some polymer showed more effect than others depending on the sample and uptake levels.

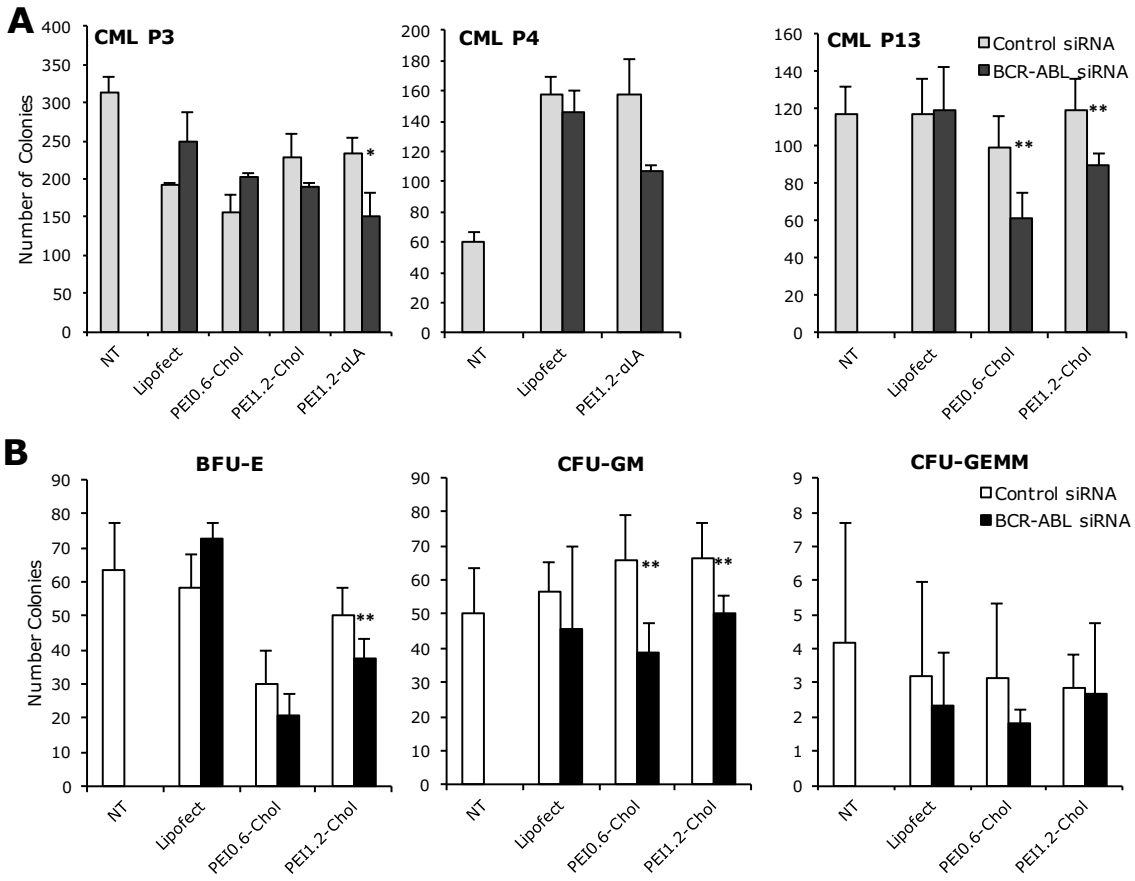

Figure 5.6. Effects of treatment with BCR-ABL siRNA/polymer complexes on colony formation in MN cells. Colony counts were performed two weeks after transfection after siRNA transfection at 60 nM siRNA. A) showed the total number of colonies of treated cells from patient 3, 4 and 13 (P3, P4, P13). B) shows number of colonies of patient samples 13 (P13) per the class of human hematopoietic progenitors detected: Burst-forming unit-erythroid-E (BFU-E); Colony-forming unit-granulocyte, macrophage (CFU-GM); and, Colony-forming unit-granulocyte, erythroid, macrophage (CFU-GEMM). Asterisks represent the level of significance, where * is $p< 0.5$ and ** is $p < 0.01$. Significant reduction of total number of colonies in three different patient samples. Significant difference was detected in BFU-E and CFU-GM colony types.

5.4 DISCUSSION

In this study, we investigated the use of lipid-modified polymers for siRNA delivery into cells from patients with CML to evaluate the potential of siRNA in leukemia therapy in more relevant cell models. Samples from CML patients were provided frozen (MN cells after Lymphoprep™ isolation) or from fresh bone marrow aspirates. Samples were

processed (thawed and/or MN cells isolated) and a library of nine lipid-modified polymers and Lipofectamine® 2000 was used for siRNA delivery. Analysis of cell association/internalization of siRNA particles was first performed, and from these studies, the best performing polymers were selected for subsequent BCR-ABL siRNA delivery for evaluation of mRNA silencing and CML cell survival by the cell-forming colony (CFC) assay.

Regarding the use of frozen samples, some challenges were encountered: after cell thawing, majority of cells were nonviable cells (assessed by trypan blue) which could have caused interference in the reading of results in the different assays performed. Even though efforts to reduce the number of dead cells were performed, such as centrifugation of cell suspension for long time and low speed (80 g, for 10 min) to separate cells from debris-, large number of dead cells remained. At the moment of cell transfection with siRNA complexes, the high number of dead cells could have interfered with the transfection process by competing for the complex binding (which was evident by the lower uptake levels in frozen cells in comparison with fresh samples). Moreover, although it is expected that the RNA from dead cells will degrade quickly, it may still be possible that dead cells contributed to the poor quality of RNA in the RT-PCR analysis and silencing studies, which in turn may have given erroneous RNA estimations of the BCR-ABL transcripts. Finally, dead cells in the background of CFC dishes may have made colony counts more challenging. Fresh cells, on the other hand, gave higher viable cell yields, which was optimal for larger scale polymers screening where multiple assays were needed for a more comprehensive understanding. Since availability of fresh CML patient samples was rare (one sample every 3 or 4 months for University of Alberta Hospital), methods to eliminate dead cells from frozen samples may improve quality of future experiments. For example, a second Ficoll paque centrifugation could be performed to remove dead cells or use of a dead cell removal kit before cell transfection.

However, number of viable cells recovered will probably still be a limiting factor.

Previous studies from our lab evaluated the hydrodynamic size by dynamic light scattering of complexes prepared with different lipid-substituted PEIs and siRNA; the results showed that complexes prepared with PEI2.0-αLA have an approx. size of 120 nm [32] while complexes prepared with PEI0.6-Chol and PEI1.2-LAs have a size of around 100 nm [38], which shows that lipid-substituted PEIs with molecular weights between 0.6 and 2 kDa are expected to have a relative uniform size of ~100 nm, so that changes in uptake efficiency are not expected to be due to size variations. Different lipid substituents, however, are expected to render a unique morphology and characteristics to the siRNA complexes that are may affect the interactions with the cell membranes. Microscopy analysis suggest that interaction of PEI0.6-Chol and PEI1.2-αLA with the cell membrane may be different. Confocal microscopy analysis suggests that siRNA fluorescence (in K562 cells and CML patient cells) with PEI0.6-Chol was more diffused whereas siRNA fluorescence with PEI1.2-αLA is more condensed. TEM images agreed; PEI0.6-Chol complexes showed a polymeric network that interacted with a large area of the outer cell membrane, whereas the polymeric network created with PEI1.2-αLA was more condensed and interacted with a smaller area of the cell membrane. Cholesterol used for PEI substitutions has a polycyclic, largely hydrophobic structure, but its polar hydroxyl group makes it amphiphilic. Due to its amphiphilic nature and high abundance in the plasma membrane, cholesterol present in complexes may have different type of interactions with components from the cell media and plasma membrane: The polar moieties can interact with a polar group from a membrane lipid or a protein, whereas the apolar section can interact with the side chains of branched amino acids or aromatic side chains from amino acids from proteins across the cell membrane [39]. Cholesterol can also form interactions with lipids in the plasma membrane, especially with microdomains enriched with sphingolipids such as sphingomyelin to form lipid rafts, and

