# University of Alberta

Improving Gene Delivery Efficiency by Lipid Modification of Cationic Polymers

by

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# Dedication

My loving parents, Mom and Dan who wanted this more than anybody…

My brother and sisters Ernesto, Annabel, Barbara and Bernadette who found a way to draw a smile in my life.

Javier Cuervo the morning bird who guides my soul and sends me to the sky to fly away.

My supervisor and friend Dr Hasan Uludag who allowed me to pursue this chapter of my life and never gave up on me. Thank you.

The ones that shared my thoughts without judgment, Tamara Fuentealba, Johana Gomez, Oscar Khazam, Paola Scholte and Luis Torres.

The people who made a beautiful work environment Meysam Abbasi, Basak Acan Clements, Ross Fitzsimmons, Charlie Hsu, Cesary Kusharsky, Breanne Landry, Xiaoyue Lin, Nesrine Mostafa, Laura Rose, Guiling Wang.

# ABSTRACT

This thesis explores the capabilities of cationic polymers modified with lipids of different carbon chain length to deliver DNA molecules to primary cells and transformed cell lines. Our studies focus on two different polymers: polyethylenimine (PEI) and poly(L-lysine) (PLL). Firstly, PEI and PLL were conjugated to palmitic acid (C16). The delivery of plasmid DNA to rat bone marrow stromal cells (rat-BMSC) was evaluated by using a Green Fluorescent Protein gene expressing plasmid (pEGFP-N2) as a reporter system. The rationale for lipid substitution is to give the polymer an amphiphilic character so as to improve the transfection efficiency of native polymers by improving the DNA/polymer translocation through the phospholipid-rich cell membranes. In the case of PLL-C16, transfection efficiency was significantly increased (5 fold) as compared to native PLL, and it was significantly higher than commercially available cationic lipids (Lipofectamine$^{TM}$ 2000 and Fugene$^{TM}$).

We further explore the use of other lipids with variable chain lengths (carbon chain length ranging from 8 to 18 saturated and unsaturated) in order to identify other candidates to enhance the gene delivery properties of the PLL. Lipid-modified PLL of high molecular weight (25 vs. 4 kDa) was found to be more effective in delivering plasmid DNA in rat-BMSC. We noted that C14-, C16- and C18-substituted PLL gave the most effective DNA delivery. Moreover, a correlation between the extent of lipid substitution and the plasmid DNA delivery efficiency was found Additionally, transgene expression by BMSC significantly

increased when amphiphilic PLLs were used as compared to native PLL. The modified polymers were able to transfect the cells up to 7 days, after which the expression decreased.

Encouraged by the successful transgene expression agents obtained by modifying low molecular weight PEI with the same series of lipids described above, we explored the possibility of modifying low molecular weight PEI (2 kDa) with longer lipids; saturated fatty acid (C22), *trans* fat (C18:1T) and essential fatty acids (C22:1, C22:6 and C18:3). Transfection efficiency proved to be cell dependent. Only the transformed 293T cells were able to express GFP compared to human-derived BMSC. The highest transfection efficiency was found with highly unsaturated lipid-substituted PEI (C18:3 and C22:6) and were able to increase transgene expression overtime (6 days). Furthermore, internalization studies indicated that effective transfection of these carries do not follow any known endocytosis pathway instead the DNA/carrier penetrates the plasma membrane directly.

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# List of Abbreviations

AF: Alexa Flour

AFM: Atomic Force Microscopy

BA: Behenic Acid

BMSC: Bone Marrow Stromal Cells

bPEI: branched Polyethylenimine

$BR_{50}$: Half Binding Ratio

C8: Caprylic Acid

C14: Myristic Acid

C16: Palmitic Acid

C18: Stearic Acid

C18:1: Oleic Acid

C18:2: Linoleic Acid

CA: Caprylic Acid

$CDCl_3$: Deuterated Chloroform

$D_2O$: Deuterated water

DCC: Dicyclohexylcarbodiimide

DCM: Dichloromethane

$ddH_2O$: Distilled/deionizd water

DHA: Docosahexeanic Acid

DMEM: Dulbecco’s Modified Eagle Medium

DMF: N,N'-dimethylformamide

DMSO: Dymethyl Sulfoxide

DNA: Deoxyribonucleic acid

DP: Degree of polymerization

EA: Erucic Acid

ELA: Elaidic Acid

EMSA: Electrophoretic Mobility Shift Assay

FBS: Fetal Bovine Serum

FITC: Fluorescent Isothiocyanide

FL: Forward Light Scattering

FRET: Fluorescence Resonance Energy Transfer

GFP: Green Fluorescent Protein

GFP-Av: Adenovirus expressing Green Fluorescent Protein

HBSS: Hank's Balanced Salt Solution

h-BMSC: human-Bone Marrow Stromal Cells

$^{1}$H-NMR: Proton Nuclear Magnetic Resonance

hrs: Hours

HSF: Human Skin Fibroblast

$IC_{50}$: Half Inhibitor Concentration

Kb: Kilobase pair

kDa: Kilo Dalton

LA: Linoleic Acid

LLA: Linolenic Acid

lPEI: Linear Polyethylenimine

MA: Myristic Acid

μL: Microliters

mg: Miligrams

mL: Milliliters

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

mV: Milivolts

NHS: N-hydroxy succinimide ester

MβCD: Methyl-β-cyclodextrin

MW: Molecular Weight

$N_2$: Nitrogen

NaCl: Sodium Chloride

N:P: Nitrogen:Phosphate

PA: Palmitic Acid

PA-Cl: Palmitoyl Chloride

PDMAEMA: Poly(2-(dimethylamino) ethyl methacrylate)

PAMAM: Poly(amido amine) dendrimers

PA-NHS: Palmitic Acid N-hydroxysuccinimide ester

PEI: Polyethylenimine

PEG: Polyethylene glycol

pEGFP: Plasmid Enhanced Green Fluorescent Protein

PLL: Poly(L-lysine)

rat-BMSC: rat-Bone Marrow Stromal Cells

siRNA: Small interfering RNA

TEA: Triethylamine

TNBS: 2,4,6-Trinitrobenzosulfonic Acid

UV: Ultraviolet

# 1. SCOPE

The main objectives of this research are two fold: i) to modify the commonly used nonviral vectors, poly-L-lysine (PLL) and polyethylenimine (PEI), by conjugating endogenous lipids of variable carbon chain lengths and saturations, and ii) to study the effect of these modifications on the carrier's ability to deliver and transfect foreign DNA into different cell types, specifically rat and human bone marrow stromal cells (BMSC) and human embryonic kidney 293 cells. The overall goal of the thesis work is to modify ineffective (PLL) and highly toxic carriers (PEI) with fatty acids like moieties, so that these new carriers could mimic the interactions of endogenous molecules with the lipid-rich membranes. It is expected that the lipophilic character of the proposed carriers will improve their delivery efficacy without compromising cell physiology.

**Chapter 2** of this thesis reviews the progress and the state-of-the art in the design of a specific type of nonviral carriers, namely cationic polymers modified with lipophilic moieties (i.e., fatty acid substituted) and hydrophobic moieties (i.e., alkyl substituted). Polymer-related carriers, especially short chain peptides modified with lipophilic substituents was covered in this Chapter, since they provide a means to better elucidate the functional performance of hydrophobic substituents. This literature provides preliminary evidence for the beneficial effect of lipophilic or hydrophobic moieties as modifying agents of cationic polymeric carriers and, specifically, how they can modulate extra- and intracellular trafficking for enhanced transgene expression.

The ability of the cationic polymers PLL and PEI and their conjugates with palmitic acid (PA, C16) to deliver a plasmid encoding for Enhanced Green Fluorescent Protein (pEGFP) to rat-BMSC was investigated in **Chapter 3**. PA is a natural occurring lipid utilized by the cells to control intracellular trafficking of proteins in particular membrane-crossing proteins. Therefore, PA conjugation was proposed as a strategy to increase DNA delivery and this Chapter developed

chemical conjugation schemes to graft the chosen lipid to the chosen polymers. It is expected that grafting PA to the polymers will transform these carriers into a better system for gene delivery. By means of improving payload packing, protection against enzymatic degradation, superior cell association, and/or reduced cytotoxicity, the modified carriers are expected to enhance transgene expression.

The studies described in **Chapter 4** were designed with the aim of exploring the effect and feasibility of substituting PLL with several endogenous lipids of variable chain lengths. The latter included the naturally found fatty acids, myristic (C14), palmitic (C16), stearic (C18), oleic (18:1) and linoleic (18:2) acid, and the unnatural caprylic acid (C8). PLL of variable molecular weights were employed to explore the effect of lipopolymer molecular weight on the transfection efficiency. The motivation for this study was the uncertainty regarding palmitic acid as the ideal lipid for PLL substitution. It was our intent to identify most suitable lipid(s), if any, for gene delivery into rat-BMSC. Furthermore, the relationship between the gene delivery efficiency and the extent of lipid substitution was studied in this Chapter.

The results of lipid modifications on high MW PEI (**Chapter 3)** suggested that there is room for improvement in the quest for the ultimate gene delivery agent. This finding motivated us to improve an alternative polymer, which is much less toxic but also inefficient transfection agent, namely the low MW (~2 kDa) PEI. This chapter explored the potential of longer chain lipids, including a saturated lipid (behenic acid, BA; C22:0), a *trans* fat (elaidic acid, ELA, C18:1T) and a series of essential fatty acids including erucic acid (EA; C22:1), α-linolenic acid (LLA; C18:3) and docosahexaenoic acid (DHA; C22:6). We evaluated the capabilities of the new materials in two cell lines; human embryonic kidney cells (293T) and human bone marrow stromal cells (h-BMSC). Additionally, we probed the internalization mechanism and intracellular trafficking of the carriers. To this end, we studied the effect of different drugs designed to block specific

intracellular pathways. The collective sum of these studies is described in **Chapter 5**.

Finally, in **Chapter 6** we draw the general conclusions of this theses work and propose new directions to thrust this research endeavor.

# 2. LIPID AND HYDROPHOBIC MODIFICATION OF CATIONIC CARRIERS ON ROUTE TO SUPERIOR GENE VECTORS

Incani V, Lavasanifar A, Uludağ H

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## 2.1. Introduction

Complete sequencing of the human genome is helping to identify disease-causing mutations and elevating gene therapy into a major thrust for treatment of human diseases. For a successful intervention, therapeutic genes need to be effectively transported and expressed in host cells. Expression of a desired gene is intended to alter protein production, modify aberrant gene expression, to inhibit cell proliferation, produce cytotoxic proteins or prodrug-activating enzymes for treatment in cardiovascular[1] and neurological[2,3] diseases, wound healing[4], cancer[5,6,7], macular degeneration[8] and infectious diseases[9]. Gene therapy has been investigated in clinics since 1990, and as many as 1347 clinical protocols[10] have advanced to phase I-III trials, although the number of 'successful' trials has been disappointingly low. The development of safe and efficient gene delivery systems was identified as a significant impediment. An effective delivery system is expected to safely deliver nucleic acids to a specific target, protect against degradation, and allow transgene expression at therapeutic levels for a desired duration. Non-viral carriers, based on synthetic compounds to deliver the genes intracellularly, are not plagued by the safety concerns, associated with viral carriers[11], but their effectiveness remains a concern. Non-viral carriers can usually bind to cell surface molecules and effectively enter cells on their own, but display reduced internalization when packaged with nucleic acids, and ultimately yield relatively lower transgene expression. In order to deliver on the promise of non-viral carriers, it is imperative to understand the barriers impeding efficient transgene expression and to elucidate structure-function relationships for effective carriers. This calls for better understanding of mechanisms of internalization, stability of delivered nucleic acids, effective crossing of cellular membranes, and binding and dissociation of nucleic acids from carriers. Polymer-based carriers present an advantage among others non-viral carriers, since they are readily amenable for engineering for specific functions.

## 2.2. Polymeric Gene Carriers

Non-viral carriers are based on cationic molecules (**Figure 2.1**) that can associate with nucleic acids and form nanoparticles suitable for cellular uptake and trafficking (**Figure 2.2**). Cationic polymers spontaneously form polyplexes with nucleic acids through electrostatic interactions between cationic amines of the polymer and anionic phosphates of the nucleotides. Compacting nucleic acids into small particles is a necessary prerequisite for efficient delivery into cells. The typical size of the polyplexes is ~150 nm, which can be modulated with addition of functional groups on polymers[12]. Primary and secondary amines are strong nucleophiles (high p*K*a) that can be also used to covalently bind targeting ligands[13,14], drugs[15] or other functional (e.g., pH sensitive) groups[16]. Tertiary amines are less nucleophilic but may buffer and prevent pH decline in endosomal compartments where nucleic acids may undergo degradation. Sequestering $H^+$ in endosomes results in an influx of $Cl^-$, which causes osmotic swelling leading to endosomal rupture by water influx. This process, known as the proton sponge effect[17], is hypothesized to facilitate nucleic acid release into the cytoplasm. Although cationic groups are primarily responsible for nucleic acid condensation, they can be employed for functional substitution. Modifications with small hydrophobic or more bulky lipid groups have been employed to facilitate cellular delivery, given the lipophilic nature of cell membranes. The fertile field of polymer functionalization will be discussed in section 2.4.

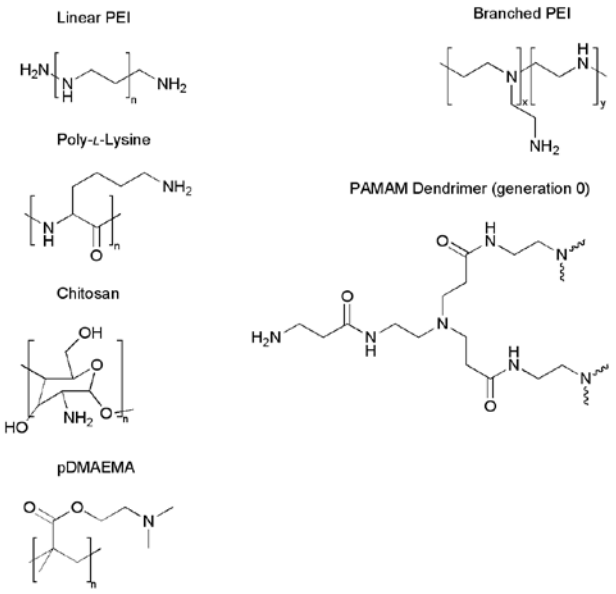


**Figure 2.1**. Prototypical cationic polymers employed as carriers of nucleic acids.

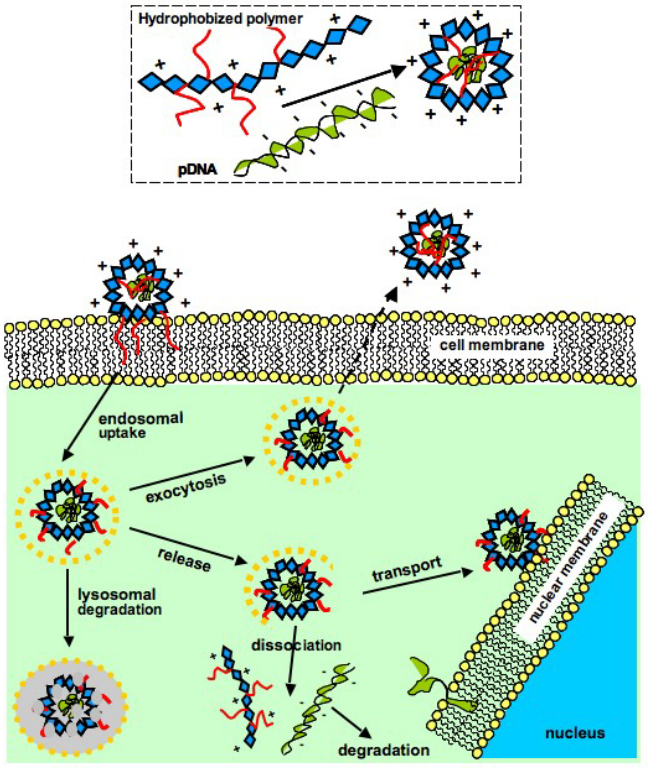


**Figure 2.2.** Schematic representation of possible sequence of events occurring after the cellular uptake of hydrophobized nanoparticles.

## 2.3. General Mechanisms of Cellular Uptake and Internalization

Endocytosis was established as the main mechanism of internalization for non-viral particles[18-20]. The endocytosis mechanism can be classified into two broad categories, phagocytosis (uptake of large particles), which is restricted to specialized cells and pinocytosis (uptake of fluid and solutes) that occurs in all cells (**Figure 2.3**). The latter pathway can take place by several mechanisms: clathrin-mediated endocytosis and non-clathrin-mediated endocytosis that includes macropinocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis[21]. The specific uptake mechanism of carrier/nucleic acid polyplexes may depend on the size of vesicles[22], type of coating[21] and specific cell type[23] encountering the polyplexes.

The clathrin-mediated pathway is an energy-dependant process, in which the cargo is carried to the cellular destination as a clathrin-coated vesicle rich in receptor-ligand complex in the 100-150 nm range[24]. In caveolae-mediated endocytosis, small (50-60 nm) hydrophobic membrane invaginations (i.e., caveolae) engulf extracellular material in cholesterol and glycosphingolipid rich vesicles. They are commonly found in endothelial cells and can internalize large molecular complexes such as toxins, bacteria and some viruses[25-27]. Unlike clathrin-mediated uptake, caveolae do not display a drop in pH upon uptake and the cargo can avoid lysosomal degradation. Clathrin- and caveolae-independent endocytosis occurs through small vesicles (40–50 nm) that are generally referred to as freely diffusing rafts on cell surface. The lipid composition of these vesicles provides specific sorting of membrane proteins and/or glycolipids based on their cellular destinations[28]. Macropinocytosis is characterized by formation of large, irregular primary endocytic (as large as 5 μm) vesicles after closure of ruffling membrane domains. Macropinosomes are structures that frequently move inwards towards the cell center and, unlike other types of vesicles; they do not have a

specific coat and do not concentrate receptors. Due to relatively large size of these vesicles, macropinocytosis is an efficient route for nonselective endocytosis of macromolecules[29].