with regions enriched with glycerophospholipids such as phosphatidylcholine. Cholesterol can also interact with other cholesterol molecules present in the cell membrane, as well as with proteins across the lipid bilayer that contain specific transmembrane domains [39], such as such as caveolin-1 (lipid transport) [40]. Translocation of linoleic acid and all other long chain fatty acids across the plasma membrane on the other hand, is achieved by the assistance of albumin (major protein in serum) [41] [42]. Albumin besides allowing the passive transport of fatty acids it may also stimulate cellular uptake of fatty acids through a direct interaction with cell membrane proteins as well as lipid rafts. Fatty acids might be liberated from albumin by adaptor molecules like FAT/CD36 at the plasma membrane. Upon binding of fatty acids, FAT/CD36 might be shifted into lipid raft platforms in which the translocation process across the membrane bilayer may occur [43]. The mechanism by which naturally occurring lipids are internalized by the cell plasma membrane may suggest possible ways of entry of lipid-modified polymers/siRNA complexes; however, lipid substituent, degree of lipid substitution, particle size, charge, interaction with serum proteins and lipid composition of the membrane of targeted cells will together play a role in the cell membrane internalization. In fact, a study undertaken in our lab analyzed how the degree of substitution of short propionic acid (PrA; C3) onto 1.2 kDa PEI can alter the siRNA delivery efficiency of the complexes. This study found that a high degree of PrA substitution caused a deleterious effect on the surface hydrophobicity and cationic charge of the complexes that impeded the siRNA uptake and silencing effect, whereas low/moderate substitutions on complexes gave higher surface hydrophobicity and higher surface charge density that resulted in enhanced cellular siRNA uptake and silencing effect [44]. Studies that investigate the differences between lipid substituents such as between α-linolenic acid and cholesterol will give more clues in terms of ways of interaction and internalization across the cell plasma membrane.

Uptake analysis (flow cytometry and confocal microscopy) with $CD34^+$ CML cells from three patients showed that siRNA delivery with PEI-Chol polymers had similar uptake levels to that of K562 cells, showing an early indication that our polymers may work similarly in both patient and cell lines. Due to limitation of $CD34^+$ cell numbers, a wide range of polymers could not be screened in these cell population as well as in-depth silencing studies, therefore MN cells population was used for the subsequent studies. In CML P3, although silencing of *BCR-ABL* by RT-PCR was not detected, flow cytometry assessed siRNA uptake and CFC assay were correlated as PEI1.2-αLA gave the highest uptake and showed significant reduction in the number of colonies (35%), while PEI1.2-Chol, the second most effective in siRNA uptake, gave a small but non-significant reduction on the colony numbers. In CML P4 sample, uptake analysis agreed with RT-PCR and CFC results as PEI1.2-αLA, gave highest uptake, showed a significant BCR-ABL mRNA silencing effect (45%) and reduction in the colony numbers (32%, although not significant vs scrambled control siRNA treatment). PEI1.2-Chol also, among the polymers that gave high uptake, showed a significant silencing at the mRNA level (17%) but this polymer was not tested in the CFC assay. In CML P12, the highest uptake given by PEI1.2-LA correlated with the silencing at mRNA level (37%). Although PEI1.2-LA was not tested in the CFC assay, one would expect to see also a decrease in the number of colonies for this polymer. Surprisingly, although uptake with PEI0.6-Chol and PEI1.2-Chol was not the highest and they did not show a reduction at the mRNA level, they did show a significant reduction in the colony numbers (38% and 26%, respectively). In summary for CML fresh patient samples, BCR-ABL mRNA levels decreased significantly by 45%, 37%, 17% (in 2 of 3 patient samples tested; two polymers showing silencing in one patient sample and one polymer in another patient sample). Colony numbers decreased by 35%, 32% (NS), 38%, 26% (in 3 of 3 samples). Moreover, specific colony numbers for the BFU-E type decreased by 24% and CFU-GM decreased by 24% and 41% (one sample). For the CML frozen patient samples analyzed and the limitations

discussed above, the uptake effect with lipid-modified PEIs was lower than in fresh samples, and the RT-PCR results (samples tested for uptake and RT-PCR were not performed on the same patient sample) agreed with uptake results as not significant reduction at the BCR-ABL mRNA was seen.

Results using the commercial transfection reagent Lipofectamine® 2000 showed it gave the highest siRNA uptake in CML frozen patient samples among all the carriers tested, gave an average uptake level in CML fresh patient samples (in comparison with the PEI carriers), and showed brighter siRNA particles in confocal microscopy than siRNA complexes from PEIs, which can be explained by a higher number of siRNA particles concentrated in a smaller area (more aggregation). Internalization by confocal was not evident but TEM suggests internalization of complex material. However, no mRNA silencing or decreased in the colony numbers were detected in any of the samples tested after treatment with BCR-ABL siRNA delivery of Lipofectamine®. These results with Lipofectamine® 2000 may suggest that the high aggregation of siRNA complexes around the cell may have not allowed the internalization and release of siRNA inside CML patient cells. These results with Lipofectamine® 2000 agreed with a previous study from our lab that investigated and compared a lipid-modified polymer that consisted of palmitic acid (PA) as the lipid moiety and poly-L-lysine (PLL) as the polymeric backbone (PLL-PA) with Lipofectamine® 2000 for plasma DNA (pDNA) delivery to bone marrow stroma cells. The confocal microscopy analysis showed that complex sizes of labelled plasmid DNA and Lipofectamine® 2000 was much larger (3-17 μm) likely due to aggregation in comparison with the size of pDNA/PLL-PA complexes, which were less than 700 nm in size and were present in single or smaller aggregates. This aggregation formed with Lipofectamine® 2000 may explain that despite the high plasmid delivery (better or comparable to PLL-PA) Lipofectamine® 2000 gave 2 or 5-fold lower transfection efficiency (green fluorescence protein expression) than PLL-PA carrier [45]. Only one

study used Lipofectamine® 2000 for siRNA delivery (final siRNA concentration of 50 nM) in CML CD34+ cells to inhibit the expression of BCR-ABL and Hsp90 genes. In this study, the knockdown of both targets was confirmed by Western Blot and led to a cell inhibition rate of up to 80% when both genes were targeted simultaneously [26]. However, the amount of Lipofectamine (e.g. siRNA:lipid weight ratio) used in their study was not stated. In our hands, Lipofectamine® 2000 worked well with the K562 cell line (i.e. delivery and silencing) but its silence efficiency did not translate to the CML MN cells.

The vast majority of studies of siRNA in CML found in the state of art are concentrated in finding new potential targets for CML treatment, mainly using siRNAs for target screenings assays in cells lines and in a few occasions with CML patient cells (**Chapter 1**). For studies that targeted BCR-ABL expression by siRNA in CML patient cells: (i) one study with CML MN cells transfected with BCR-ABL siRNA by electroporation (357 nM, Est.) decreased the BCR-ABL mRNA level between 55% and 79%, whereas CML $CD34^+$ cells treated in the same way decreased similarly their mRNA levels between 50% and 78%. However, the inhibition in the colony formation for both MN and $CD34^+$ cells was not significant [18], and (ii) another study also used electroporation (800 nM) for BCR-ABL siRNA delivery into CML $CD34^+$ cells to decrease in BCR-ABL protein (percentage of decrease in protein expression was not quantified) [19], but the consequences of this decrease were not evaluated. Other two studies used lipid-based transfection reagents: (iii) delivery of BCR-ABL siRNA with DOTAP transfection reagent (54 pM, a surprisingly low concentration) showed a 36% decrease at the BCR-ABL mRNA level in CML MN cells [22], (iv) while transfection with TransMessenger (286 nM, Est.) decreased BCR-ABL mRNA between 23% to 67% in CML MN cells [21]. In the latter case, cell proliferation decreased by 45% and apoptosis increased by 2-folds in comparison with the control groups [21].

Other studies also performed siRNA-mediated silencing in CML patient cells that

targeted genes other than BCR-ABL: (i) cells from patients in blast crisis were transfected with a Lyn kinase siRNA (357 nM, Est., electroporation), which had a cell growth inhibition between 50% and 90% three days after siRNA transfection. In the same study Lyn siRNA-mediated silencing also led to a 60% decrease in the number of CFU-GM colonies [23]. (ii) siRNA silencing of autophagy genes ATG5 and ATG7 (3571 nM, electroporation) in $CD34^+$ CML cells significantly enhanced the inhibitory effect of imatinib in colony formation [20]. (iii) Similarly, silencing of autophagy ATG4B gene by siRNA (HiPerFect, 400 nM) in CD34+ CML cells decreased the colony formation by 30% and enhanced the inhibitory effect of imatinib in the colony formation [46]. (iv) Proliferation rate of MN primary CML cells after silencing of PPP2R5C (a regulatory B subunit of protein phosphatase 2A) by siRNA (electroporation, 2140 nM Est.) was significantly decreased at 72 h [25]. (v) Double silencing of the Hsp90 chaperone and BCR-ABL (50 nM, Lipofectamine® 2000) significantly enhanced the cell proliferation inhibition (80% at 96 h) in CD34+ CML cells in comparison with cells treated with either siRNA alone (60% to 70% at 96 h) [26]. Overall, all these studies used commercial transfection reagents with siRNA concentrations ranging from 50 nM to 3571 nM, and for those studies that targeted BCR-ABL, the decrease in BCR-ABL mRNA ranged between 23-79% in comparison with control-siRNA groups. The cell growth inhibition rates found in the above studies were between 50% and 90% (for all genes targeted), and the inhibition of the ability to form colonies ranged between 30% and 60% (all studies except those that targeted BCR-ABL).

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6. OVERALL CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

6.1 Overall conclusions and discussion

RNAi technology represents a promising alternative for the treatment of chronic myeloid leukemia (CML) when targeted therapies, such as tyrosine kinase inhibitors, fail due to drug resistance development and CML stem cells are (or become) insensitive to these drugs. This thesis focuses on the evaluation of lipid-modified polyethyleneimine (PEI) as non-viral siRNA carriers in chronic myeloid leukemia cells to induce therapeutic effects and create alternative therapeutic solutions that overcome the current challenges.

siRNA-mediated silencing in CML is predominantly used for discovery and identification of new target genes for the development of conventional drug molecules [1]. However, the evaluation of siRNA as a siRNA-based drug itself has only been evaluated recently mainly because of the lack of a suitable vehicle that delivers the siRNA across the cell membranes efficiently and that may be translated for clinical use. In fact, for the specific case of siRNA use in CML drug research development, the number of studies focused on the identification of new molecular targets outweighs the number of studies that design and make use of the siRNA carriers as potential siRNA-based drugs (23 studies on siRNA targets vs. 6 studies on siRNA delivery, **Chapter 1,** [2]). One reason for this limitation is the challenge of creating a delivery vehicle that stabilizes and delivers the siRNA across the membrane of cell lines and primary cells [1], [3], [4]. Furthermore, the interaction and internalization of siRNA particles through the membrane of cells that grow in suspension is generally known to be more difficult to achieve than the attachment-depended cells because of their lower cell membrane area, lower interaction of siRNA particles with cell membrane and structural differences of

their cell membrane that contribute to this challenge (**Chapter 1** and **2**). Therefore, more efforts in the design of delivery carriers for suspension-growing CML cells may bring this delivery technology closer for applications of siRNAs as therapeutics.

The chosen target gene in this thesis work was the *BCR-ABL* gene as this is the main driver of CML induction and maintenance, by activating multiple downstream signaling pathways that increase proliferation and inhibit apoptosis, and it orchestrates the disease progression, along with other mutations, from chronic phase to blast crisis [5], [6]. Silencing of this directly relevant gene was used here as a proof of principle for siRNA silencing and the delivery method, and it represented a drug-gable target to evaluate the therapeutic effect in CML cells induced by this system.

The difficulty of delivering nucleic acids to suspension growing cells was appreciated in our hands. While PEI2 substituted with long chain lipids, such as linoleic acid (LA, C18:2) gave robust silencing [7] in the attachment-dependent breast cancer cell line MDA-MB-231, a siRNA uptake comparison between MDA-MD-231 cells and K562 cells using the same lipid-substituted PEIs revealed that MDA-MD-231 cells had ~45-times fold greater siRNA uptake than K562 cells (**Chapter 2**). Moreover, a similar LA-substituted PEI (PEI2-LA, lipid substitution of 2.1 LA per PEI molecule) was explored in K562 cells for siRNA delivery and, despite giving high siRNA uptake levels (similar to commercial PEI25 polymer), it did not yield GFP-silencing in GFP-positive cells, even when wide range of siRNA:polymer ratios (2 to 12) and siRNA concentrations (36 to 140 nM) were tested. The preparation of siRNA complexes was in saline solution in a small volume (60 µl) in those studies. Unlike the PEI2-LA effect, lower MW PEI (PEI1.2) substituted with palmitic acid (PA, C16:0) (PEI1.2-PA, lipid substitution of 2 PA per PEI) conferred higher GFP silencing and milder toxicity (63% GFP silencing at 72 nM siRNA, ratio 8 with 60% cell recovery) in comparison with PEI25 (54% GFP silencing and 20% cell recovery). In addition, an increase in the transfection efficiency was noted when

complexes were prepared in OptiMEM/RPMI in larger volumes (100 µL, not shown), perhaps by reducing the complexes aggregation and toxicity at the time of transfection [8]. BCR-ABL siRNA delivery with PEI1.2-PA polymer decreased the BCR-ABL mRNA by 20% (100 nM siRNA, ratio 4), and increased significantly cell apoptosis 2 and 3 days after BCR-ABL siRNA treatment. This study demonstrated the proof-of-principle for the potential of PA-substituted polymers for a functional therapeutic outcome. Higher siRNA:polymer ratios (12, as opposed to ratios of 2 to 8 for breast cancer cells) and higher level of lipid substitution were found beneficial for siRNA transfection in suspension K562 cells. A higher cationic charge at higher polymer:siRNA ratios [9] may increase the interaction of siRNA/polymer complexes with the cell membrane of suspension cells, ultimately leading to increased transfection efficiency; excess cationic charge from complexes however, may lead to unwanted cytotoxicity [9], [10].