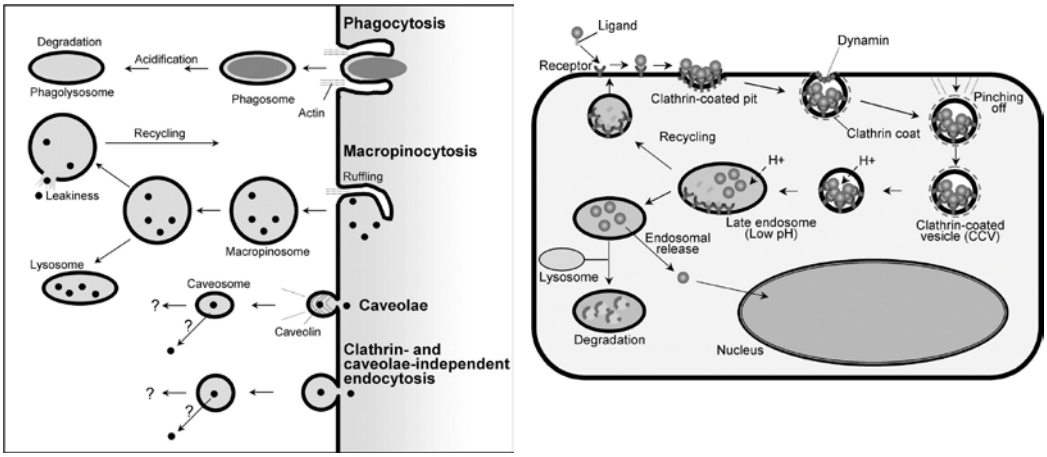


**Figure 2.3.** Different endocytosis pathways in nonviral gene delivery. Adopted from Khalil et al., Pharmacol. Rev. 2006, 58, 32-45.

Arginine-rich peptides were shown to translocate in a manner that does not involve the classical endocytic pathway,[30] and depend on the length of the peptide chain[31]. When complexed with pDNA, however, the complexes are internalized by the clathrin-mediated pathway. Uptake of polyplexes formed with longer amino acid polymers (e.g., poly-L-lysine, PLL) was suggested to proceed via clathrin-mediated endocytosis. Goncalves[20] *et al.*, showed that internalization of 70-200 nm histidinylated-PLL/DNA polyplexes could proceed by either clathrin-mediated endocytosis or by macropinocytosis. However, gene expression was seen only after clathrin-mediated uptake and stimulation of macropinocytosis afforded minimal protein expression in HepG2 cells. On the other hand, polyethylenimine (PEI)/DNA polyplexes were internalized by either clathrin-mediated or by caveolae-dependent endocytosis[32]. Only polyplexes from the caveolae-mediated uptake escaped the lysosomal compartment and effectively transfected the A549 and HeLa cells. Others have shown the clathrin pathway to be critical for PEI polyplexes in several cell lines[33]. The PEI/DNA internalization in some cells could be aided by heparan sulfate proteoglycan (HSPG) or syndecan-mediated phagocytosis[34]. This process was proposed to involve

complex binding to transmembrane HSPGs, which then induces clustering of cholesterol-rich rafts on the cell surface. This clustering triggers protein kinase C phosphorylation, followed by the binding of actin skeleton through the linker protein, resulting in tension fibers that facilitate the complex phagocytosis.

The polyplex size was shown to determine the entry pathway and subsequent intracellular routing. Particles up to 500 nm were shown to be internalized by B16 cells, while smaller particles distributing throughout the cell interior and larger particles primarily localizing to cell periphery. The involvement of clathrin- and caveolae-mediated uptake was dependent on the particle size. Although the caveolae were considered too small to accommodate larger particles, the authors speculate that electrostatic interactions and recruitment of internalization machinery was helpful to accommodate large particles in caveolae. Differences in transfection efficiency were noted depending on polyplex size[35]. The larger PEI polyplexes (1000 nm) transfected cells faster than smaller poly(2-dimethylamino)ethyl methacrylate (pDMAEMA) polyplexes (200 nm), possible due to increased sedimentation. Inhibition of caveolae-mediated uptake significantly reduced uptake of PEI polyplexes, but not that of pDMAEMA polyplexes. In the case of pDMAEMA polyplexes, caveolae-mediated pathway appeared to be important for effective gene expression and clathrin-mediated pathway did not necessarily promote delivery to cell nucleus.

## 2.4. Lipid and Hydrophobic Modifications of Cationic Carriers for Improved Delivery

Hydrophobic moieties are desirable in gene carriers since lipids are the main component of cell membrane and synthetic carriers may utilize lipid-like moieties for facilitated interactions with plasmid membrane and subsequent entry into cells. Under physiological conditions, the biological activities of several cellular proteins also require association with internal membranes. This membrane localization is dependent on the co- or post-translational attachment of lipid

residues via -$NH_2$, -SH or -COOH residues of proteins[36,37]. Covalently modified lipid-proteins play an important role in various biological processes[37], such as protein-protein and protein-lipid interactions, and as protein anchors to membranes[36]. **Table 2.1** contains a list of lipid and hydrophobically modified carriers that are subject of this review.

### 2.4.1. Influence of Lipid and Hydrophobic Modification on Nucleic Acid Binding and Polyplex Properties

In compacting nucleic acids into nanoparticles suitable for cellular uptake, carrier properties such as MW and charge density can be adjusted for optimal complexation. The N/P ratio (ratio of protonable amines to phosphates) used to formulate complexes is critical as well as the pH and ionic strength of the formulation medium. Given the beneficial effect of hydrophobic modification on transgene expression (**Section 2.5**), the particle properties obtained after hydrophobic modification is actively explored (**Table 2.2**). Although the size and charge of the particles are routinely assessed, the structural details and especially the location of hydrophobic substituents in the particles (i.e., buried inside vs. surface coating) remain to be thoroughly investigated.

#### 2.4.1.1. Lipid Modified Peptides

Using lipopeptides prepared from lysine and histidines ($CK_nH_m$, **Figure 2.4**), the C16-substituted peptides were more effective in condensing pDNA than the C12-peptides; the longer chain length was hypothesized to result in more compact and stable particles. Analogous results were observed when H was replaced by the hydrophobic residues tryptophan (W) and tyrosine (Y)[39-41]. The hydrophobic residues were proposed to facilitate formation of intermolecular ion pairs between multiple K residues and the phosphate backbone. In contrast, increasing the charge (N/P) ratio decreased the particle size (e.g., from 1186 to 423 nm with C16-$CKH_2$)[42]. Incorporation of various acyl moieties to the mastoparan peptide

(MS: INLK-ALAA-LAKK-IL-$NH_2$; **Figure 2.5**) gave this peptide a pDNA binding ability that was independent on the acyl chain length or lipophilic structure.

**Table 2.1.** Hydrophobically modified polymeric carriers explored with different nucleic acids and cell lines. Due to space limitations, synthetic polymers (Poly(DMAEMA-co-IPAAm), Poly(DMAEMA-co-PAA), coPolyvinyl ethers, Dextran-spermine, and PHEA-spermine) were not discussed in the text. Abbreviations for amino acids in peptides: R: arginine; C: cysteine; W: tryptophan; Y: tyrosine; K: lysine; H: histidine.

| Carrier | Modification | Gene/Plasmid | Target | Evaluation | Reference |
|---|---|---|---|---|---|
| $R_{4, 8, 12, 16}$ | C12, C18, cholyl, cholesteryl | PGV-C2-Luc | COS-7 | In vitro | 44 |
| $R_8$ | C18 | pCMV-Luc | NIH/3T3 | In vitro | 30 |
| $R_9$ | Cholesteryl | siVEGF/pCMV-Luc | CT-26/293T | In vivo/vitro | 45 |
| MS and HM | C2, C8, C10, C12, C16, D, cholesteryl | PGV-C-Luc | COS-7 | In vitro | 43 |
| $CWK_{3-18}$ | C2 | pCMLV-Luc | HepG2/COS-7 | In vitro | 39 |
| $CWK_{18}$, $CYK_{18}$ | C2 | pCMLV-Luc | HepG2 | In vitro | 41 |
| $CK_{1-3}H_{1-5}$ | C12, C16 | pCMV-Luc | COS-7 | In vitro | 42 |
| PLL | C8, C14, C16, C18, C18:1, C18:2 | pEGFP-$N_2$ | HSFC, BMSC | In vitro | 69, 70 |
| b-PEI | C2 | pGL3-Luc | MDA-MB-231/HEK293/C2C12 | In vitro | 51, 52 |
| b-PEI-PEG | C2, C3, C4 | pCMV-EGFP | COS-1 | In vitro | 50 |
| b-PEI | C1, C2, C12, C16 | gWIZ β-gal | COS-7 | In vitro | 83 |
| b-PEI, b-PEI-PEG | C1, C16 | pCMV β-gal, pEGFP-C1 | A549/A431 | In vivo/vitro | 89 |
| b-PEI | C14, Cholesteryl | pSV β-gal | 293 | In vitro | 87 |
| b-PEI | C8, C18, C18:1, C18:2 | siRNA | B16 | In vitro | 23 |
| b-PEI | Cholesteryl | pCMV-Luc | CT-26/ 293T | In vitro | 53 |
| b-PEI | Cholesteryl | p2CMVmIL-12, pIRESmIL-12 | CT-26 | In vivo/vitro | 54 |
| b-PEI | Cholesteryl | pCMS-EGFP | Jurkat | In vitro | 56 |
| b-PEI | Cholesteryl | siRNA | PC-3 | In vivo/vitro | 55 |
| l-PEI | Cholesteryl | SMD2-LucDITR | HepG2 | In vitro | 82 |
| l-PEI | Cholesteryl | pmIL-12e/pCMS-EGFP | MCF-7/Renca/B16-F0 | In vitro | 57 |
| PAMAM | C12 | pGL3-Luc | CV1 | In vitro | 60 |
| PAMAM | C1, C12 | pBudCE4.1/LacZ/CAT | COS-7 | In vitro | 59 |
| PAMAM-OH | C1 | pGL3-Luc | 293T | In vitro | 58 |
| PLL dendrimer | C14 | pSVβ-gal | COS-7, BHK-21 | In vitro | 94 |
| PLL dendrimer | C14 | pGL3-Luc, FITC-ODN, pCI-Luc, RNAi | HeG2, HeLa, CHO, COS-7, Jurkat, ECV304, C2C12 | In vitro | 95 |
| Chitosan | C1 | RSV-α3 | COS-1, Caco-2 | In vitro | 96 |
| Chitosan | C4, C8, C12, C16 | pcDNA 3.1-CAT | C2C12 | In vitro | 61 |
| Chitosan | C18 | pEGFP-C1 | A549 | In vitro | 62 |
| Chitosan | Deoxycholic acid | pCMV-CAT/pCMV-Luc/pEGFP-N1 | COS-1/COS-1/293T | In vitro | 65, 74, 87 |
| Glycol Chitosan | C1, C16 | pCMV sport β-gal | A431, A549 | In vivo/vitro | 63 |
| Glycol Chitosan | 5β-cholanic acid | pCMV-Luc | COS-1 | In vivo/vitro | 66 |
| Poly(DMAEMA-co-IPAAm) | BMA | pCMV-LacZ | COS-1 | In vitro | 99, 100 |
| Poly(DMAEMA-co-PAA) | BMA | siRNA | HeLa, ATCC, CCL-2 | In vitro | 101 |

**Table 2.2.** Effect of hydrophobic or lipid substitution on the size of complexes formed between the nucleic acids and carriers. The indicated particle sizes were obtained at specific N/P ratios where cell uptake of the complexes was assessed.

| Base Polymer | Substituent | Particule Size (nm) | | |
|---|---|---|---|---|
| | | *Unmodified* | *Lipid-Modified* | *Ref.* |
| $CWK_{3,8,13,18}$ peptide | C2 | N/A | 53-231 | 39 |
| $CWK_{18}$, $CYK_{18}$ peptide | C2 | N/A | 78 | 41 |
| $CK_{1-3}H_{1-5}$ peptide | C12, C16 | N/A | 250-300 | 42 |
| $R_8$ | C18 | 2917/101 | 5148/126 | 44,30 |
| PLL (25 kDa) | C16 | 126 | 112 | 47 |
| | | N/A | 100-160/350[a] | 46 |
| b-PEI (25 kDa) | C2 | 40-60 | 80-120 | 51 |
| | | 243 | 250-270/450750 | 52 |
| b-PEI (25 kDa) | C12, C14, C16 | N/A | 235-480 | 49 |
| b-PEI (750 kDa)-PEG (8 kDa) | C2, C3, C4 | N/A | 84-124 | 50 |
| b-PEI (1.8 kDa) | Cholesterol | N/A | ~50/26-62 | 53, 54 |
| | | | 100-250 | 55 |
| b-PEI (1.8 and 10 kDa) | Cholesterol | 84-383[b] | 110-205 | 56 |
| l-PEI (25 kDa) | Chol. (L, B and T) | > 200 | 250-275 | 57 |
| Chitosan (50 kDa) | C4, C8, C12, C16 | N/A | 261-343[c] | 61 |
| Chitosan (450 kDa) | C18 | N/A | 102-318 | 62 |
| Quaternized-Chitosan | C16 | N/A | 200-500 | 63 |
| Chitosan (5-200 kDa) | Deoxycholic acid | N/A | 130-300[c] | 64 |
| Chitosan (1-5 kDa) | Deoxycholic acid | 68-82 | 86-209 | 65 |
| Chitosan (250 kDa) | 5β-cholanic acid | N/A | 277 | 66 |

[a]Particle size for circular or PCR DNA and linear DNA, respectively. [b]Particle size for BPEI 1.8 kDa. [c]Aggregates with no DNA.

On the other hand, incorporation of acyl C10 to a α-helix model peptide (HM; $[LARL]_3$-$NH_2$; **Figure 2.5**) imparted a weaker pDNA binding as compared to C2- and C16-incorporation, for reasons not clear yet[43].

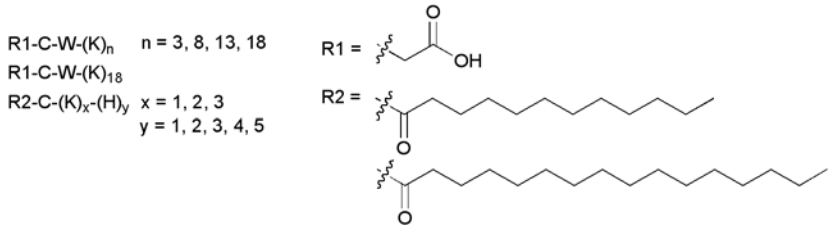


**Figure 2.4.** Lysine and histidine rich peptides modified with lipophilic moieties[39,41,42].

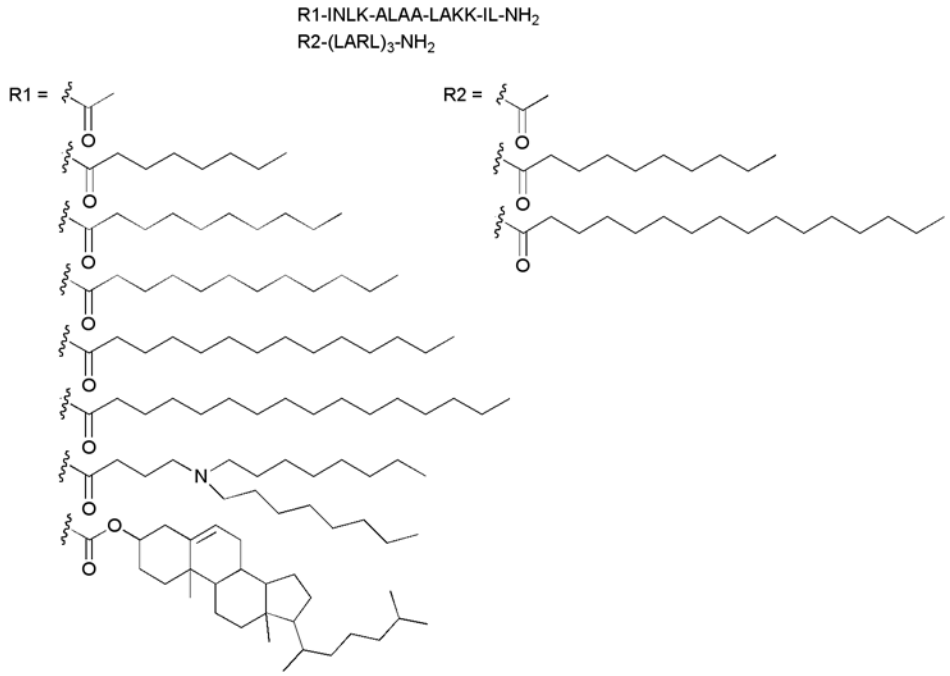


**Figure 2.5.** Mastoparan (INLK-ALAA-LAKK-IL-NH2) and 12 a.a. α-helix model peptide ((LARL)3-NH2) modified with lipophilic moieties[43].

Large particle sizes were obtained after pDNA condensation with C18-substituted $R_8$ or virus-derived Rev (TRQA$(R)_2$N$(R)_4$WRERQR; **Figure 2.6**) peptides, which was a result of aggregation[44]. Other authors[30] used C18-$R_8$ and efficiently condensed pDNA into stable and smaller polyplexes with no free pDNA exposed to the medium - a critical issue for preventing pDNA degradation. The surface charge of the particles formed with C18-$R_8$ was relatively low, possibly due to partial shielding of the cationic charge by the C18 substituent. With $R_9$, the bulky cholesterol substitution did not affect pDNA binding of $R_9$, and no further

changes in the polyplex structure were reported[45].

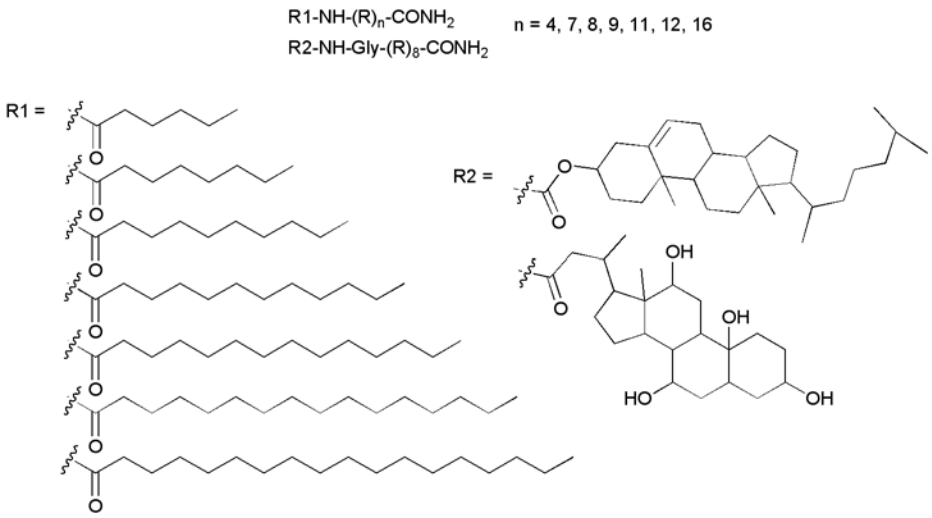


**Figure 2.6.** Arginine-rich peptides modified with lipophilic moieties[30,44,45,67].

### 2.4.1.2. Lipid Modified Poly-L-lysine (PLLs)

Our group[46] investigated condensation abilities of N-acyl C16 substituted PLL (25 kDa) (**Figure 2.7**) with different types of pDNA molecules. A circular plasmid DNA formed smaller spherical particles (100-160 nm) than an equivalent linear DNA isoform (~350 nm, **Figure 2.8**). The particle size decreased with increasing carrier:DNA ratio for some combinations of carriers and DNA isoforms (e.g., C16-PLL with circular DNA), other combinations (e.g., C16-PLL with linear DNA) did not give a predictable pattern, so that N/P ratio might not be the sole determinant of particle size. The C16-PLL[47,48] gave smaller particles (~112 nm) as compared to the particles formed with the native PLL (~126 nm).

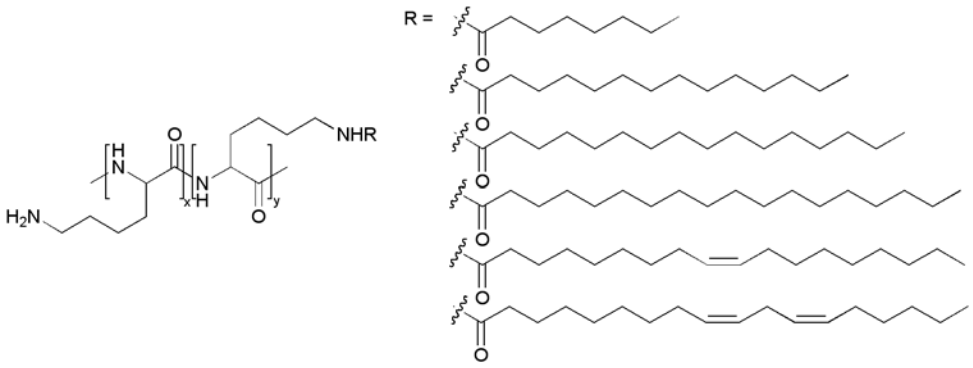


**Figure 2.7.** Poly-L-lysine (PLL) modified with lipophilic moieties[68-70].

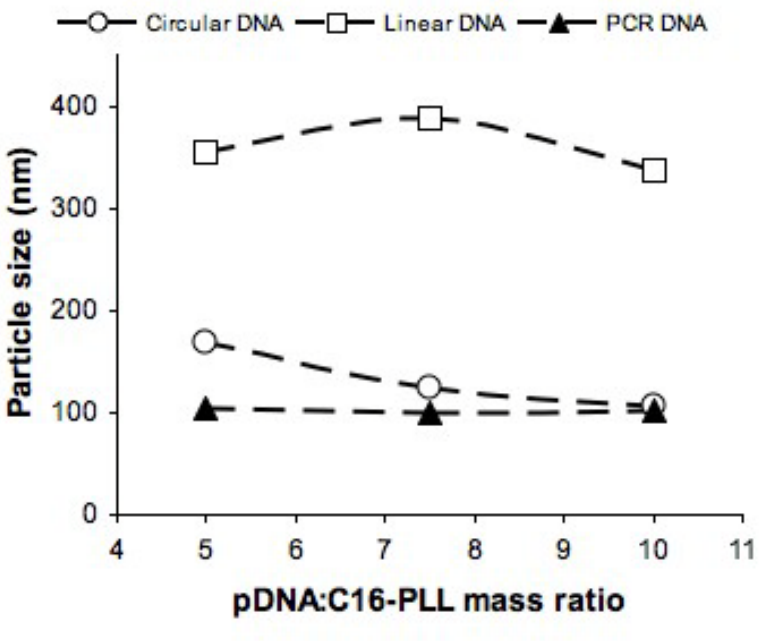


**Figure 2.8.** Mean particles sizes (nm) obtained with C16-PLL at three (1:5, 1:7.5 and 1:10) pDNA:carrier mass ratios. The pDNA molecules used were a circular pDNA (4272 base pairs), a linearized version of the circular pDNA (4272 base pair) and linear, PCR-amplified fragment form the circular pDNA (2209 base pairs)[46].

### 2.4.1.3. Lipid and Hydrophobically Modified PEIs

The 750 kDa b-PEI derivatives substituted with alkyl and acyl moieties (C12, C14 and C16) (**Figure 2.9a**) gave similar size particles (235-480 nm). The increased alkyl substitution generally increased the particle size, although acyl substituents were less sensitive to such a change. Increasing N/P ratio decreased the overall particle size, maximizing transfection[49]. In contrast, acylation of the same polymer with shorter C2, C3 and C4 chains (**Figure 2.10**) had a significant effect on the size and surface charge of polyplexes; as the chain length and degree of substitution increased, so did the particle size (i.e., from 84 to 124 nm). Acylation reduces the net positive charge of polymers, which could weaken pDNA binding and promote polyplex dissociation[50]. Similar trends were also observed with acylated (C2) 25 kDa b-PEI[51,52], where increasing acylation of PEI led to larger particles. Increased aggregation due to increased hydrophobicity might have also contributed to the increase in particle size.

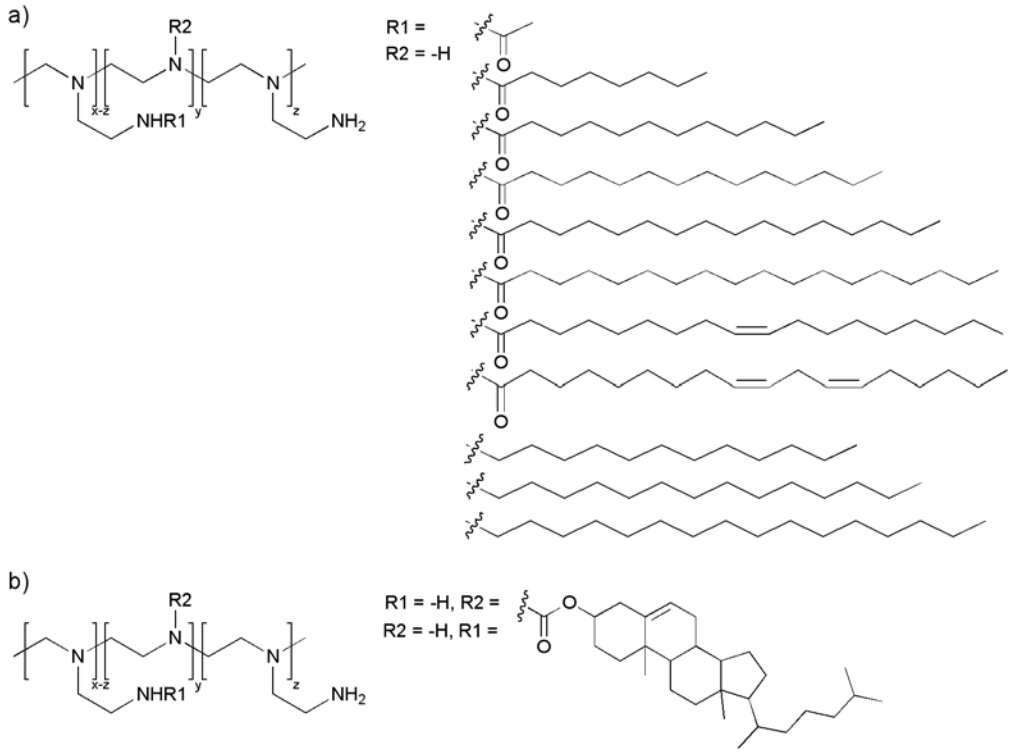


**Figure 2.9.** Branched PEI with lipophilic or hydrophobic moieties[49,50,53,56,73,88].

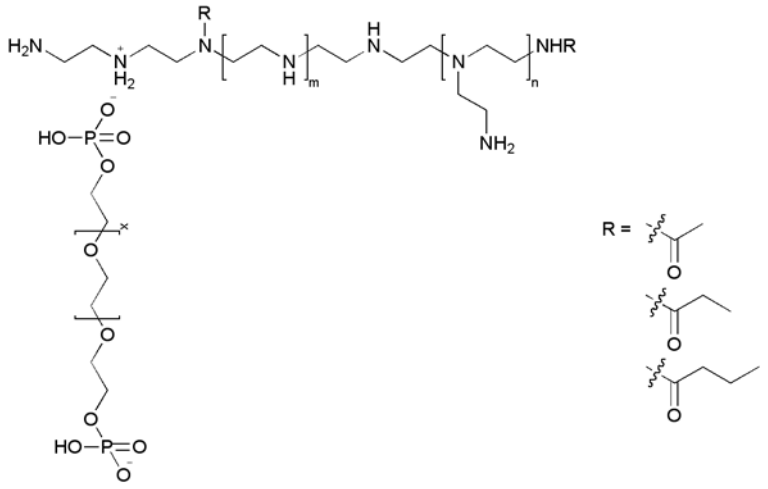

**Figure 2.10.** Branched PEI 750 (PEI-PEG-bis-P) modified with lipophilic moieties[50].

Several groups explored highly-lipophilic cholesterol for polymer modification. Grafting the primary amines of b-PEI (1.8 kDa) with cholesterol (**Figure 2.9b**) created a water-soluble lipopolymer that was able to condense pDNA into 26-149 nm particles[53,54]. Cholesteryl-PEI formed ~100 nm particles with siRNA, with decreasing particle size as the carrier:siRNA ratio increased. However, complexation with native PEI was not investigated so that the specific effect of cholesteryl on siRNA condensation cannot be assessed[55]. On the other hand, pDNA polyplexes formed with b-PEI (1.8 and 10 kDa) carrying cholesteryl on secondary amines[56] (**Figure 2.9b**) had reduced particles sizes (110-205 nm). The authors pursued this mode of modification to preserve the primary amines for more effective pDNA condensation. Furgeson[57] *et al*., generated different geometries of PEI-cholesteryl conjugates, including linear (L; cholesteryl substituted at terminal end of l-PEI), T-shaped (T; cholesteryl substituted at secondary amines on l-PEI backbone), and a combination of the two (LT) (**Figure 2.11a**). Irrespective of the substitution geometry, the pDNA particles were >300 nm after modification. The increase in particle size observed could be due to intermolecular H-bonding between neighboring polyplexes resulting from a conformation change within the cholesteryl-l-PEI conjugates. Polyplexes with T-l-PEI had a smaller size than the particles from L-l-PEI and LT-l-PEI, suggesting

better interaction of cholesteryl moieties that are placed on the polymeric backbone and possibly better cellular internalization. A general trend for increasing surface charge was observed in the following order: T-l-PEI > LT-l-PEI > L-l-PEI > b-PEI > l-PEI. An abrupt increase was particularly observed for T-l-PEI and LT-l-PEI and the authors suggested that reorientation of the cationic and hydrophobic units in an aqueous environment for this effect[57].

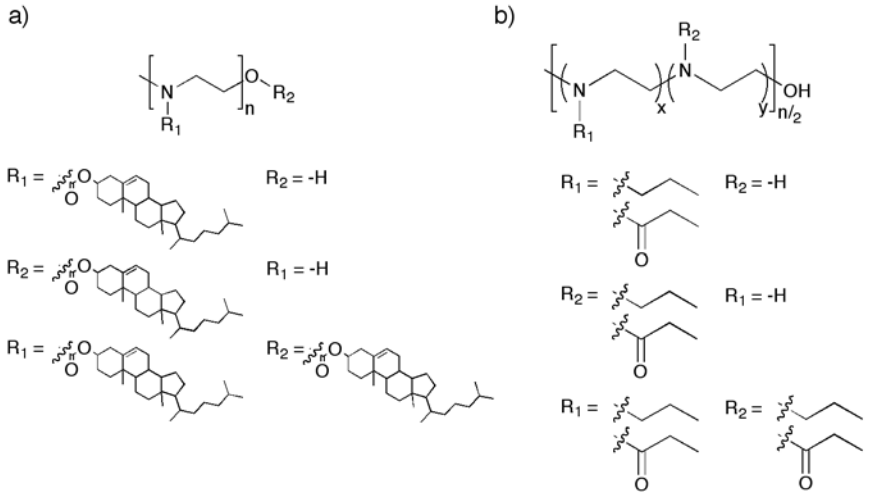


**Figure 2.11.** Linear PEI modified with (a) lipophilic and (b) lipophilic and hydrophobic moieties[57,82].

## 2.4.1.4. Hydrophobically Modified Dendrimers

Quaternization (q) of the interior tertiary amines of PAMAM-OH dendrimers with methyl groups (C1) led to formation of relatively smaller particles with permanent charges as compared to unmodified PAMAM particles[58] (**Figure 2.12a**). The particle size was dependent on the polymer: pDNA ratio and the degree of C1 substitution. At low charge ratio and C1 content (i.e., 57%), q-PAMAM-OH polyplexes formed very large particles (680 nm), but increasing the charge ratio led to smaller particle size (123 nm). On the other hand, particles formed between pDNA and highly q-PAMAM-OHs were <157 nm at all polymer:pDNA charge ratios, suggesting that these particles might be suitable for receptor-mediated endocytosis. The particles did not acquire cationic surface charge even at high

carrier ratios, thus cellular interaction was hindered relative to cationic particles formed with native polycations. Viullaume[59] *et al*., also quaternized PAMAM tertiary amines with C12 (side chain) and C1 (side chain and backbone) (**Figure 2.12a**). q-PAMAM with C1 either on the side chain or the backbone formed disperse particles of 55-350 nm compared to q-PAMAM C12/C1 conjugate that formed smaller particles of ~35 nm (i.e., C12 facilitated smaller particles). The surface charge of polyplexes formed with pDNA:q-PAMAM C1 side chain and q-PAMAM C12/C1 were weakly positive, resulting in unstable particles. Additionally, increasing the quaternization content of C1 side chain or with C12 led to less tight binding to pDNA (compared to q-PAMAM C1 backbone), due to a dilution of the total charges and/or steric hindrances of bulky substituents. Higher generation PAMAM C12 (**Figure 2.12b**) had higher ability to form pDNA polyplexes compared to unmodified PAMAM. It is likely that the larger number of amines and the C12 substituent contributed to tighter binding to pDNA by multivalent ionic bonds as well as through hydrophobic interaction. The increased hydrophobic content improved polyplex stability, based on polyplex de-stabilization with heparin[60].