A new lipid substituent, α-linoleic acid (αLA, C18:3; PEI1.2-αLA with ~1 αLA per PEI) was used in **Chapter 3** with the purpose of improving the balance between silencing effect and cytotoxic features. Comparative experiments showed that PEI1.2-PA (used in **Chapter 2**) gave 60% GFP silencing with 28% cell recovery (75 nM, ratio 8) and PEI1.2-αLA gave 54% GFP silencing with 41% cell recovery. This suggested that PEI1.2-PA and PEI1.2-αLA achieved similar levels of silencing but with PEI1.2-αLA displaying lower cytotoxicity. Therefore, the level of performance for PEI1.2-PA, although promising, was not considered overly significant in going forward towards clinical utility. Furthermore, it was surprising to find that PEI1.2-αLA at effective siRNA concentrations (40 nM or more) resulted in increasing silencing levels from day 2 up to day 9 after one dose of GFP siRNA, reaching silencing levels of 80% on days 2 and 9 (80 nM siRNA, ratio 12). Moreover, BCR-ABL siRNA delivery with PEI1.2-αLA gave 50% BCR-ABL mRNA silencing (60 nM siRNA, ratio 12). Cell viability studies (MTT assay) showed a 20% reduction in cell viability in comparison with control siRNA on day 4 (60 nM siRNA, ratio 12). The

viability of cells treated with control-siRNA/PEI1.2-αLA complexes was 48% on day 2 and 60% on day 4, showing an initial degree of toxicity due to transfection; however, cells progressively recovered their viability after day 2 of treatment. The structural composition of αLA in comparison to PA, i.e., slightly longer carbon (C18 vs C16, respectively) and increased unsaturation (3 double bonds in αLA vs no double bonds in PA) makes the former lipid less flexible. This may lead, once grafted onto PEI, to a less compact structure and looser binding to siRNA due to increased steric hindrance that may facilitate the siRNA release once the particles are inside the cell. On the other hand, siRNA/PEI1.2-PA may form more compact complexes due to PA's flexible structure (saturated fatty acid) and higher lipid substitution on PEI1.2 (2 PA per PEI vs 1 αLA per PEI), which may form a much stronger interaction with the cell membrane that may affect negatively the integrity of the cell membrane and cell viability.

Furthermore, in **Chapter 4** a higher αLA substitution performed on PEI2 (PEI2-αLA, 2.7 αLA per PEI) gave a 54% GFP silencing (60 nM siRNA, ratio 12) with a 62% cell recovery. These results compared better with the effect of PEI1.2-αLA from **Chapter 3**, which gave 54% GFP silencing and 41% cell recovery (75 nM siRNA, ratio 8, with ~1 αLA per PEI). Increased level of lipid substitutions of PEI2-αLA appeared to increase the GFP silencing effect proportionally. Differences in the polymer synthesis protocol may explain the different cytotoxic effects of the αLA-substituted PEIs: 1) Lipid-modified polymers with lipid substitutions between 1 and 2 are usually easier to precipitate, therefore allowing the removal of excess (unbound) lipids, polymer; whereas polymers with lower lipid-substituted polymers (less than 1) are less easily precipitated. The latter can therefore still include some unreacted lipid and polymer molecules that may negatively affect the cell viability, and 2) removal of water molecules from the acid chloride solvent before reaction, and temperature control (reaction made in ice) were improved steps expected to increase the reaction efficiency that could avoid the creation

of impurities that may induce cytotoxicity. The changes of molecular weight of PEI (from 1.2 to 2 kDa PEI) are not expected to change the transfection efficiency given that the percentage of substitution was very similar in both cases (for PEI1.2-αLA, 1 lipid in 10 amines of PEI1.2 was 10% substitution, and for PEI2-αLA 2.72 in 15 amines of PEI2 was 18%). Therefore, PEI2-αLA was considered a promising candidate for further experiments in K562 cells and CML primary samples.

As an alternative to aliphatic lipid substitutions on PEI, Cholesterol (Chol) substitutions on low molecular weight PEIs (0.6, 1.2 and 2 kDa PEI) showed high transfection efficiencies at higher lipid substitution and lower molecular PEI weight (personal communication Dr. Remant KC, unpublished). In **Chapter 5**, PEI0.6-Chol (1.1 Chol per PEI) and PEI1.2-Chol (1.2 Chol per PEI) used for BCR-ABL siRNA delivery (60nM siRNA, ratio 12) induced a 47% decrease in cell viability on day 3 and 73% decrease on day 6 in comparison with control siRNA group in K562 cells. Toxicity after transfection was 30% on day 3 but fully cell viability recovery was observed after day 6. A 40% decrease in the colony formation was observed at the same dose with 40% BCR-ABL mRNA silencing. For the case of GFP silencing, PEI0.6-Chol gave a 50% GFP silencing on day 3, with a cell recovery of 37% (personal communication, Dr. Remant KC). The rigidity (resistance of a particle to deform) of lipid-substituted polymers can affect the cellular uptake efficiency [11]. Sun *et al.* found that the more rigid the particles are the better the cell uptake will be in comparison with less rigid 'soft' particles. The reasoning behind this observation is that the more flexible nanoparticles may undergo more deformation, requiring more binding energy to overcome the bending energy from deformation to complete the internalization [11]. This may suggest that the use of more rigid lipids such as unsaturated lipids (αLA) and cholesterols may give better cellular uptake in comparison with saturated lipids (PA) because of the less energy required for their transportation through the cell membrane. Studies comparing the rigidity of

nanoparticles resulting from different lipid substituents on polymers would be useful to elucidate this characteristic. In summary, the identified characteristics of lipid-modified polymers that were found to be beneficial for efficiency siRNA delivery and transfection in suspension CML cells are: i) use of larger volumes and cell media for complex formation in order to prevent aggregation and toxicity, ii) higher siRNA:polymer ratios (12, in comparison with attachment-dependent cells that use ratios of up to 8) to ensure the tight interaction with cell membrane, and iii) lipid substitution between 1-2.5 lipids per PEI to have balance an optimal balance of the lipophilic-cationic moieties that control transfection efficiency and cytotoxicity.

The siRNA transfection of lipid-modified polymers on an *in vivo* leukemia (K562) model was evaluated in **Chapter 3**. In the *in vivo* model used here, a wide variation in the growth of tumors in mice was observed: in some cases, there was no tumor formation and often tumors did not enter the exponential growth phase. The use of Matrigel® did not improve the tumor engraftment and growth. The low implantation success rate of 40% together with the different and slow tumor growth rates did not allow a very clean assessment of the BCR-ABL siRNA effect. Regardless of the heterogeneity of the tumor growth, a retardation of tumor growth was observed after the treatment with BCR-ABL siRNA/PEI1.2-αLA in comparison with the treatment with control-siRNA/PEI1.2-αLA and untreated tumors. This tumor retardation was found to be dose-dependent as higher siRNA dose gave higher tumor retardation, but some toxicity from the transfection was perceived in the treatment with control-siRNA/PEI1.2-αLA at the highest dose used. We were able to locate only one published report in the literature that attempted delivery of nucleic acids (in these case antisense oligonucleotides) with non-viral carriers (transferrin receptor targeted lipopolymers) to K562 xenografts models [12], which may correspond well with the difficulty of growth this CML xenografts and the challenge of the delivery of nucleic acids to in vivo tumor

CML models.