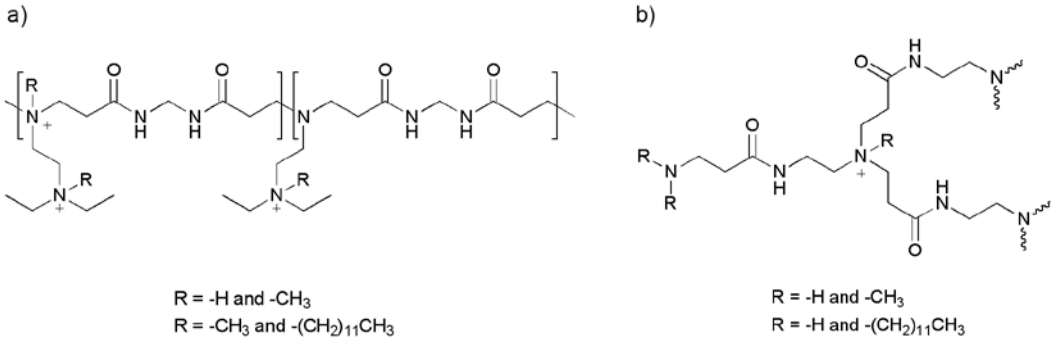


**Figure 2.12.** PAMAM modified with hydrophobic moieties[58-60].

## 2.4.1.5. Lipid and Hydrophobically Modified Chitosans

The natural polysaccharide chitosan has been modified by several groups. Liu[61] *et al*., alkylated (C4, C8, C12, C16) chitosan and obtained pDNA condensates of

261-346 nm with increasing size at longer chain lengths (**Figure 2.13a**). The hydrophilic domains were hypothesized to provide cooperative binding with pDNA in addition to electrostatic interactions. Acylation of chitosan with C18 (**Figure 2.13a**) led to pDNA polyplexes that displayed maximal size (~318 nm) at a critical N/P ratio, after which a decrease in size was observed at higher N/P ratios due to polyplex stability[62]. The surface charge, on the other hand, increased as the N/P ratio was increased.

Uchegbu[63] *et al*., observed an increasing particle size as the q-chitosan MW increased. Modification with C16 alkylation vs. acylated q-chitosan (**Figure 2.13b**) resulted in larger spherical condensates (200-500 nm) with neutral or positive surface charge compared to unmodified q-chitosan. Amine substitution did not appear to have a profound effect on pDNA affinity even when the amines were substituted with electron withdrawing groups (-CONH-). Grafting deoxycholic acid (DA) to chitosan (**Figure 2.13a**) led to self-aggregates in the absence of pDNA with a size range of 130 to 300 nm depending on the degree of polymerization[64]. The increase in size was attributed to the change of shape of aggregates from a bamboo-like cylindrical structure (i.e., DA-chitosan of 5 kDa) to a poorly organized bird nest-like structure (i.e., DA-chitosan of 200 kDa), because of the chain rigidity arising from the parent chitosan and bulky DA. Higher MW DA-chitosan condensed pDNA more compactly as compared to low MW DA-chitosan. Consistently, DA substitution[65] resulted in larger self-aggregates (e.g., 200-240 nm) that increased with the degree of substitution. The introduction of DA on chitosan enhanced pDNA condensation capacity and the particle size accordingly decreased (i.e., 68-209 nm). This phenomenon might be due to rearrangement and/or reformation of the new supramolecular structure during polyplex formation. A recent study suggested that hydrophobic modification of glycol chitosan with β-cholanic acid (**Figure 2.13b**) was absolutely necessary for condensation of hydrophobized pDNA (i.e., pDNA ion-paired with an aliphatic amine)[66], where unmodified chitosan was unable to condense the pDNA.

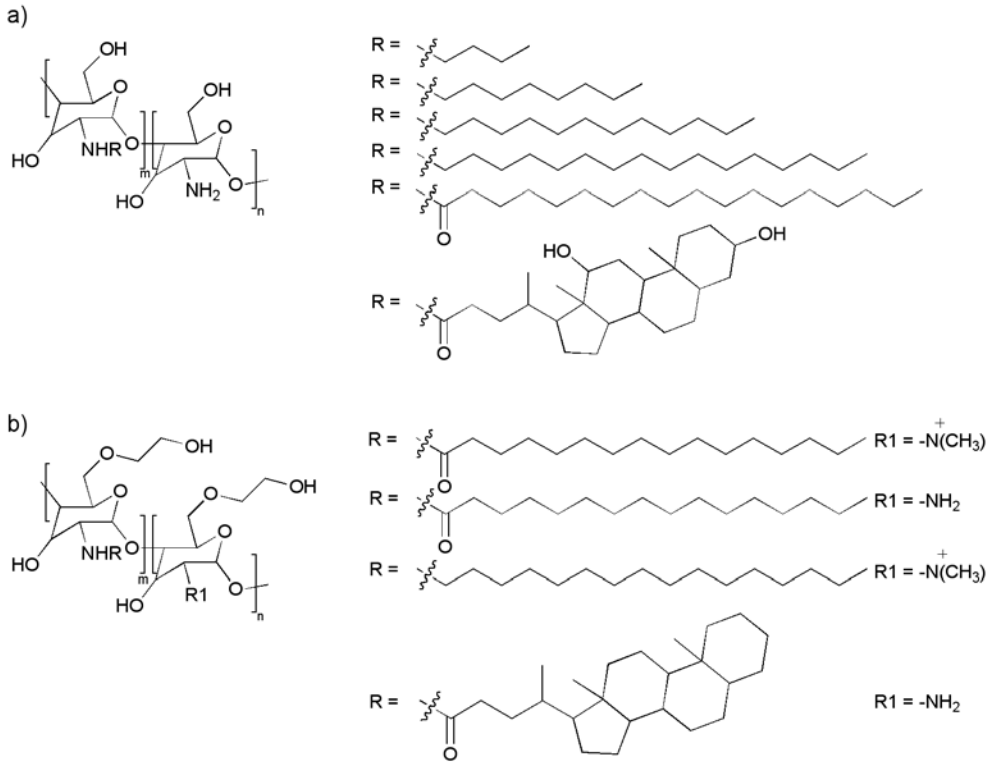


**Figure 2.13.** Chitosan (a) and glycol chitosan (b) modified with lipophilic or hydrophobic moieties[61-66].

## 2.4.2. Internalization of Polyplexes Formed with Modified Carriers

### 2.4.2.1. Lipid Modified Peptides

Khalil[30] *et al.*, investigated the mechanism of cellular internalization of $R_8$ and C18-$R_8$ carriers in NIH3T3 cells by blocking specific endocytosis pathways. Internalization of $R_8$ alone (without a cargo) was only slightly affected, if any, by lowering the temperature (inhibitor of endocytosis), presence of sucrose (specific inhibitor of clathrin-mediated endocytosis) or N-ethylmaleimide (NEM, inhibitor of all vesicular transport). On the other hand, low temperature, sucrose and NEM significantly inhibited the internalization of C18-$R_8$, indicating its active transport. These results suggested that $R_8$ internalization occurs mainly through pathways different from the classical endocytosis, while that of C18-$R_8$ may occur

mainly through the clathrin-mediated endocytosis. In contrast, internalization of both polyplexes was significantly inhibited by low temperature or sucrose incubation, indicating internalization via the clathrin-mediated pathway. The presence of the cargo seemed to have altered the internalization pathway. While C18-$R_8$ condensed pDNA effectively and detected either at the cell surface or intracellularly, $R_8$ did not efficiently condense the pDNA and yielded only low amounts of pDNA in the cytosol. C18 groups apparently favored hydrophobic interactions for a more stable polyplex, leading to higher cellular uptake. Along the same lines, Pham[67] *et al.*, systemically explored changes in cell-localizing ability of oligoarginines after fatty acid modifications (**Figure 2.6**). C14-$R_7$ demonstrated the optimal cellular association among the synthesized lipopeptides and gave a 7-fold increased uptake as compared to $R_7$. Further lengthening of the lipid chain to C16 decreased the association efficiency with the cells. Although the authors did not study the mechanism(s) of internalization, the uptake of lipid-substituted polyarginines was hypothesized to follow a similar pathway to that of polyarginines (i.e., seemingly endocytosis-independent), but only at enhanced levels. However, both lipid substitution and pDNA cargo are likely to alter the entry pathway.

### 2.4.2.2. Lipid Modified PLLs

C16 substitution greatly enhanced pDNA delivery into bone marrow stromal cells (BMSC) and human skin fibroblast cells (HSF). [47, 48, 68] In the presence of Brefeldin A, a known inhibitor of cellular endocytosis at the trans-Golgi network, a significant reduction in cell-associated pDNA was observed, suggesting active uptake of the polyplexes. The pDNA particles condensed with C16-PLL were initially present at the cell periphery, whereas the particles were adjacent to the cellular nuclei after 5 h. The uptake was dependent on the N/P ratio[47,48] and different types of DNA (c-DNA, l-DNA and pcr-DNA)[46] were effectively delivered with the C16-modified PLL.

Cellular delivery of pDNA with lipophilic-PLL was dependent on polymer MW; although, lipid-substituted 4 kDa PLL enhanced cellular uptake, this was much lower than the delivery with 25 kDa derived polymers. A positive correlation between the lipid content and cellular uptake was evident with lipid-substituted PLLs,[69,70] where highly substituted polymers enhanced the uptake (**Figure 2.7**). An inverse correlation between lipid content and polyplexes dissociation (heparin induced) was additionally observed, suggesting that hydrophobic content of polymers was critical for polyplex dissociation[69]. The increased stability with lipid substitution might have been the underlying basis for increased uptake[69], reminiscent of C18-substituted oligoarginine[30].

Sensitivity of the polyplexes to nucleases is one critical barrier that lowers the gene delivery efficiency. As observed with permeabilized cells, cytoplasmic nuclease activity has an inhibitory effect on intracellular transport of pDNA, particularly DNase I (found in cytoplasm as well as serum and extracellular space) and DNase II (typically found in endosomes)[71,72]. We observed that all PLL carriers, independent of the nature of the lipid substituent, equally protected the pDNA from degradation *in vitro*. Recovery of intact pDNA from the cells was possible up to 7 days after treatment with the polyplexes of C14-, C16- and C18-PLLs.

### 2.4.2.3. Lipid and Hydrophobically Modified PEIs

Lipid-substituted b-PEIs were systematically evaluated for siRNA delivery in B16 melanoma cells[73] (**Figure 2.9**). In line with improved complexation, particularly oleoyl (C18:1) and C18-substituted b-PEI displayed better resistance to heparin-induced dissociation and siRNA protection against serum nucleases. All polymers, including native b-PEI, routinely displayed >90% siRNA-positive cells, indicating high efficiency of the polymers for siRNA delivery. A reduction in siRNA uptake was observed with a reduction of polymer concentration used to prepare polyplexes, even when all siRNA had been condensed into particles.

Whether excess polymer continues to alter the polyplex properties or facilitates complex uptake remains to be clarified.

Forrest[51] and Gabrielson[52] *et al.*, observed a 4-fold increase in uptake of the polyplexes formed with 76 and 96% acetylated b-PEI, but further modification of b-PEI decreased cellular uptake in several cells. The addition of acetyl groups will enhance the lipophilicity of polyplexes, thereby increasing uptake due to increased polyplex association with cell membranes. Despite a reduced charge density, acetylated PEI was able to form polyplexes with a positive z-potential necessary for nonspecific association with cell membrane; however, the polyplexes were more easily dissociated as compared to native PEI. It is possible that charged cellular or endosomal membranes could dissociate the polyplexes prematurely, leaving some pDNA free in the cytosol (results supported by FRET methodology)[51,52].

Using an siRNA against vascular endothelial growth factor (VEGF)[55], increased delivery was obtained in PC3 cells with cholesteryl-substituted PEI (~64% vs. ~10% with b-PEI). The internalization of pDNA with cholesteryl-l-PEI (in MCF-7 cells) appeared to involve Low Density Lipoprotein Receptor[57], since saturation of this receptor inhibited the efficiency of uptake.

### 2.4.2.4. Hydrophobically Modified Dendrimers

Mixed results were obtained with quaternization of PAMAM molecules. The increased level of quaternary content (C1) in the backbone appeared to have a beneficial effect due to better pDNA condensation, leading to smaller particles and higher surface charge. This result is in agreement with literature indicating that small size (< 150 nm) polyplex is crucial for nonspecific adsorptive endocytosis and nuclear trafficking [59]. In contrast, Lee[58] *et al.*, observed that although q-PAMAM-OH with C1 on the backbone efficiently condensed pDNA compared to PAMAM-OH, its surface charge was anionic, thus cellular

interaction with this carrier was reduced. The nature of the PAMAM was different between the two studies (i.e., difference in generation number) so that this might have affected the effectiveness of C1 substitution for pDNA condensation.

### 2.4.2.5. Lipid and Hydrophobically Modified Chitosans

Hydrophobic modification of chitosan facilitated pDNA entry into cell membranes due to perturbations caused by electrostatic as well as hydrophobic interactions. Moreover, alkyl-chitosan required less amount to condense pDNA (indicating facilitated binding), and gave higher transgene expression due to increased uptake and higher unpacking of the cargo[61]. Hu[62] *et al.,* also formed stable polyplexes with pDNA with C18-substituted chitosan, which better protected the cargo against enzymatic degradation. With q-chitosan, modification with alkyl or acyl C16 groups was necessary to enhance cellular uptake when the degree of polymerization (DP) of chitosan was 73-171. As DP increased, the cell association decreased with q-chitosan[63]. In contrast, increasing DP of DA-chitosan led to bigger self-aggregates capable of condensing pDNA and enhancing cell association of pDNA. The combined characteristic of the amphiphilic substituent (membrane destabilization) and the chitosan (buffering capacity) might have been responsible for the enhanced gene delivery[65,74]. Low level of substitution of chitosan with β-cholanic acid was sufficient to condense hydrophobized pDNA into particles with nearly neutral surface charge. These particles were efficiently internalized probably through endocytosis, unlike the native chitosan since it could not condense the pDNA[66].

## 2.5. Transgene Expression by Modified Carriers

### 2.5.1. Lipid Modified Peptides

A significant improvement in transfection efficiency was observed for C18-substituted HIV-1 Tat (48-60) and FHV coat (35-49) peptides[44] in COS-7 cells,

even though the ability for translocation and protein delivery was unaffected[31]. Same improvement in transgene expression was also observed with N-terminal C18-$R_8$ and C18-$R_{12}$ as compared to unmodified peptides. The transfection efficiency with C18-modified $R_n$ was lower when $n<8$ or $n>12$, but all C18-peptides showed higher transfection efficiency than the corresponding oligoarginines[31]. C12-$R_8$ and cholesteryl-$R_8$ were also effective transfection reagents, and similar results were later reported with C18-$R_8$ in NIH/3T3 cells[30]. The authors suggested that C18 moieties helped condense pDNA into stable polyplexes that were highly internalized due to better absorbtion onto cellular surfaces as a result of ionic and hydrophobic interactions. The addition of the endosomolytic agent chloroquine to C18-peptides showed no improvement, indicating the inherent ability of C18 polyplexes to disrupt the endosomes. Cholesteryl-$R_9$ polyplexes prepared by another group also readily transfected 293T cells with pDNA, and suppressed VEGF production (by ~55%) after siRNA delivery in CT-26 cells, unlike $R_9$[45]. *In vivo* studies yielded an effective VEGF siRNA delivery with cholesteryl-$R_9$, based on marked suppression of tumor growth, reduced tumor vascularization and lower levels of VEGF *in situ*.

The transfection efficiencies with C12, C14, C16 and dialkylcarbamoyl-substituted MS peptide[43] were 3-6 fold higher as compared to pDNA treated COS-7 cells. Chloroquine increased the efficiencies of substituted MS peptides, so that endosomal entrapment was an impediment for these carriers in the chosen cell model. This was unlike the $R_n$ peptides; the ability for endosomal escape may be significantly influenced by the nature of base carrier and not lipid substitution alone. In the case of C2, C10, and C16-HM series of peptides, only C10-HM showed significantly gene transfer, indicating an optimal acyl chain length for delivery. The C10-HM apparently had considerable perturbation activity against endosomal membranes.

Another class of peptides whose length and amino acid composition significantly influenced transgene expression was lipophilic K-rich peptides ($K_{3-36}$) with single

C and W residues[39]. Peptides of 13-18 Ks and possessing a single hydrophobic residue (W) enhanced gene transfer in HepG2 cells: a 40-fold reduction in particle size and 1000-fold increase in luciferase expression was observed for alkyl-$CWK_{18}$ in HepG2 cells compared to $K_{19}$, whereas lipophilic peptides with K<8 either failed to condensate pDNA or formed large particles incapable of transfecting cells. These results suggested the efficiency pDNA condensation to be closely related to delivery efficiency, as discussed in **Section 2.4**. However, a significant difference was observed with COS-7 cells; alkyl-$CWK_8$ and $K_{19}$/pDNA polyplexes mediated significant gene expression despite their inactivity in transfecting HepG2 cells. The size requirements for the polyplexes might have been less strict in COS-7 cells. Given that K length of alkyl-$CWK_{18}$ and $K_{19}$ were nearly equivalent and that a single C did not affect transgene expression or cytotoxicity, the authors hypothesized that the inclusion of single W increased binding to pDNA, and condensed pDNA into smaller particles suitable for pinocytosis. The same group[41] subsequently changed the alkyl-peptide sequence from W to Y and found indistinguishable physical properties and equivalent levels of gene expression in HepG2 cells.

Analogous peptides[42] C16-$CK_2H_2$ were found to better sustain transgene activity as compared to C12-$CK_2H_2$ in COS-7 cells, which correlated with superior pDNA condensation, polyplex stability and nuclease resistance. Increasing K residues in these peptides (i.e., from C16-$CK_1H_2$ to C16-$CK_{2\text{-}3}H_2$) increased the transfection activity, possibly due to increased surface charges on polyplexes. In contrast, increasing the H residues ($H_2$ to $H_5$) led to less efficient complexation and delivery, even though additional Hs might have helped with endosomal escape. The best lipopeptides (i.e. C16-$CK_2H_2$ and C16-$CK_3H_2$) showed higher transfection efficiencies than the PLL and b-PEI (750 kDa).

### 2.5.2. Lipid Modified PLLs

Having ~16 C16 substitutions per PLL improved transgene expression in BMSC

up to 5-fold compared to unmodified PLL (25 kDa), where the maximal level of expression was found on day 3 after which a reduction was noted[48,68]. Under conditions that allowed robust HSF growth, C16-PLL provided robust transgene expression up to 10 days, a period that might be sufficient for applications that require transient expression[47]. Dosing BMSC multiple times with C16-PLL polyplexes and adding Lipofectamine™ 2000 to polyplexes improved transfection efficiency. It is possible that prolonged incubation of particles in the media caused adverse changes in complex properties; therefore providing fresh particles have increased intracellular delivery of polyplexes. In addition, cationic lipid groups might have had better association with amphiphilic C16-PLL leading to a favorable outcome, as seen with other systems[75]. A positive correlation between transfection efficiency and the extent of lipid substitution was noted with modified PLLs, C18-PLL particularly giving the highest transfection levels[70].

There was no obvious correlation between the extent of substitution and cytotoxicity, but additional toxicity was evident at times as a result of lipid substitution. Similar results were obtained with HSF cells, where C14 and C18-PLL gave the highest levels of expression over a 10-day period[69]. Prolonged presence of intact pDNA in the cytosol was evident[76,77], which is likely to result in higher nuclear localization of exogenous DNA due to its persistence around the nuclear membrane increasing its chance of nuclear uptake during cell division. A correlation between the extent of lipid substitution and initial transfection efficiency was evident, but a significant drop in the transgene expression was noted on the long run consistent with the transient nature of non-viral delivery[78].

Other research groups also prepared hydrophobically modified PLL but did not test these polymers on their own. The polymer was incorporated into different systems, such a lipid vesicle[79], terplex[80] or formulated as a nanoemulsion[81]. Although these systems have achieved high levels of transfection, studies with the lipid-substituted polymer as the single transfection agent were not conducted; therefore we will not discuss these findings.

### 2.5.3. Lipid and Hydrophobically Modified PEIs

Increasing the degree of acetylation of 25 kDa b-PEI (up to 57%) enhanced the transfection efficiency as compared to unmodified PEI in MDA-MB-231, HEK293, and C2C12 cells[51,52] without affecting cell viability. However, further acetylation decreased the polymer's buffering capacity (inhibiting endosomal release) as well as the gene delivery efficiency. Cellular proteins or endosomal membranes could have dissociated the polyplexes more easily in this case, leaving unprotected pDNA in the cytosol[51,52]. Increasing the length of the acyl group (from C2 to C4) and the degree of acylation also improved transfection efficiency in COS-1 cells[50]. Particularly, 30% modified C4-PEI-PEG proved to be the most efficient carrier, but higher modifications (e.g., 40-50%) decreased the gene delivery properties as before. Although these substitutions decreased the pKa, buffering capacity and cationic nature of the carrier, it increased its lipophilicity for cell membrane interactions and inhibited aggregation (i.e., due to PEG) needed for optimal particle delivery. An appropriate balance between the lipophilicity and hydrophilicity was apparently struck with the C4-PEI-PEG[50]. In line with these results, complete alkylation (C3) of l-PEI[82] reduced transfection compared to unmodified l-PEI (22 kDa) in HepG2 cells. However, complete acylation or combination of acyl/alkylation (**Figure 2.11b**) increased gene expression. Acylation might have ensured a higher solubility or flexibility than alkylation for this outcome[82]. This subtle difference between the two means of substitution is critical to note in designing more effective carriers.

While a q-PEI (25 kDa) improved pDNA binding, the transfection efficiency was decreased >20-fold, suggesting an impaired proton sponge effect[83,84]. Additionally, C12 and C16 substituted on primary vs. tertiary amines groups of PEI influenced transfection efficiencies. Partial substitution of primary amines with both alkyl residues resulted in marked decline in transfection. When the tertiary amines of PEI were quaternized with C12, the carriers displayed higher transfection efficiency as compared to primary amine-modified PEI. Suh[85] *et al.*, and Johnson[86] *et al.*, proposed that hydrophobic groups attached to primary

amines of PEI form clusters to avoid water exposure, adversely affecting the protonation of amine groups. In contrast, the hydrophobic residues on tertiary amines of PEI were suggested to be located towards the polymer interior, minimizing the propensity for clustering and not influencing the protonation ability to a significant degree.

Similar substitutions (C12, C16) on 2 kDa PEI (**Figure 2.9a**) created effective gene carriers from the ineffective 2 kDa PEI. The obtained transfection efficiencies exceeded that of 25 kDa PEI under some conditions[83]. In an independent study[87], decreasing the total number of lipids substituted (10, 16 and 26 PEIs per C14) on 2 kDa b-PEI enhanced transfection in 293 cells in the absence of serum. In the presence of serum, these polymers were as efficient as 25 kDa b-PEI with negligible cytotoxicity. We independently showed the beneficial effect of various lipids on 2 kDa b-PEI as well, but a significant increase in toxicity was evident for the lipid-substituted polymers[88]. In a limited set of lipids substituted PEIs, no clear candidate emerged as the most effective lipid even though linoleoyl (C18:2) appeared to give more effective transfections as compared to other lipids[88].

Quaternization of primary amines with C1 (**Figure 2.14**), acylation with C16 (q-PEI-C16 or PEI-C16) or PEGylation (C16-PEI-PEG) did not improved transfection efficiency in A431 and A549 cells. Although these carriers were less efficacious than the native b-PEI (25 kDa), gene expression *in vivo* showed different results after systemic administration: PEI-C16 > PEG-PEI-C16 > q-PEI-C16 > q-PEI[89]. Other results showed that N-acylation and N-alkylation with C12, C14 and C16-PEI did not improve transfection efficiency when compared to native b-PEI (25 kDa) in NIH3T3 fibroblast cells[49]. Having as little as 2-4% modification was sufficient to decrease the PEI's efficiency. Although a lower effectiveness was generally observed upon modification, the authors claimed a positive trend favoring acylation over alkylations. Since these hydrophobic polymers showed greater stability against competitive exchange with polyanions,

it is likely that there will be a reduced propensity to release the DNA once inside the cell[49].

Unpublished studies from authors' group did not indicate a benefit of aliphatic lipid substitution on 25 kDa b-PEI for pDNA delivery, perhaps a reflection of the strong transfection ability of native b-PEI. On the contrary, better knockdown of integrin $\alpha_v$ by siRNA on B16 cells was accomplished with C18 and C18:1-substituted 25 kDa b-PEI[73]. These polyplexes mediated an additional 30-45% reduction in surface integrin $\alpha_v$ as compared to native b-PEI. C18-PEI provided significant enhancement of siRNA silencing over C18:1-PEI in this system, which was attributed to the higher stability of the polyplex and better protection from nuclease degradation[73].

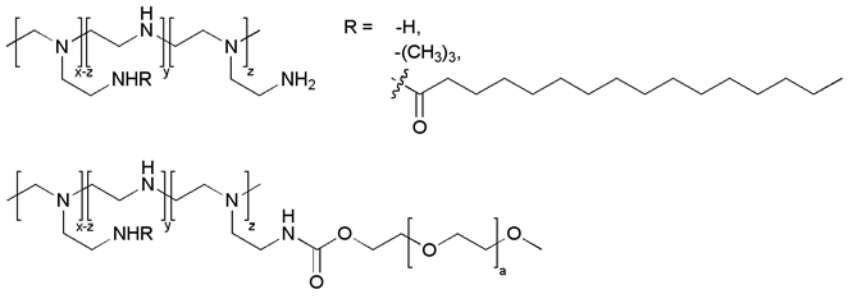


**Figure 2.14.** Branched PEI and PEI-PEG modified with hydrophobic and lipophilic moieties[89].

Transfection efficiency of cholesteryl-PEI (1.8 kDa) was significantly enhanced in CT-26 and 293T cells[53,54]. Micelle formation due to hydrophobic interactions was suggested to enhance the cationic charge density of the polymers. Cholesteryl-PEI was also effective *in vivo* (intratumoral injection of IL12 plasmid into CT-26 tumors in BALB/c mice), with long-term benefits on survival and tumor progression as a result of effective IL12 expression. Unfortunately, no data with the unmodified PEI was provided, so that the beneficial effect of cholesteryl substitution could not be confirmed *in vivo*[87]. In a subsequent study[56], cholesteryl anchored to secondary amines of b-PEI (1.8 and 10 kDa) transfection was effective with the low MW b-PEI, but it did not make a significant impact with

the high MW b-PEI, which was expected considering the low degree of substitution and relatively larger size of the latter b-PEI. The cholesteryl-conjugated 1.8 kDa b-PEI was also explored for siRNA delivery in VEGF silencing[55,90], as a means to inhibit tumor growth and metastasis in a variety of animal models[91,92]. Intratumoral injection of cholesteryl-PEI/siRNA reduced tumor growth by ~65% in 28 days[55]. However cholesterol-PEI grafted via secondary amines did not show a remarkable gene delivery. Instead, a slight increase in toxicity was observed at the optimal N/P ratio used for transfection[56].

The transgene expression efficiency of cholesteryl-l-PEI with different geometries[57] was in the order of L-l-PEI > T-l-PEI > l-PEI > b-PEI > LT-l-PEI. The beneficial effect of cholesteryl substitution was attributed to less compact binding of pDNA (facilitating intracellular dissociation), and increased endosomal escape. The authors hypothesized that L-l-PEI provided the best outcome due to: increased water solubility by decreasing the H-bonding between neighboring l-PEI backbones, favorable protection of cholesteryl conjugate by the hydrophilic-interactions of the two neighboring strands, and increased buffering capacity after cleavage of cholesteryl by lipase.

### 2.5.4. Hydrophobically Modified Dendrimers

Studies on PAMAM substituted with dialkyl C12 (**Figure 2.12b**) groups have shown a correlation between the dendron generation (G1 to G4) and the gene delivery efficiency. The C12-PAMAM dendron was able to condense pDNA into more stable polyplexes as compared to unmodified PAMAM[60]. In contrast, quaternization of PAMAM's tertiary amines either on the backbone or side chains with alkyl C1 or C12 yielded carriers with a relative low transfection activity[59]. Polyplexes based on quaternized C1 amines on the backbone displayed higher transfection efficiency and lower cytotoxicity activity as compared to the combined with quaternized C1/C12, quaternized C1 side chain alone and unmodified PAMAM. In fact, a limited amount of C1 groups appeared to have a

beneficial effect probably because of better DNA condensation, and small particle size (50-100 nm). Further alkylation of side chains with C1 and C12 significantly decreased transfection probably due to high cytotoxicity of the carrier[59]. On the other hand, improvement in toxicity and polyplex formation was observed as quaternization (C1) of PAMAM-OH backbone was increased (97% > 78% > 52% > 27%). This result suggested that creating a permanently charged polycation by quaternization reduces cytotoxicity. However, these dendrimers showed a decreased transgene expression since q-PAMAM-OH particles had a neutral surface charge, possibly reducing the interactions with cellular membranes[58].

The PLL dendrimers with 8 and 16 free amino groups and substituted with C14 were effective in transgene expression with COS-7 and BHK-21 cells, without affecting cell viability and without a significant impediment of endosomal entrapment[93,94]. Epithelial (HeG2, HeLa and CHO), fibroblast (COS-7 and C2C12), and lymphoid T cells (Jurkat) were more easily transfected than the endothelial cells (ECV304) with C14-PLL dendrimers. The differences in transfection varied from a 10-fold to >1000-fold enhancement in different cells. Additionally, a modest suppression of expression was observed with these carriers with a custom iRNA. This is probably associated to the confounding factors of the synthesized iRNA and not to the capability of the carrier delivery[95]. It is important to mention that lipid-poly(L-lysine) dendrimers polyplexes were not compared against the unmodified dendrimer. Therefore, the impact of the lipid chain could not be truly understood.

### 2.5.5. Lipid and Hydrophobically Modified Chitosans

Quaternized chitosan oligomer with 40-50% of C1-substitution showed enhanced transgene expression in both COS-1 and Caco-2 cells as compared to unmodified polymer[96]. The formation of smaller particles by the q-chitosan could have aided the delivery. The presence of serum proteins had minimal effect on transfection, which indicated minimal polyplex dissociation by serum[97], and cell-dependent

differences in transfection efficiency were noted[98]. As with other cationic polymers, the quaternized (C1) and N-acylated or N-alkylated C16 glycol chitosan gave cell binding and gene transfer efficiency that was dependent on the degree of polymerization[63]. For the acylated polymer, a DP of 117 or less was necessary to form smaller polyplexes and efficient gene transfer. Cell viability was reduced by the C16 acylation of chitosan, however, biocompatibility of these carrier was restored with the C1 substitution on primary amines. In contrast, alkylated polymer was less efficient at gene transfer because of its relatively high toxicity. Although the addition of C1 and C16 resulted in a broader spectrum of transfection, the absolute degree of substitution appeared to have no bearing on the gene transfer activity. It is possible that these studies have not uncovered the lower limit of substitution for effective transfection. However, it was clear that the hydrophobic chains enhanced gene expression. After intravenous administration, C1-/C16-glycol chitosan effectively mediated gene expression in the liver and heart, more so than the commercial polymeric carriers. These results suggested that C1-/C16-glycol chitosan could be used for gene delivery specifically to these organs[63]. The alkylation of chitosan enhanced gene delivery to C2C12 cells, which was increased upon elongating the alkyl side chain from C4 to C8. This higher transfection efficiency was presumably due the increased cellular interactions with the hydrophobic polyplex, and/or easier polyplex dissociation[61]. Increasing the lipid chain to C18 also enhanced transgene expression in A549 cells; the possibility of formation of a core-shell micelle structure with C18 moieties near the shell of the micelle might have favored the endosomal escape of the polyplex.

The size and structure of chitosan also influenced the transfection of COS-1 cells with DA-chitosan[74]. Without serum, transfection efficiency was increased as the polymer MW was increased from 5 to 40 kDa, after which a steep decrease was noted for a 200 kDa polymer. Low transfection efficiencies were attributed to low or high polyplex stability given by the lowest or highest MW polymer, respectively. In the presence of serum, however, the transfection efficiency was

consistently increased with the MW. The serum proteins may act as an inhibitor of polyplex formation, so that high MW polymers may be beneficial to resist this phenomena[64]. However, low MW chitosan oligosaccharides could be also made effective in 293 cells after DA substitution; the hydrophobized polymers enhanced DNA condensation and formed core-shell type particles that can increase cell membrane-carrier interactions and/or destabilization of the cell membranes as well as affording better DNA protection. Particularly, a 3 kDa chitosan modified with ~5 DA units was found to be an effective carrier without adversely affecting cell viability[65]. 5β-cholanic acid was another substituent suitable for enhanced gene delivery[66].

## 2.6. Conclusions and Future Prospects

Compared to viral particles, cationic polymers are generally believed to mediate gene delivery with lower efficiency. In order to improve delivery, a new generation of carriers will be needed and hydrophobic modifications will play an integral part in this endeavor. This review has attempted to highlight the advances recorded with lipophilic carriers and their *in vitro* and, in some cases, *in vivo* transfection behavior. Grafting small amount of hydrophobic or lipophilic moieties in most cases aided condensation of the payload into stable, compact and smaller polyplexes and subsequently enhanced cell internalization compared to unmodified carriers. The cellular uptake of the cargo was not always confirmed with such polymers, and this will be needed to confirm and/or better elucidate the mechanism of action of lipophilic moieties. The experimental evidence with these polymers also indicated that, besides the electrostatic interactions, hydrophobic interactions might be critical for enhancing DNA binding as well as cell association. The beneficial effect was observed to depend on the MW or degree of polymerization, polymer to payload ratio, nature and degree of substitution and the functional group modified. As the chain length and/or degree of substitution increased so did the polyplex size and cellular uptake in some cases. This increase may be reflective of less compact polyplexes due to weaker binding leading to

larger particles and increased sedimentation. Transgene expression could be enhanced in this case due to faster release of the cargo from the particles. The same mechanism (i.e., reduced affinity) was used as the reason for reduced delivery/transfection in some studies. It will be important to consistently elucidate changes in properties of polyplexes, so that the detrimental or beneficial consequence of lower nucleic acid affinity is thoroughly revealed. A reduced surface charge on polyplexes was expected after hydrophobic modification; therefore, larger amount of polymer per nucleic acid was required for efficient payload condensation. Incorporation of lipophilic or hydrophobic groups into the carrier should also affect interactions with endo/lysosomal membranes resulting in a more efficient escape. An undesirable aspect of this might be increased toxicity since plasma membrane disruption was also expected to be correlated with the endosomalytic activity. Although some studies are beginning to address this issue, more extensive investigations are needed to better elucidate the dependence of membrane disrupting activity on the molecular details of the lipid substituent, as well as the polymer backbone. It is likely that an optimal substitution might be identified in this respect where the right amount/nature of substituent can lead to sufficient de-stabilization of cellular membranes for polyplex uptake and endosomal release, without excessive membrane lytic activity that might compromise the normal cellular physiology.