The creation of a fibronectin (FN) grafted polymeric surface to investigate the influence of leukemic cell adhesion on siRNA treatment with lipid-modified polymers was investigated in **Chapter 4**. Components of bone marrow environment, such as FN, are known to protect and reduce the therapeutic effect of anti-cancer drugs of leukemic cells, therefore the creation of this system was useful to evaluate whether lipid-modified polymers can also transfect the FN-adhered K562. FN covalently grafted on plasma treated polytetrafluoroethylene (PTFE) films showed significant K562 cell adhesion and growth in comparison with the unmodified surfaces. Moreover, siRNA therapy using lipid-modified polymer was similarly effective on FN-adhered K562 cells and on K562 cells grown in suspension, indicating that lipid-modified polymers induce siRNA-mediating (GFP) silencing on K562 cells regardless of their adhesion state. The developed cell-adhesive system here could be employed to assess the influence of FN-mediated CML cell attachment on siRNA/drug therapies, but this was not further explored due to time constraints. While specific targets for a therapeutic outcome remain to be investigated, the proposed system could be useful to investigate the role of other extracellular matrix proteins and specific siRNA therapies to control undesirable leukemic growth.

A group of polymers previously identified for effective siRNA delivery to K562 cells, composed of low molecular PEIs substituted with αLA, LA and Chol at different lipid substitutions, was screened for siRNA transfection in CML primary samples (**Chapter 5**). With a final concentration of 60 nM siRNA and ratio 12, PEI1.2-αLA (2.46 αLA/PEI), PEI1.2-Chol (1.21 Chol/PEI), and PEI1.2-LA (2.55 LA/PEI) reduced the BCR-ABL mRNA expression by 17% to 45% and inhibited the formation of colonies by 24% to 41% in comparison with control siRNA. A 20% or less decrease in colony formation with cells treated with control siRNA in comparison with untreated cells suggested a low toxicity effect after transfection. In comparison with the effects seen in K562 cells with Chol-PEI

(40% BCR-ABL mRNA silencing and 40% decrease in colony formation), the effect of Chol and αLA polymers in CML primary cells translated very similarly. The fact that no single polymer was universally effective in all patient samples, may suggest the patient-to-patient variability in terms of therapeutic response. These results showed that a much lower dose of BCR-ABL siRNA could be used with lipid-modified polymers as compared to literature reported values (as discussed in **Chapter 5**, siRNA doses ranged from 50 nM to 3571 nM) in order to reduce BCR-ABL mRNA expression, CML cell survival and colonies formation. The results from this study revealed the potential of siRNA-based drugs and are encouraging for the future design of non-viral delivery system for the treatment of CML.

6.2 Future considerations

6.2.1 Efficacy siRNA/polymer nanoparticles

An increase in the siRNA dose of our treatments would naturally increase the silencing and pharmacological effects on the intended cells; however, cytotoxicity, cost, scalability to *in vivo* models, and off-targets effects are factors that will need to be taken into account in the design of siRNA-based therapies, so strategies other than increasing of siRNA concentration used for treatments need to be considered. The siRNA formulations effective in the 10-50 nM range would be desirable for clinical translation [2]. One important challenge that needs to be overcome with any siRNA therapy are the off-target effects. One type of off-target effect is sequence-specific and can result from the partial sequence complementarity of the siRNA guide strand with sequence motifs from 3' untranslated regions of mRNA present in the cell. This imperfect pairing, which resembles the function of microRNAs, can produce unspecific transcript silencing that could potentially lead to false positive results [13], [14]. Another off-target effect is the inflammatory response, where monocytes recognize pathogens (in this case siRNA, or even delivery vehicles such as cationic lipids) by means of their toll-like receptors (TLR)

localized in the endosomes (TLR7/8). The activation of these receptors results in an unwanted production of cytokines [14], [15]. Some factors that contribute to the potency of off-target effects and immune activation have been identified and recommendations to mitigate these effects are available, which may include chemical modifications [16], and control of specific sequence designs [17], [18] and are expected to not reduce the siRNA silencing potency and specificity.

6.2.2 Assays for better assessment of outcomes in CML primary cells

Hematopoietic stem cells (HSCs) maintain normal hematopoiesis and have the capacity of self-renewal and differentiation along the hematopoietic lineages [19]. Long-term hematopoietic stem cells (LT-HSC) are the stable pool of the most primitive multipotent cells of the hematopoietic stem cells (HSC) [19]. As with human hematopoietic stem cells, CML leukemic stem cells (LSC) share the same characteristic surface markers $CD34^{+}CD38^{-}$ [19], [20]. Presence of residual BCR-ABL^{+} cells in the $CD34^{+}CD38^{-}$ HSC fraction has been found in CML patients who had achieve complete cytogenetic and molecular remissions after TKI treatment [21], [22]. Long term persistence of these cells is believed to be due the presence of BCR-ABL^{+} LT-HSCs, which have a high capacity of self-renewal to induce CML [20]. Under these circumstances, most CML patients need to continue TKI treatment throughout life to prevent relapse [23], [24].

Effect of siRNA delivery with lipopolymer delivery systems should therefore be evaluated in future studies in the CML $CD34^{+}CD38^{-}$ stem cells fraction. Specialized assays are required for an optimal evaluation of the therapeutic effects. MTT was also employed in earlier studies (**Chapter 2** and **3**), and although this assay was useful for fast proliferating K562 cells, it was not suitable on slower proliferating primary cells. Therefore, more specialized *in vitro* and *in vivo* assays would allow better assessment of the pharmacological effect after siRNA treatment of CML primitive cells. Colony-

forming cells (CFC) assay employed in **Chapter 5** were introduced as a more relevant assessment tool for both K562 cell line and CML primary cells, and in the case of CML primary cells, this assay also allowed the identification of the different types of progenitors. Other assays specific for the growth and maintenance of hematopoietic ($CD34^+CD38^-$) stem cells could be useful in the future to analyze other biological characteristics such as self-renewal capacity, and engraftment capacity. Long-term culture-initiating cell, (LTC) assays are specifically designed to allow survival, self-renewal, proliferation and differentiation for continuous generation of myeloid cells from a small sample of primitive hematopoietic cells *in vitro* when co-cultured in a feeder layer of competent fibroblasts feeder layers [25]. Once these cells, called LTC-initiating cells (LTC-IC), are re-plated in methylcellulose they generate a new cohort of progenitor cells or colony-forming cells (CFCs) [25]. This will allow the quantification of CML stem cells with the capacity to produce progenitor cells. Moreover, LTC-IC assays performed using the limiting dilution assay (decreasing cell seeding numbers) can be used to measure the frequency of the CML leukemic stems cells [26], [27]. So that LTC-IC dilution assays could be performed in CML stem cells treated with specific siRNA complexes to evaluate the active cell frequency contributing to cell self-renewal and survival in comparison to untreated cells.

To evaluate the long-term ex vivo effect of siRNA treatment on survival and long-term engraftment of CML $CD34^+$ cells, the most physiologically-relevant mouse model currently available could be used: NOD/SCID/γc (non-obese diabetic/severe combined immunodeficiency/mutation of IL-2 receptor γ-chain deficient) mice we found to support superior engraftment of human hematopoietic stem cells in comparison to other mice models previously used [28], [29]. Human $CD34^+CD38^-$ CML cells from bone marrow samples can be exposed to siRNA *in vitro* and transplanted intravenously into irradiated NOD/SCID/γc mice. The engraftment of human $CD34^+$ in murine BM and spleens can be

evaluated by quantifying the percentage of human $CD45^+$ enriched cells from the BM and spleens of mice 4-10 weeks after human cell transplantation [26], [30], [31]. Moreover, $CD45^+$ cells could further be plated in CFC assay to evaluate the capacity of cell colony formation [32]. This *in vivo* model would be useful to test the effectiveness of the siRNA/lipopolymer treatment on the engraftment capacity of primitive human CML stem cells in immunodeficient mice.