The transfection efficiency has been modulated by three variables, namely the degree of substitution, chain length of the substituent and functional group modified. Depending on the nature of polymer, modification of a specific functional group in the polymer might cause formation of large aggregates not suitable for internalization. In the absence of aggregation, higher transfection efficiencies were achieved by inclusion of *N*-acyl and, to a lesser extent, *N*-alkyl groups. It is possible that this behavior might depend on the nature of the base polymer, and extensive literature on this issue does not exist. Acylation might result in higher solubility and/or flexibility in polyplexes, but how this impact the gene delivery remains to be investigated. The structure and density of the amine

groups could affect transfection efficiency. Increasing the number and charge density typically improves gene transfer; however, such an increase also promotes toxicity. Therefore, the beneficial effect of the amine quaternization in polymeric carriers remains ambiguous. The overall performance of lipophilic or hydrophobic modification polymers can be puzzling at times. A delicate balance between hydrophilic and hydrophobic components is crucial for the design of new polymeric carriers, as well as thorough understanding of the changes in polyplex properties that becomes critical for transgene expression. Although it might be tempting to develop certain expectations for the behavior of lipid and hydrophobically modified polymers, several issues need to be clarified on a case-by-case basis (**Table 2.3**) and it will be misleading to translate the experience of one system to another. This is vital to pursue polymeric gene carriers that will be one day employed in a clinical setting.

**Table 2.3.** Possible effects of lipid and hydrophobic substituents. Some of the effects summarized below have been previously investigated in the context of specific carriers; while other have not been explore to-date. These effects of substituents make a significant impact on the performance of carriers and should be elucidated for new carriers designed by the researchers.

| **Event** | **Issue** |
|---|---|
| Interactions with cargo | Contribution/s of substituents to nucleic acid binding and condensation; surface coating of polyplexes as a barrier against surrounding molecules; polyplex stability in a medium of competing molecular species. |
| Cellular internalization | Role of substituents in plasma membrane crossing of polyplexes; active endosomal transport after specific receptor binding vs. passive uptake via membrane fusion and intracellular passage; changes in cell surface receptors as a result of substituents |
| Endosomal Escape | Facilitated endosomal escape due to substituent interactions with membranes; role of polymer backbone in endosomal escape; role of membrane lytic activity on cellular physiology. |
| Intracellular transport | Association with intracellular membranes; sub-cellular compartmentalization as a result of substituent targeting; changes in polyplex dissociation as a result of substituent; increased degradation of cargo in unstable polyplexes vs. enhanced perinuclear transport of more stable polyplexes. |
| Nuclear uptake and transgene expression | Facilitated transport through nuclear membrane as a result of substituents; polyplexes dissociation by cationic proteins (histones) and transcription factors; changes in gene expression due to enhanced or reduced stability of polyplexes. |

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# 3. PALMITIC ACID SUBSTITUTION ON CATIONIC POLYMERS FOR EFFECTIVE DELIVERY OF PLASMID DNA TO BONE MARROW STROMAL CELLS

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## 3.1. Introduction

Cells derived from the bone marrow environment are extensively utilized in human gene therapy protocols[1,2]. Bone marrow stromal cells (BMSCs) represent a source of stem cells that are readily accessible from individual patients[3]. They provide a clinically acceptable cell source for therapy without the ethical concerns associated with the embryonic stem cells. The transplantation of BMSCs to a host can be performed without systemic immunosuppressants, an important consideration that has limited extensive application of gene therapy. Significant efforts were recently devoted to BMSC expansion *ex vivo*[4], and it is now practical to achieve a clinically sufficient cell mass by using current cell culture technologies. Moreover, BMSC can be manipulated during *ex vivo* culture to direct them into different lineages, including osteoblastic, chondrogenic, adipogenic, and myogenic phenotypes[5]. Manipulating BMSC into specific phenotypes enables *ex vivo* construction of functional tissues for tissue replacement in a host. Undifferentiated BMSC, due to their plasticity and an ability to induce neovascularization, can also reverse functional degeneration in tissues, as was recently demonstrated in the case of cardiac muscle degeneration[6].

Genetic modification of BMSC has been primarily achieved with viral carriers. Retroviruses and adenoviruses have been used in a clinical setting to deliver therapeutic genes[7], since they are regarded as the most effective carriers for a prolonged gene expression period. The long-term gene expression relies on integration of viral genes into the host genome. The undesirable consequences of viral integration process (i.e., haphazard activation or silencing of host genes), as well as the immunogenicity of viral particles have recently raised safety concerns about viral carriers[8,9]. Unacceptable safety outcomes in a recent clinical trial have led to a moratorium on the use of retroviruses in clinical setting. Non-viral carriers are preferred for gene delivery because of better safety profiles, but the use of non-viral carriers for BMSC modification has been limited. Only cationic lipids were used to modify the cells in the pre-clinical setting and, an important

class of non-viral carriers, namely cationic polymers, remains to be investigated in detail for BMSC modifications. Cationic polymers are able to interact with plasmid DNA (pDNA) via electrostatic means and protect the pDNA against extracellular nuclease degradation. Cationic polymers enable the entry of pDNA molecules into the cells by condensing the string-like pDNA into compact nanoparticles[1,10]. Polymeric carriers capable of effectively delivering pDNA into BMSC will greatly facilitate utilization of BMSC in gene therapy.

In this study, we investigated the potential of two cationic polymers, polyethylenimine (PEI)[11] and poly-L-lysine (PLL)[12], to deliver exogenous genes to BMSCs. The cationic charges on these polymers were considered sufficient to neutralize the anionic charge of pDNA, and to condense it into structures suitable for cellular uptake. Adding hydrophobic moieties to cationic polymers was shown by others to improve the transfection efficiency in immortalized cell lines[13,14], presumably due to better compatibility of pDNA/polymer complexes with the lipid bilayer of the plasma membrane. On the basis of this observation, we explored the utility of a naturally occurring lipid, palmitic acid (PA), to enhance the ability of PEI and PLL for gene transfer. PA is utilized by mammalian cells to control the intracellular trafficking of proteins[15,16]. The propensity of proteins to associate with cellular membranes is enhanced after palmitoylation because of hydrophobic association of the palmitoyl moiety with the hydrophobic membranes. We reasoned that imparting a hydrophobic character to the cationic polymers would enhance their ability to cross the plasma membrane and, hence, facilitate intracellular delivery of the pDNA cargo. Here, we show that PA substitution on PEI does not improve the capacity of this polymer to act as DNA carriers, but PA substitution on PLL makes this polymer a very effective DNA carrier whose efficiency was equivalent to an adenoviral carrier.

## 3.2. Material and Methods

### 3.2.1. Materials

Branched PEI ($M_w$ 25 kDa, $M_n$ 10 kDa), PLL hydrobromides ($M_{wvis}$ 25, 9, 4 and 1 kDa), homopolymers of arginine, histidine and asparagine, triethylamine (TEA), anhydrous dimethylsulfoxide (DMSO), anhydrous *N*,*N'*-dimethylformamide (DMF), palmitoyl chloride (PA-Cl; 98%), palmitic acid *N*-hydroxysuccinimide ester (PA-NHS), 5% (w/v) 2,4,6-trinitrobenzosulfonic acid (TNBS), Hanks' Balanced Salt Solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypsin/EDTA were obtained from SIGMA (St. Louis, MO). Anhydrous ethyl ether, dichloromethane (DCM) and absolute ethanol were purchased from Fisher Scientific (Fairlawn, NJ). Linear PEIs with $M_w$ 2 and 25 kDa were obtained from Polysciences (Warrington, PA). Dulbecco's Modified Eagle Medium (DMEM; high glucose with L-glutamine), Penicillin (10,000 U/mL), and Streptomycin (10,000 μg/mL) were from GIBCO (Grand Island, NY). Deuterated chloroform ($CDCl_3$) and water ($D_2O$) used as $^1$H-NMR solvent were from Cambridge Isotope Laboratories and ALDRICH (Milwaukee, WI), respectively. Dialysis tubing with a MW cut-off of 12-14 kDa was purchased from Spectrum Laboratories (Gardena, CA). Lipofectamine-2000$^{TM}$ was from Invitrogen (Carlsbad, CA). Fetal Bovine Serum (FBS) was from PAA Laboratories Inc. (Ontario, Canada). Ester of Cy5.5 (Cy5.5-NHS) was obtained from AMERSHAM (St. Laurence, QC) and used for labeling of plasmid DNA following manufacturer's protocol. Fluorescein isothiocyanate (FITC) was purchased from PIERCE (Rockford, IL). An adenovirus expressing the GFP gene was prepared as described before[17].

A 4.7 kb plasmid incorporating an enhanced green fluorescent protein (pEGFP-$N_2$) and a kanamycin resistance gene was obtained from BD Biosciences, and replicated in kanamycin resistant DH5alpha E.coli strain grown in Luria-Bertani medium. After purification with a Qiagen Plasmid Giga Kit, the plasmid

concentration and purity were determined by UV spectroscopy according to the manufacturer's protocol. The plasmid was dissolved in 10 mM Tris-HCl/1 mM EDTA (pH = 8.0) at 0.4 mg/mL for use in this study.

### 3.2.2. Synthesis and Characterization of PA-Substituted Polymers

The PEI-PA and PLL-PA were synthesized by N-acylation of the corresponding polymers with PA-Cl and PA-NHS, respectively (**Figure 3.1**). To obtain PEI-PA, 1 g of PEI was dissolved in DCM (~5.9 mL) under $N_2$ at 5 $^{\circ}$C. After addition of TEA (0.24 mmol), PA-Cl dissolved in DCM was gradually added to the PEI solution over a 30 min period (see **Figure 3.2** for exact mole ratios used). The solution was stirred at 5 $^{\circ}$C for 12 hrs under $N_2$. The product was concentrated in vacuo at room temperature and washed (×3) with excess ethyl ether. The residue was dried under vacuum at ambient temperature. To obtain PLL-PA, the preactivated PA-NHS was dissolved in 1 mL of DMF and added dropwise to 25 mg of PLL in 1 mL of DMSO. The mixture was allowed to react for 2 hrs at room temperature under $N_2$. Excess ethyl ether was added to precipitate the polymer product, which was washed with ethyl ether, and dried under vacuum overnight at room temperature.

The composition of the reaction products was determined by a 300 MHz $^1$H-NMR spectroscope (Bruker 300 AM; Billerica, MA). The solvents used for PEI-PA and PLL-PA were $CDCl_3$ and $D_2O$, respectively. The proton shifts specific for PA (δ ~ 0.8 ppm; -C<u>H</u>$_3$), PLL (δ ~ 4.3 ppm; –(CO-C<u>H</u>-NH$_2$) and PEI (δ ~ 2.5-2.8 ppm; -HN-C<u>H</u>$_2$-C<u>H</u>$_2$-NH-) were integrated, normalized for the number of Hs in each peak, and used to obtain PA substitutions on polymers.

### 3.2.3. TNBS Assay[18]

The polymer samples to be analyzed along with the standards were prepared in

distilled/deionizd $H_2O$ ($ddH_2O$). A 20 μL of sample (in duplicate) was added to the disposable cuvettes, followed by 130 μL of 0.1 M phosphate buffer (pH = 7.0) and 850 μL of 1 mM TNBS in borate buffer (pH = 9.0). After incubating for 1 h at 37 °C, the optical density of the samples was measured at 367 nm.

### 3.2.4. Electrophoretic Mobility Shift Assay (EMSA)

The samples for EMSA were prepared by mixing 3 μL of 0.1 M HEPES buffer (pH 6.5) with 4 μL of 0.4 mg/mL plasmid solution, and 8 μL of serially-diluted concentrations of a polymer solution. The mixture was incubated for ~30 min at room temperature, after which 3 μL of 6×-sample buffer (50% glycerol, 1% bromophenol blue, and 1% xylene cyenol FF in TBE buffer) was added, and the samples were loaded onto the 0.5% agarose gels containing 0.05 mg/mL EtBr. Electrophoresis was performed at 130 V and ~ 52 mA for 45 min, and the resulting gels were photographed under UV-illumination. The pictures were digitized, and analyzed with the Scion image analysis software to determine the mean density of plasmid bands. The measured density was plotted as a function of polymer concentration and the polymer concentration required for 50% reduction in plasmid density ($IC_{50}$) was determined. A polymer-specific binding ratio, $BR_{50}$, was determined by dividing the plasmid concentration with the $IC_{50}$ and it was used as a quantitative measure of plasmid-binding efficiency of each polymer. A lane in which the agarose gel contained the plasmid without any polymer added was used as a reference, and each polymer was tested at least in 2 independent EMSA assays.

### 3.2.5. BMSC Isolation and Expansion[19]

The BMSC were isolated from both femurs of ~8 weeks-old female Sprague-Dawley rats and pooled to obtain a single cell suspension. The marrow was removed by cutting the femur at distal and proximal ends with a bone-cutter, and aspirated using a 5 mL syringe with an 18G needle. The bone marrow was flushed

out with ~15 mL of DMEM containing 10% FBS, 50 μg/L ascorbic acid, 100 U/mL penicillin and 100 μg/L of streptomycin (referred as basic medium). The cells were centrifuged (600 g for 10 min), suspended in fresh basic medium and seeded in a single 75 $cm^2$ flask (FALCON). After a medium change on day 3, the cells were trypsinized on day 7, and expanded on 75 $cm^2$ flasks (1:4 dilution). The BMSC passaged between 2 and 4 generations were used in this study and were grown in either multiwell plates (6-, or 48-well plates from Corning) or 75 $cm^2$ flasks (FALCON) for testing below.

### 3.2.6. Polymer Binding to Cells

The PEI and PEI-PA samples were labeled with 1 mM FITC according to the manufacturer's directions, and dialyzed against 0.1 M phosphate buffer to remove the unreacted FITC. TNBS assay was then used to estimate the polymer concentrations in dialyzed samples. The PLL and PLL-PA samples were also labeled with 0.1 mM FITC, but were precipitated with absolute ethanol, and centrifuged at 1600 rpm for 15 min to collect the labeled polymers. The polymers were allowed to dry in vacuum, and reconstituted in HBSS at a desired concentration for the cell binding study.

To assess polymer binding to BMSC, the medium from a confluent layer of cells in 48-well plates was removed, and the cells were rinsed with HBSS (×2). DMEM (200 μL) was added into each well (in triplicate) followed by the addition of 20 μL of FITC-labeled polymer solution. Cells were incubated for 2 hrs under standard tissue culture conditions. The medium was then removed, and the cells were rinsed with HBSS (×2) to remove the unbound polymers. Fluorescence associated with BMSC was measured with a plate reader ($\lambda_{ex}$ =485 nm, $\lambda_{em}$ =525 nm; Thermo Labsystems, Waltham, MA). The actual amount of cell-bound polymer was calculated based on a standard curve generated with the original FITC-labeled polymer solution.

In a modification of the earlier procedure, FITC-labeled polymers were incubated with the cells for ~22 hrs, after which PI was added to the medium to give a final concentration of 10 μg/mL. After 15 min, the BMSCs were trypsinized and percentage of cells displaying FITC-labeled polymer and PI was assessed by flow cytometry.

### 3.2.7. MTT assay

The cytotoxicity of the polymers was tested on BMSCs in 48-well flat-bottomed microplates. In a typical assay, a 2 mg/mL polymer solution in HBSS was serially diluted (×2) with HBSS, and 20 μL of polymer solutions were added to the wells (in triplicate) containing 200 μL of basic medium. After 4 hrs incubation at 37 $^{\circ}C$ in a humidified 95/5% air/$CO_2$ atmosphere, the media was replaced with 500 μL basic medium, and 100 μL of MTT solution (5 mg/mL) was added to each well. After 2 hrs incubation, the basic medium was removed, the cell monolayer was washed 500 μL of HBSS (×2), and the MTT crystals were dissolved with 500 μL of DMSO. The optical density in each well was measured at 570 nm using a microplate reader.

### 3.2.8. DNA Transfer into BMSC

To assess the ability of polymers to transfer pEGFP into BMSCs, 20 μg of pEGFP-$N_2$ solution (in 300 μL) was dialyzed against 0.1 M phosphate buffer (pH = 7.0) to remove free amines in solution. A 100 μL of Cy5.5-NHS dye solution (1 mg in 300 μL) was added to this solution and allowed to react for 2 hrs at room temperature. The reaction was stopped by dialysis against TBE buffer. Five μL of this solution was mixed with a desired concentration of polymer solution, and incubated for 30 min before addition to cells in 6-well plates. After incubation for either 4 or 24 hrs, the cells were washed with HBSS (×2), trypsinized and the uptake of Cy5.5-labeled pEGFP was detected by flow cytometry ($\lambda_{ex}$=485 nm, $\lambda_{em}$=525 nm; FACScalibur; BD Dickenson). The results were expressed as the

percentage of cells exhibiting significant fluorescence over that of control samples (i.e., cells not exposed to labeled pEGFP). As a reference carrier, Lipofectamine-2000$^{TM}$ (2-20 μL) was also mixed with the labeled pEGFP (2 μg), incubated with the cells, and cell uptake was analyzed as earlier.

### 3.2.9. Transfection of BMSC

The polymer/pEGFP polyplexes used for transfections were prepared by mixing equal volumes of pEGFP (in 150 mM NaCl) and polymer solutions (in HBSS) at desired concentrations (see Figure legends). After 30 min incubation, the polyplexes were added to the cells grown on 25 cm$^2$ FALCON flasks to give 2 μg of plasmid per flask. The BMSCs were incubated for 24 hrs with the polyplexes, after which the cells were either trypsinized for assessment of EGFP expression or the medium was replaced with fresh medium for longer time cultures. Flow cytometry was performed on a BD FACscan where the cell fluorescence was detected in FL1 channel. The instrument settings were calibrated for each run so as to obtain a background level of EGFP expression of ~1% of cells for control samples (i.e., cells not transfected).

### 3.2.10. Statistical Analysis

Where shown, the results are summarized as mean ± standard deviation (SD) of the indicated number of replicates. Variations between the group means were analyzed by the Student's *t*-test.