6.2.3 BCR-ABL silencing in other Leukemias

Acute lymphoblastic leukemia (ALL) is a malignant neoplasm of the lymphocyte precursors cells. Philadelphia (Ph) chromosome in patients with acute lymphoblastic leukemia (ALL) is present in 2% to 5% of children, 20% to 40% of younger adults, and up to 50% in older adults (>55 years) [33]. TKIs are considered as a complement to chemotherapy and as a bridge to allogeneic stem cell transplantation (SCT) [33]. Intensive treatment and SCT are believed to be necessary for a more curative therapy, but most of elderly patients are not fit for chemotherapy and/or SCT [34]. In patients ineligible for SCT, there is a unmet need of novel drugs that eliminate the BCR-ABL^+ clones for a long-term survival of patients with Ph^+ ALL [33]-[35]. In acute myeloid leukemia (AML), Ph^+ is present in approximately 0.5% to 3% patients. Although not a standardized treatment (due to small Ph^+ AML population), TKI therapy is sometimes combined with chemotherapy before SCT transplantation. Even though BCR-ABL is not considered a driver mutation in AML as in the case of CML, BCR-ABL might still confer a proliferative advantage to the BCR-ABL^+ AML clones [36]. Furthermore, blockage of CD99 cell surface receptor by CD99 specific antibody is a promising therapy in AML as it induced cell death; however, this treatment was not effective in a BCR-ABL^+ AML patient sample [37]. BCR-ABL siRNA therapy could be used in combination with ALL and AML chemotherapy or other therapeutic strategies to help with the elimination of BCR-ABL^+ clones.

6.2.4 Beyond BCR-ABL targeting

Progression from chronic phase to blast crisis include increased BCR-ABL expression, and additional mechanisms besides BCR-ABL that produce differentiation arrest, inappropriate activation of self-renewal capacity, loss of tumor-suppressor functions, and changes in the cell adhesion, homing and drug metabolism [5], [6], [38]. Although BCR-ABL is the disease driver, and differentiated cells depend on BCR-ABL activity for their survival (sensitive to TKI), CML stem cells do not rely on BCR-ABL activity to thrive [27], [28]. Strategies to target other mechanisms of transformation of leukemia stem cells are needed. So far, several potential new targets have been identified (**Chapter 1**). Silencing of these potential targets together with BCR-ABL silencing may prove beneficial for the eradication of leukemic stem cells. Whether the same polymers could be used for targeting other drivers of leukemic transformations is an open question. This thesis work provides ample opportunities (i.e., delivery systems) in this regard that could be useful to silence other target genes in leukemic cells for therapeutic purposes.

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APPENDIX

A. APPENDIX CHAPTER 2

Table 2.S1 Lipid-substituted polymers derived from PEI2.

Substituted Lipid	Polymer	Lipid:PEI mole ratio[a]	Lipid/PEI[b]
Caprylic acid	PEI2-CA1	0.066	1.1
	PEI2-CA10	0.1	2.4
	PEI2CA20	0.2	6.9
Myristic acid	PEI2-MA1	0.066	0.6
	PEI2-MA10	0.1	1.7
	PEI2-MA20	0.2	1.5
Palmitic acid	PEI2-PA1	0.066	0.6
	PEI2-PA10	0.1	0.8
	PEI2-PA20	0.2	1.1
Stearic acid	PEI2-SA1	0.066	0.5
	PEI2-SA10	0.1	3.6
	PEI2-SA20	0.2	4.9
Oleic acid	PEI2-OA1	0.066	1.0
	PEI2-OA10	0.1	1.7
	PEI2-OA20	0.2	2.5
Linoleic acid	PEI2-LA1	0.066	1.0
	PEI2-LA10	0.1	1.8
	PEI2-LA20	0.2	3.2

[a] Lipid:PEI mole ratios used during the reaction for the synthesis of the polymers.

[b] Actual number of lipids substituted per PEI calculated from ^{1}H NMR analysis.

Table 2.S2 PA-substituted polymers derived from PEI0.6, PEI1.2, and PEI2.

Substituted Lipid	Polymer	Lipid:PEI mole ratio[a]	Lipid/PEI[b]
Palmitic acid	PEI0.6-PAI	1	0.1
	PEI0.6-PAII	2	0.25
	PEI0.6-PAIII	4	0.3
	PEI1.2-PAI	1	0.4
	PEI1.2-PAII	2	0.6
	PEI1.2-PAIII	4	2.0
	PEI2-PAI	1	0.2
	PEI2-PAII	2	0.6
	PEI2-PAIII	4	3.0

[a] Lipid:PEI mole ratios used during the reaction for the synthesis of the polymers.

[b] Actual number of lipids substituted per PEI calculated from ^{1}H NMR analysis.