## 3.3. Results

### 3.3.1. PA Grafting to Polymers and DNA Binding

PA was grafted to PEI and PLL by using two different approaches (**Figure 3.1**). The PA-Cl was successfully used to graft 1.4-5.2 PA per PEI (**Figure 3.2**), but this approach did not yield any PA substitution on PLL under the same reaction

conditions. A variety of reaction times, and solvents were attempted with PA-Cl with no apparent PA substitution on the PLL. The PA-NHS, which contained an activated *N*-hydroxysuccinimide ester for $-NH_2$ coupling, was alternatively utilized to successfully graft 13.2-16.2 PA per PLL (**Figure 3.2**). A plateau appeared to be reached for the PA substitution for both polymers and chemistries at PA:polymer mole ratios of ~0.2 in reaction medium.

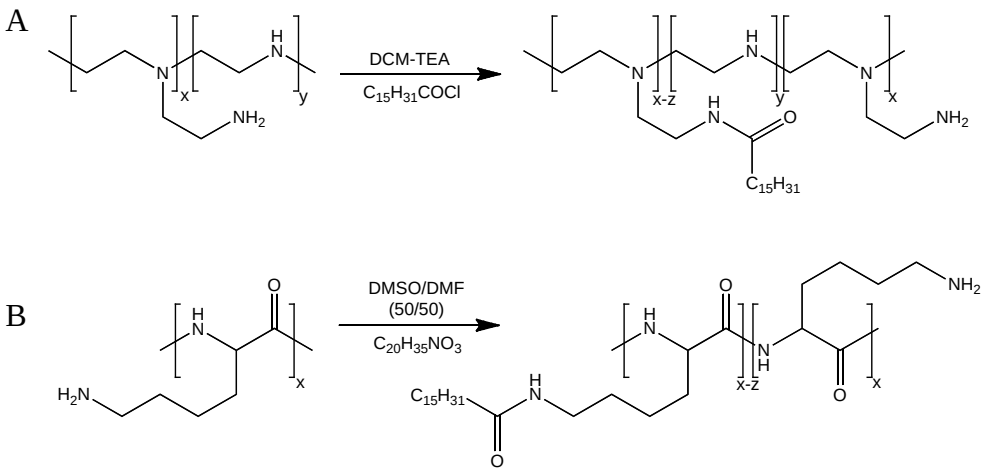


**Figure 3.1.** Reaction schemes for the PA conjugates of PEI (A) and PLL (B). PEI-PA was prepared using palmitoyl chloride in DCM, whereas PLL-PA was prepared by N-hydroxysuccinimide ester of palmitic acid 50/50 DMSO/DMF.

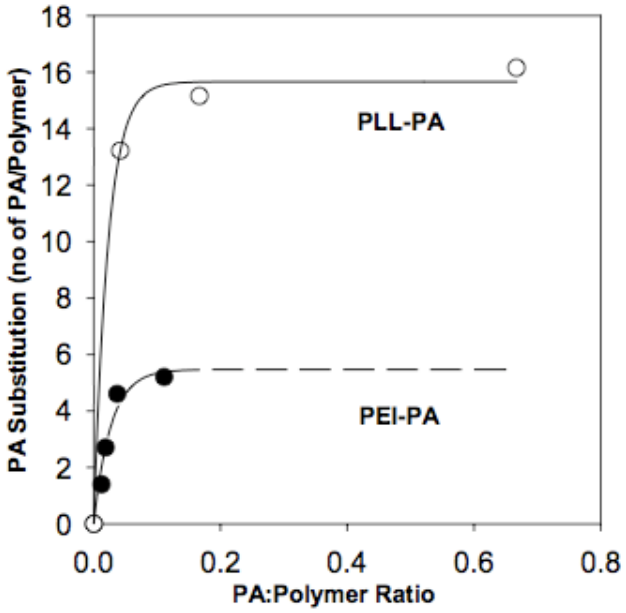


**Figure 3.2.** Extent of PA substitution on PEI and PLL conjugates prepared for this study. The extent of PA substitution was controlled by the extent of PA:polymer mole ratio in the reaction medium. The PA substitution on PLL was ~3-fold higher.

On the basis of EMSA analysis, PEI and PLL were both able to complex pEGFP and prevent its penetration into agarose gel as a function of polymer concentration (**Figure 3.3**). Whereas PEI exhibited a $BR_{50}$ of 0.051, PLL exhibited a ~6-fold higher $BR_{50}$ of 0.32. This difference was indicative of the stronger binding of PEI to a pEGFP. The PEI with PA substitutions of 1.4, 2.7 and 5.2 PA/PEI gave a gradually increasing $BR_{50}$ of 0.074, 0.085 and 0.160 (**Figure 3.3B**), indicating an inhibitory effect of PA on DNA binding of PEI. The PA substitution of PLL at 13.2-16.2 PA/PLL also increased the $BR_{50}$ to 0.52-0.56 (**Figure 3.3B**), indicating the interference of PA in DNA binding of the cationic polymer.

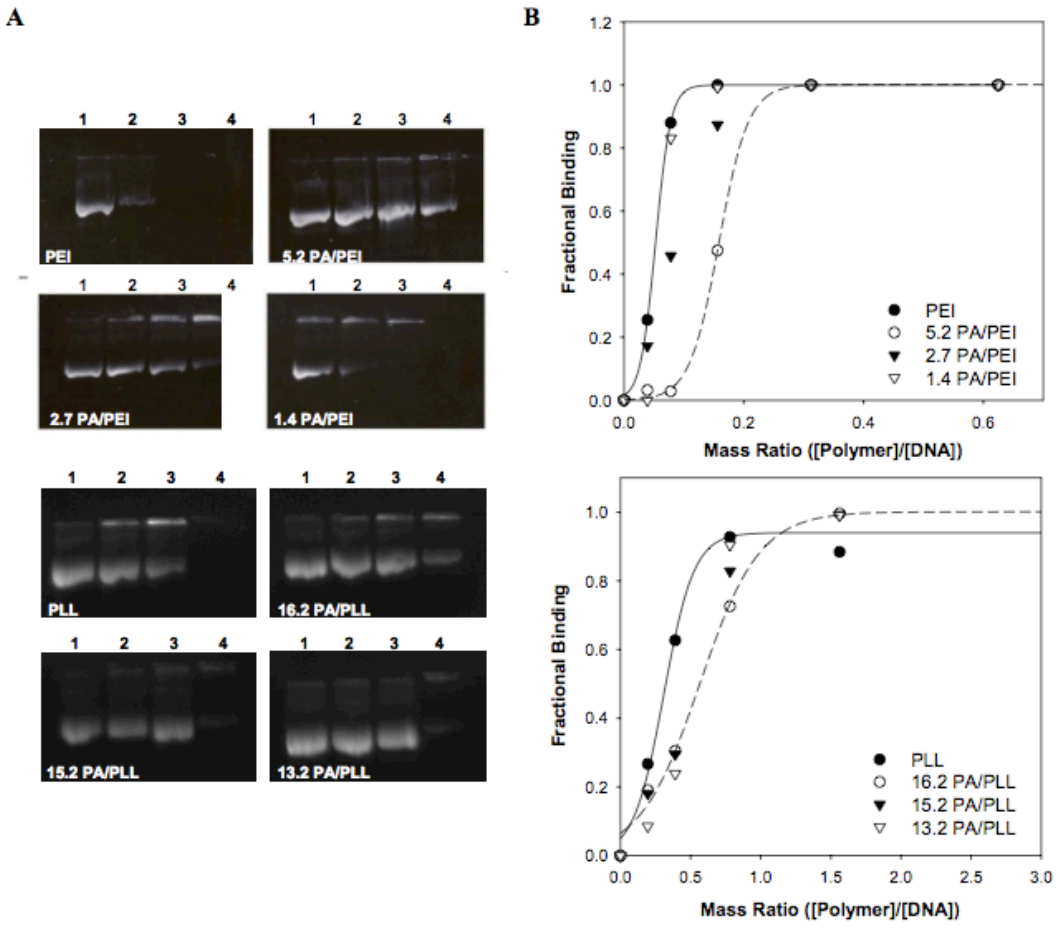


**Figure 3.3.** Agarose gel electrophoresis analysis of binding of PEI-PA and PLL-PA conjugates to pEGFP. The gel results for the individual conjugates were shown in A (1, 2, 3, 4 stands for mass ratio polymer/DNA), and densitometric analysis of the binding results was shown in B. Lines in B represent sigmoidal fits for native polymers and polymers with the highest extent of PA substitution (other fits not shown for clarity). The extent of PA substitutions on the polymers was indicated on the gel pictures, and in the graph legend. Note that for both polymers, PA substitution increased the $BR_{50}$ (i.e., mass ratio for 50% binding) as compared to the native polymers.

### 3.3.2. Binding of Polymers to BMSC and Cytotoxicity

The effect of PA substitution on polymer binding to BMSC was then investigated by using FITC-labeled polymers. Whereas no apparent difference was evident between the native PEI and the PEI with low PA substitutions, PEI with 5.2 PA/PEI exhibited a significantly higher binding to BMSC (**Figure 3.4A**). Despite the higher binding, the cytotoxicity of the PEI-PA was not altered as compared to native PEI (**Figure 3.5A**). Similarly, PA substitution on PLL increased the binding to BMSC (**Figure 3.4B**), while reducing the polymer toxicity (**Figure 3.5B**).

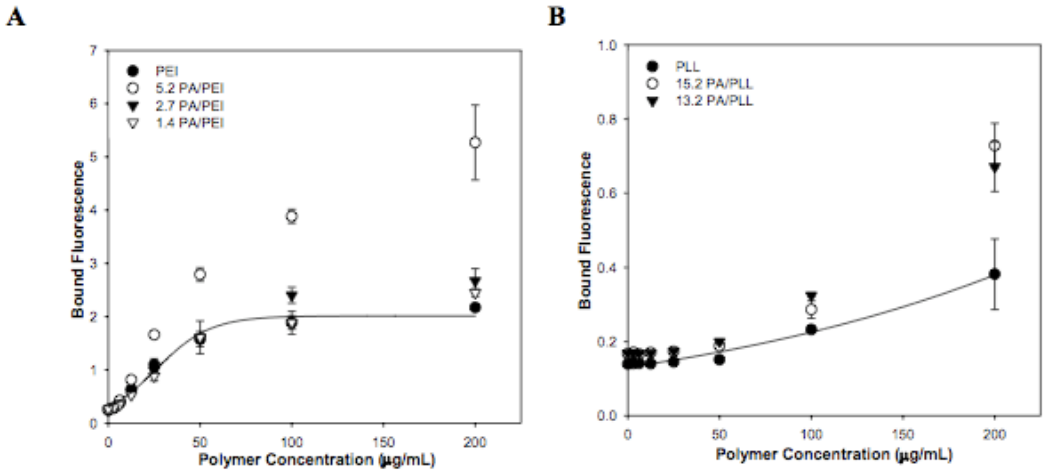


**Figure 3.4.** Binding of FITC-labeled polymers to BMSC grown in 48-well plates. A. PEI-PA at the highest PA substitution ratio (5.2 PA/PEI) exhibited a higher binding compared to lower substituted and native PEIs. B. PA substitution also increased the binding of PLL to BMSC.

To determine the percentage of BMSC that exhibited polymer uptake, flow cytometry was used to determine the FITC-positive cells after the cells were incubated with the FITC-labeled polymers for 24 hrs. The percentage of cells exhibiting polymer uptake was increased with the polymer concentration, but there was no apparent difference between the PEI and PEI-PA (5.2 PA/PEI; **Figure 3.6**). The PLL and PLL-PA polymers were also internalized with the BMSC as a function of polymer concentration, but the PLL-PA (14.2 PA/PEI) was able to enter the cells to a much higher extent than the PLL especially at low concentrations (**Figure 3.6**).

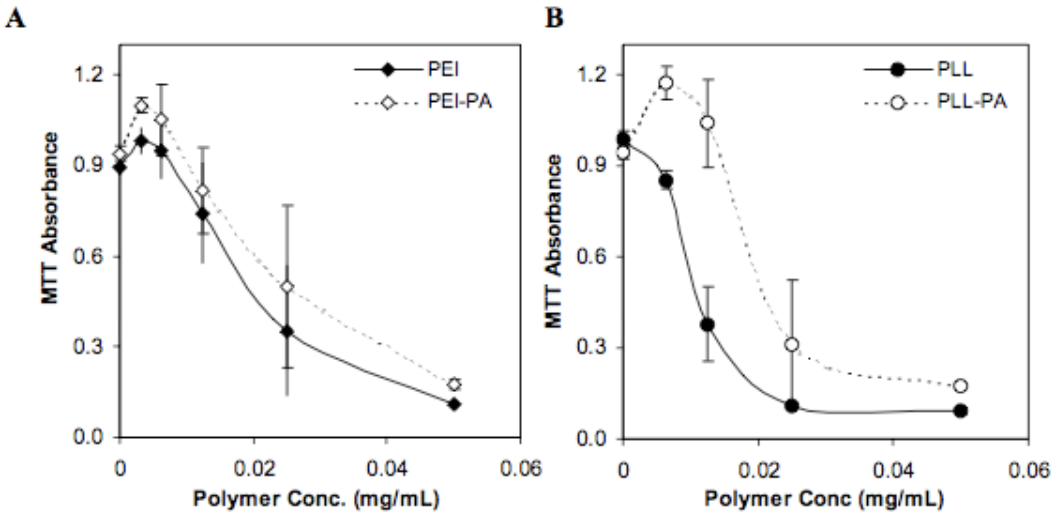


**Figure 3.5.** Toxicity of a PA-substituted PEI and PLL compared to the native polymers. Note that PA substitution (5.2 PA/PEI) did not affect the toxicity of PEI, whereas PA substituted PLL (15.2 PA/PEI) exhibited ~2-fold reduced toxicity, based on the polymer concentration required for loss of 50% viability.

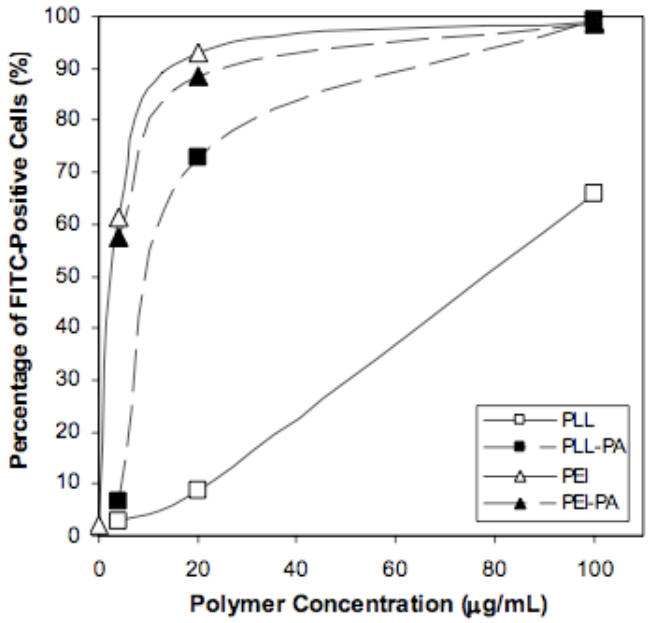


**Figure 3.6.** BMSC uptake of native PEI and PLL, and PEI-PA and PLL-PA conjugates (5.2 and 16.2 PA/polymer, respectively). The uptake was assessed by FITC-labeled polymers by using flow cytometry, after cells grown in 6-well plates were incubated with the labeled polymers for 24 hrs. The PA-substituted PEI did not exhibit a preferential uptake by the BMSC, while a dramatic uptake by PA-substituted PLL was evident.

### 3.3.3. DNA Delivery into BMSC

Incubating the pEGFP alone with BMSC did not result in significant delivery of pEGFP to cells after 4 and 24 hrs (**Figure 3.7**). The pEGFP complexed with both PEI and PEI-PA enhanced the plasmid uptake by the cells and there was no significant difference between the PEI and PEI-PA (18-22% uptake, **Figure 3.7**). The pEGFP delivery to BMSC was not significant by the PLL after 4 hrs, but slightly elevated to 4.3% after 24 hrs. The PA-grafted PLL, on the other hand, significantly increased the delivery to 35.2% of the cells after 24 hrs. As a reference reagent, pEGFP delivery with the commercially available Lipofectamine-2000$^{TM}$ was evaluated; BMSC incubated with Lipofectamine$^{TM}$/pEGFP complexes displayed the highest uptake after 4 hrs, but was lower than the PLL-PA at the 24 hrs time point ($p < 0.01$).

The relative effectiveness of PLL-PA to deliver pEGFP was compared to several other polycations after 24 hrs incubation with the BMSC (**Figure 3.8**). The PLL-PA and branched PEI were the most effective among the polymers tested, followed by the PLL of 25 kDa. The PLL with lower MWs (1.0-9.2 kDa), linear PEIs with MWs of 2.5 and 25 kDa, and three homopolymers of the amino acids asparagine, histidine and arginine, displayed only a small fraction of pEGFP delivery to the BMSC (**Figure 3.8**).