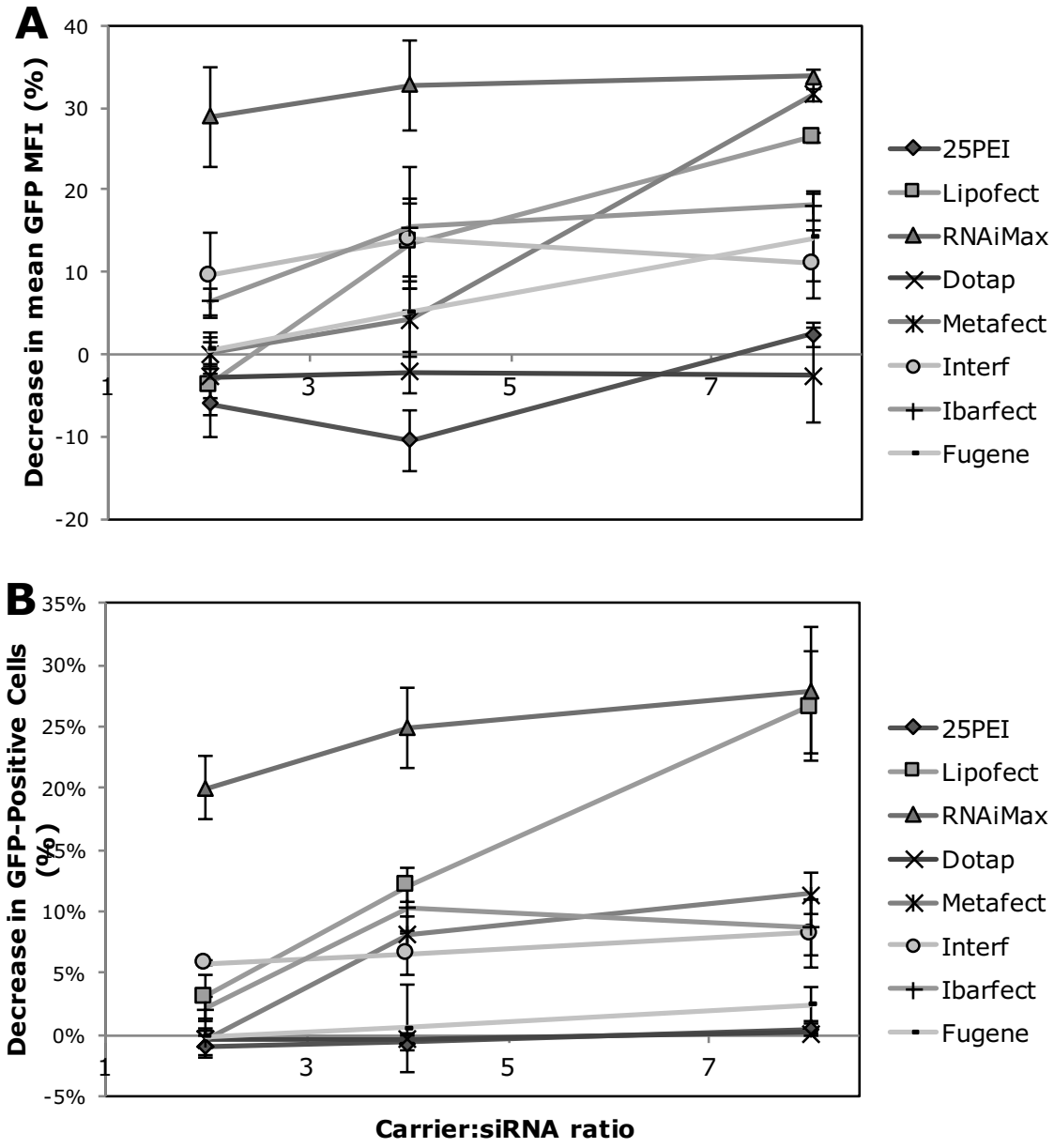


Figure 2.S1. GFP silencing in GFP-K562 cells with commercial reagents. Reduction in GFP MFI (**A**) and percent decrease in the GFP-positive population (**B**) were assessed by flow cytometry 3 days after siRNA treatment. The complexes were prepared at a carrier:siRNA ratios of 2:1, 4:1 and 8:1 and used at 24 nM GFP-siRNA concentration.

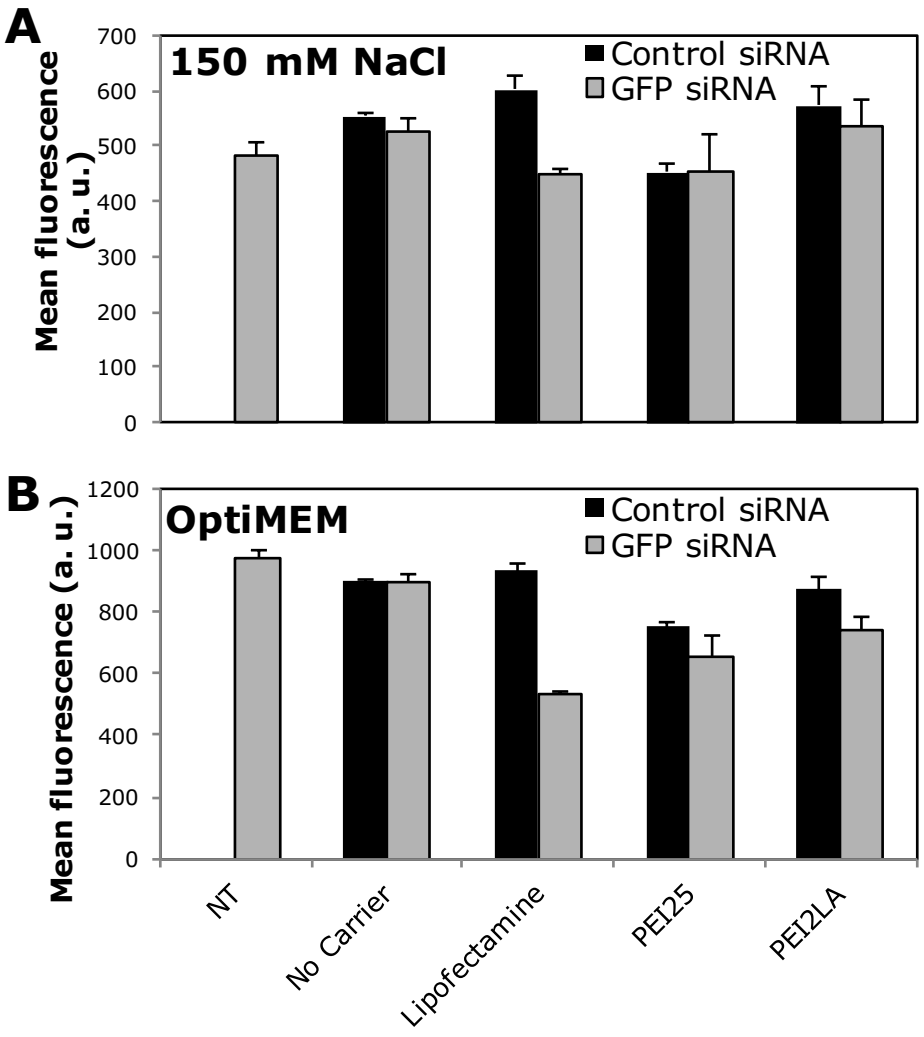


Figure 2.S2. Method of complexation affects silencing results. Mean fluorescence values (arbitrary units, a. u.) prior analysis of percent decrease in mean GFP fluorescence (Fig. 2Ai) of GFP-K562 cells 72 h after transfection with complexes prepared with control (scrambled) siRNA and GFP siRNA in either 150 mM NaCl (**A**) or OptiMEM (**B**) at a carrier:siRNA ratio of 8:1 and a siRNA concentration of 36 nM.

B. APPENDIX CHAPTER 5

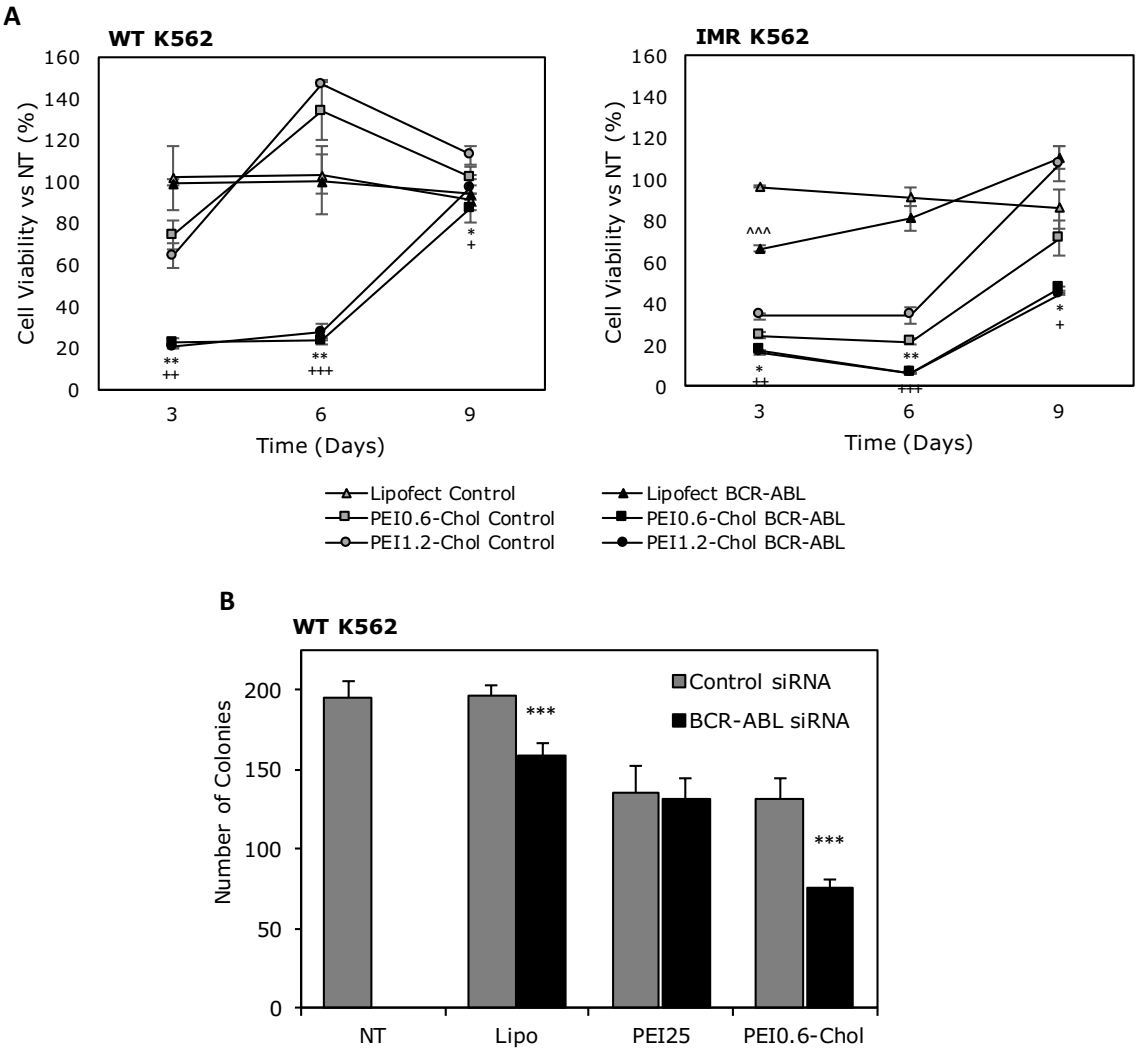
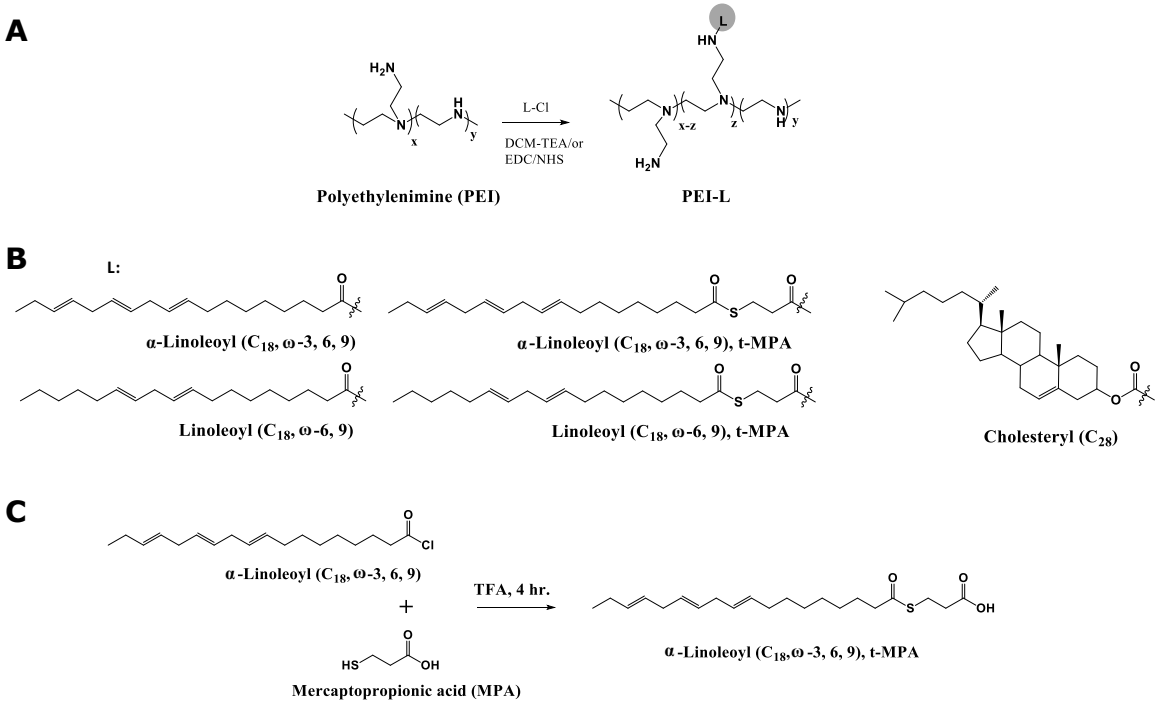


Figure 5.S1. Cell Viability and Colony formation in inhibition of K562 cells after BCR-ABL siRNA transfection with PEI-Chol polymers. A) Wild type (WT) K562 and imatinib resistant (IMR) K562 cells were transfected with Lipofectamine® 2000, PEI0.6-Chol and PEI1.2-Chol at a polymer:siRNA ratio of 12 and carrier:siRNA ratio 2 for Lipofectamine® at a siRNA concentration of 60 nM. Cell viability was assessed by MTT assay 3, 6 and 9 days after transfection. **B**) Ability to form colonies of WT K562, transfected at similar conditions as described above. Colony counts were performed after two weeks. Average ± standard deviation of three independent replicates are shown. Significant differences between control siRNA and BCR-ABL siRNA is indicated by *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, where ^ is for Lipofectamine®, * for PEI0.6-Chol, and + for PEI1.2-Chol in A, and * for both Lipofectamine® and PEI-0.6-Chol. PEI0.6-Chol and PEI1.2-Chol both demonstrated significant cell viability reduction after BCR-ABL siRNA transfection on day 3 to day 9 for WT and IMR K562 cells, whereas Lipofectamine showed a significant cell viability reduction on day 3 in IMR K562 cells but not on WT K562 cells. Moreover, PEI0.6-Chol showed ~40% inhibition in the colony formation whereas Lipofectamine inhibition was ~20%.



D

Polymer	Lipid/PEI Feed Ratio	Lipid/PEI Calculated Ratio
PEI0.6-Chol	2	1.08
PEI1.2-Chol	3	1.21
PEI2.0-Chol	4	2.36
PEI1.2-LA	6	2.55
PEI1.2-αLA	4	2.46
PEI1.2-tLA	2	1.3
PEI1.2-tαLA	4	1.96
PEI2.0-LA	6	2.31
PEI2.0-αLA	4	2.72

Figure 5.S2. Reaction scheme for the synthesis of lipid-modified cationic polymers. A) Polymers were synthesized by grafting different aliphatic and aromatic lipophiles (L) via N-acylation for 18 hr at room temperature in basic anhydrous dichloromethane (DCM) and trimethylamine (TEA) using PEI (molecular weights 0.6, 1.2 and 2 kDa) as the backbone. **B**) Structure of lipophiles used in the polymer synthesis; the lower-case number represents the numbers of carbon atoms, ω- represents the position of unsaturation in the aliphatic chain, t- represents the thioester linkage between aliphatic lipids and end-capping agent, mercaptopropionic acid (MPA). **C**) Synthesis of thioester linkage (t-MPA) on aliphatic acid prior grafting onto PEI. Aliphatic lipids (LA or αLA) were end-capped with MPA thioester linkage by reacting with each other in trifluoroacetic acid (TFA) for 4 hr at room temperature. **D**) Lipid substitution levels on modified PEIs. The lipids were grafted on PEIs at the lipid/PEI feed (mol/mol) ratios indicated. The extend of lipid substitution was calculated by H1 NMR.