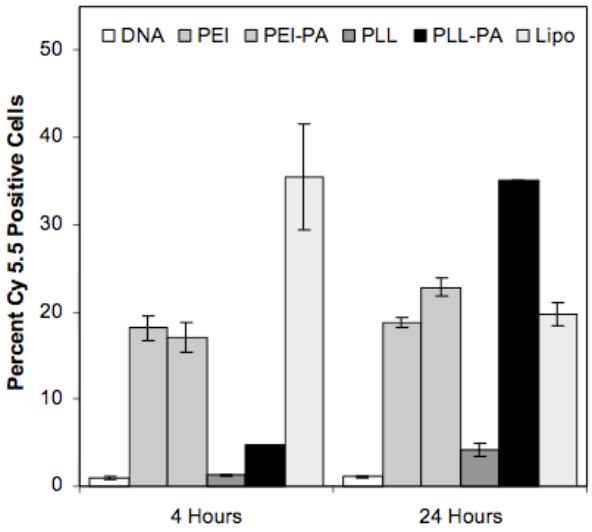


**Figure 3.7.** DNA delivery into BMSC by PA-substituted polymers. Cy5.5-labeled pEGFP was utilized without any polymer (designated as DNA in the legend), or complexed with PEI, PEI-PA (5.2 PA/PEI), PLL, PLL-PA (16.2 PA/PLL) and Lipofectamine-2000$^{TM}$. pEGFP uptake was assessed by flow cytometry after 4 and 24 hrs incubation of the complexes with the cells. There was no uptake of pEGFP in the absence of any carriers and with PLL. The pEGFP uptake with PEI and PEI-PA was similar. Lipofectamine-2000$^{TM}$ provided the highest pEGFP uptake after 4 hrs, but the PLL-PA gave the highest uptake after 24 hrs.

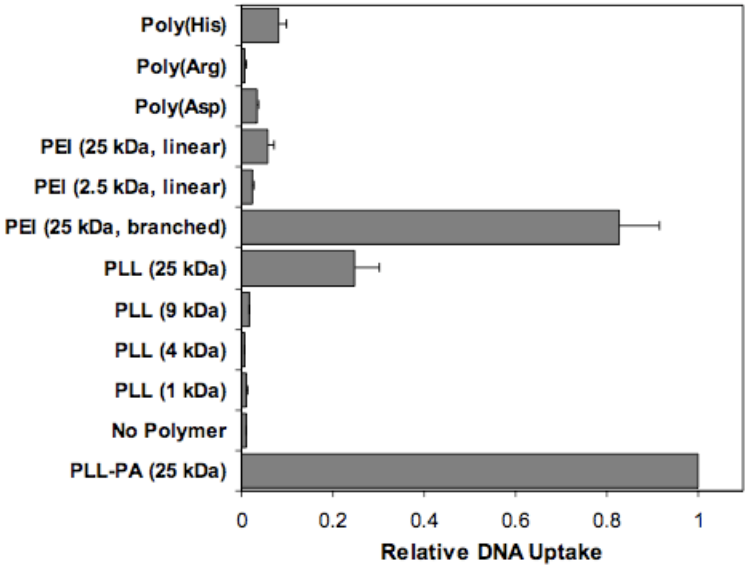


**Figure 3.8.** Uptake of pEGFP by BMSC with various cationic polymers. Cy5.5-labeled pEGFP was complexed without any polymer or the following polymers (10 μg/mL polymer): poly(histidine), poly(arginine), poly(asparagines), linear PEI with $M_w$ of 2.5 and 25 kDa, branched PEI (25 kDa), PLL with $M_w$ of 25, 9.2, 4.0 and 1.0 kDa, and PLL-PA (16.2 PA/PLL). The uptake was expressed as a fraction of PLL-PA uptake. Note that apart from PEI and PLL, no other polymers provided any significant pEGFP delivery into the BMSC.

### 3.3.4. Transfection Efficiency

The effect of PA substitution on the transfection efficiency of BMSC was then investigated. An initial study concentrated on evaluating the efficiency of PLL vs. PLL-PA, rather than PEI vs. PEI-PA, based on lack of improvement in pEGFP delivery in the latter case. The BMSC was incubated with polymer/pEGFP complexes for 24 hrs, and EGFP expression was evaluated over the subsequent 8 days (**Figure 3.9**). The transfections by pEGFP alone (i.e., without any carrier) and pEGFP/PLL was similar, with≤ 3% during this time period. PLL appeared to be beneficial on day 3, but no other benefit of PLL was noted on day 7. The transfection with pEGFP/PLL-PA was most effective, with maximal level of ~15% on day 3.

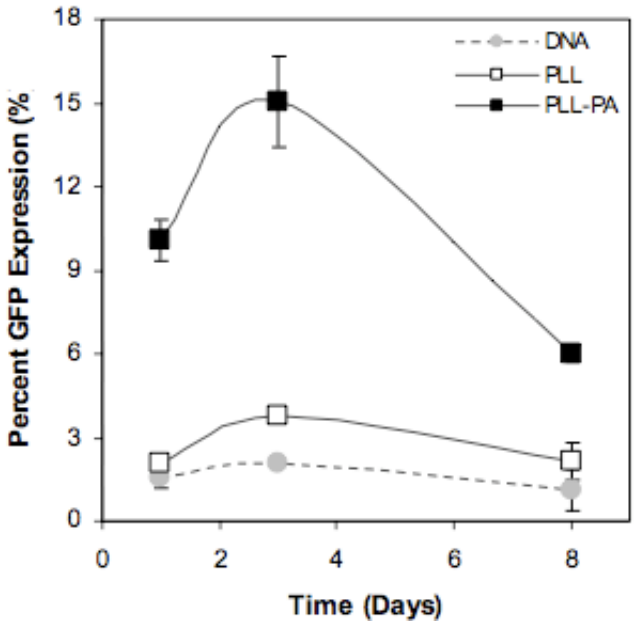


**Figure 3.9.** Transfection efficiency of PLL-PA as compared to PLL. pEGFP alone (2 μg) or pEGFP (2 μg) complexed with PLL and PLL-PA (10 μg) was incubated with BMSC in flasks (in triplicate; 2 mL medium) for 24 hrs, after which the cells were incubated with fresh medium (4 mL) without the pEGFP or the complexes. The percentage of GFP-positive cells was assessed 1, 3 and 8 days afterwards by flow cytometry. PA substitution clearly improved the transfection rate of the BMSC at all time points.

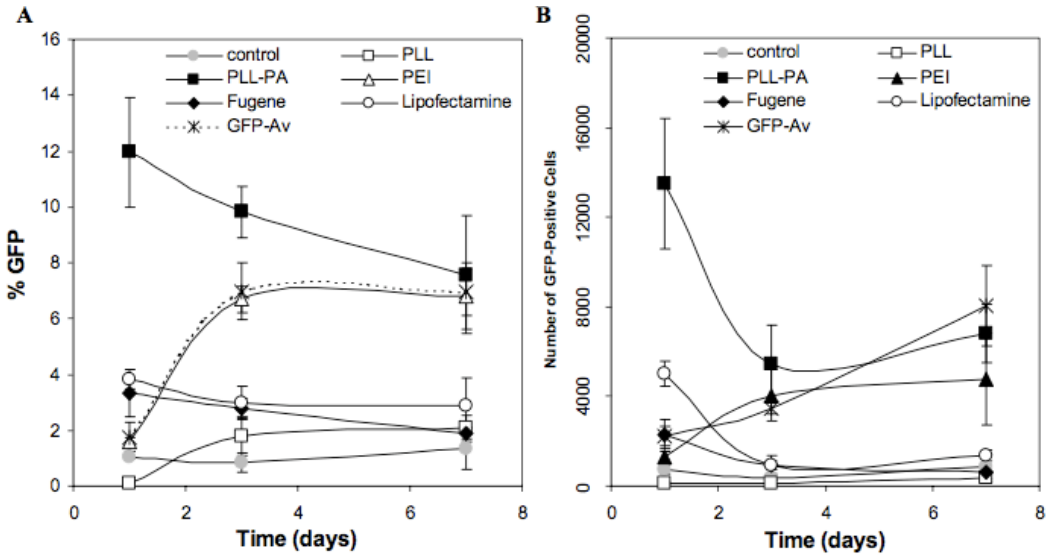


**Figure 3.10.** Transfection efficiency of various carriers over a 7-day period. The pEGFP (4 μg) was complexed with 10 μg of PLL, PLL-PA (16.2 PA/PLL) and PEI, and 10 μL of Fugene™, and Lipofectamine-2000™ for 20 min. The pEGFP without any carrier served as the negative control. The complexes and the GFP-Av were added to the BMSC in 6-well plates (2 mL medium) and incubated for 24 hrs, after which the complexes were removed, and fresh medium was added to the cells. The cells were trypsinized after 1, 3 and 7 days and used to obtain percent transfection by flow cytometry (A) or direct cell counts by a hemocytometer. The combination of total cell counts and percent transfection for each carrier was used to obtain total number of GFP-positive cells (B). Note that PLL-PA gave the highest percent transfection initially, which gradually decreased over the 7-day period. On day 7, GFP-Av and PEI were as effective as the PLL-PA based on both percent transfection efficiency and the total number of cells transfected.

A subsequent study evaluated the efficiency of PLL-PA as compared to two liposomal carriers (Fugene™ and Lipofectamine-2000™), unmodified polymers PLL and PEI, and the GFP-Av. Based on the % transfection rate from the flow cytometry assessment, PLL-PA was the most effective agent to transfect BMSC 24 hrs after incubation with BMSC (**Figure 3.10A**). There was a gradual decrease in the transfection level for the PLL-PA over the 7-day study period, at which point PEI and adenovirus provided a similar level of % transfection to that of PLL-PA. The number of cells was determined in parallel to calculate the total number transfected cells, since any differences in cell number among the carriers might make % transfection unreliable as a measure of the effectiveness of the carriers. As shown in **Figure 3.10B**, the PLL-PA gave the highest number of transfected cells on day 1, which was reduced over the 7-day study period. The number of transfected cells was increased for PEI and GFP-Av during the study

period, reaching to equivalent levels to that of PLL-PA on day 7. The numbers of cells transfected cells with PLL, and the liposomal carriers were significantly lower than the earlier three carriers.

## 3.4. Discussion

This study explored the utility of lipophilic polymers for modification of primary BMSCs derived from rats. Lipophilic polymers offer the possibility of enhancing gene transfer efficiency by facilitating transfer of polyplexes through phospholipid-rich cellular membranes. Modification of cationic polymers has been attempted before by using other lipophilic molecules. Cholesterol, an endogenous aromatic lipid, has been utilized to improve the transfection capability of branched PEI[13] and linear PEI[20] after chemically coupling the molecule to the polymers. In the case of linear PEI, cholesterol conjugation drastically increased the size of the polyplexes formed (2-4-fold)[20], which could have accounted for their improved effectiveness. Cholesterol conjugates obtained with linear PEI had 1-2 cholesterol/PEI chain for different size PEI chains ($M_w$ ~ 1.8 or 25 kDa). Smaller particles were obtained when cholesterol was conjugated to 1.8 kDa PEI[13,21]. There was some but not a dramatic improvement in cell toxicity over the parent polymer, reminiscent of our results. Cetylated PEI, where linear PEI was modified with -$(CH_2)_{15}$-$CH_3$, was also explored, but this polymer was most effective as part of a liposomal formulation in the presence of helper lipids, and its transfection efficiency alone was relatively low[22]. The cytotoxicity of the liposomal formulations with this polymer, however, was significantly improved over the conventional liposomes[22]. This was presumably because of the lower cationic density of the polymers, which is the main reason for the toxicity of non-viral vectors[23]. Unlike these results, an undesirable effect of lipophilic modification on branched PEI (~25 kDa) was observed by an independent group[24], which reported hydrophobic modifications with –$(CH_2)_{11}$-$CH_3$ and –$(CH_2)_{15}$-$CH_3$ to reduce transfection efficiency in immortal COS-7 cells. It is possible that this observation is valid for this particular cell type, since cell-to-cell

differences in transfection response have been appreciated[25,26]. It was interesting to note that modification with $–(CH_2)_{11}-CH_3$ and $–(CH_2)_{15}-CH_3$ moieties was beneficial particularly in the case of a short (2 kDa) linear PEI, where a non-effective polymeric carrier was transformed into most effective carriers as a result of this modification[24]. Prior to this study, a palmitylated PLL was described by Reddy[27] *et al*., and this polymer was shown to improve transfection efficiency of a β-galactosidase (LacZ)-containing plasmid in immortal KB cells as compared to native PLL. The obtained improvement was modest (~1.5-fold), and it was not possible to determine whether this improvement was because of a higher percentage of transfected cells or the higher expression levels per cell. That study, however, employed an additional liposomal carrier in order to transfer pDNA/polymer complexes, and no studies were conducted with palmitylated PLL as the sole transfection agent. The authors attributed to better dissociation of the acylated PLL (vs. native PLL) as the mechanism behind better transfection in that study. The collective experience form several independent groups suggested that hydrophobic modification of cationic polymers might be beneficial in improving the effectiveness of the polymers for gene transfer, but this improvement appeared to be dependent on the nature of the polymer and/or cells utilized for gene transfer.

PA was chosen for polymer modifications in this study since it is the natural lipid used for intracellular protein modification for trafficking. An amide linkage was relied upon to form stable conjugates to mimic intracellular N-palmitylation. This avoids using unnatural linkages, such as N-ethers utilized in some lipophilic polymers[24]. A relatively low level of substitution was achieved for both PEI and PLL (< 10% of available primary amines), after which aqueous solubility of the polymers was compromised. A distinct difference between the PA-substituted PEI and PLL was the lack of an effect on cell penetration in the case of PEI, but a much improved cell penetration in the case of PLL. This was also reflected in the ability of the polymers to carry pEGFP into the BMSC; whereas PA substitution did not improve the effectiveness of native PEI, PA substitution facilitated cell

delivery of the pEGFP. The improved delivery was achieved in the absence of an additional toxic effect, in fact with a slight improvement in the toxicity of PLL. It is likely that the reduction of cationic charge was not a significant factor in the observed toxicity, since this amounted to at most ~12% reduction of available amines in the case of PLL. Given the significantly increased uptake of PLL-PA (~10-fold; see **Figure 3.6**), it is likely that the reduced toxicity might arise from either; (i) a reduced concentration and/or residence time of palmitylated polymer on plasma membrane, minimizing the extent of non-specific interactions with anionic residues on cell surface and subsequent membrane perturbing activity[28,29], or (ii) altered intracellular processing of the palmitylated polymer, reducing nonspecific interactions with critical molecules of the cell metabolism[30]. The current study was not intended to investigate this issue, and future studies will be needed to elucidate the underpinnings of reduced toxicity observed with PA modification.

The PLL-PA was the most effective carrier when the ability of polymers to deliver pDNA intracellularly was considered. Only native PEI exhibited a similar extent of plasmid delivery. Linear PEIs were notable ineffective for plasmid delivery into BMSC. This was consistent with others' experience that reported superior transfection ability with branched PEI vs. linear PEI in immortal cells[24], but it was unlike several other reports that demonstrated successful gene transfer with linear PEIs[20,31,32]. A clear distinction in our studies was the actual measurement of pDNA delivery into the cells. Important differences between the linear and branched forms of the PEIs were observed in terms of their complex formation activity and association pattern with cells under confocal microscopy[33]. However, transfection efficiency was typically relied upon as a measure of DNA delivery, but several intracellular mechanisms can further contribute to transfection. Homopolymers of cationic amino acids histidine, arginine, and asparagine were also not effective for BMSC modification, although the individual amino acids has been successfully utilized to improve the performance of other carriers[34,35]. Presumably, a homopolymer of these cationic amino acids

did not have the optimal properties for DNA binding, compaction, cell surface interaction, and finally internalization by the BMSC. Polymers with relatively little pEGFP delivery (linear PEIs, homo(amino acids), and low MW PLLs) were not further evaluated in transfection studies, given the low probability of improved EGFP expression with relatively little intracellular delivery of pEGFP.

BMSC are unlike the commonly used immortal cells that undergo rapid proliferation, and provide a relatively robust cell model for evaluating the effectiveness of gene carriers. BMSC in our current culture system can be propagated for only 3-4 passages, after which senescence sets in to prevent further passage. Viral vectors have been the primary choice for modification of BMSC. Some studies reported almost complete modification of BMSC with GFP by using retroviral vectors on mouse-derived (96-98%[36]), and rat-derived BMSC [90-95%[37]). Other studies reported lower extent of GFP modifications (based on flow cytometric analysis, as used in this study) by retroviral vectors, ranging from relatively low levels of ~5% for mouse BMSC[38], to intermediate levels of 20-30% for mouse BMSC[39,40], to high levels of 60-80% for mouse, canine and human BMSC[40-42]. Other viral vectors, such a lentiviral and adenoviral vectors similarly exhibited a 25-80% transfection efficiency on BMSC[43,44]. Our experience with an adenoviral vector was at the lower than these reported values (10-15%)[17]. Although non-viral vectors have been used for modification of BMSC[45,46], little quantitative information on the transfection efficiency has been reported. Approximately ~5% efficiency was reported for a liposomal (FUGENE$^{TM}$) carrier in one report[47], where higher transfection efficiency (5-17%) was reported for another liposomal formulation (Lipofectamine$^{TM}$) in rat BMSC[48]. A cationized gelatin was not effective for modification of rat BMSC, but PEI provided 4-12% transformation depending on the N:P ratio[48]. This was consistent with our recent study that reported 5-10% transfection efficiency in rat BMSC with pEGFP/PEI formulations depending on the N:P ratio of the formulation[17]. This study reported a maximal transfection efficiency of ~15% by using PLL-PA, typically immediately (1 day) after exposure to the carriers. The PEI and GFP-Av were not

as effective as the PLL-PA initially, but provided an effective transfection agent in longer (1 week) time period. Consistent with other reports[47,48], liposomal carriers were not very effective for BMSC modifications, and our more extensive studies with different pEGFP/liposome ratios did not improve the transfection efficiency beyond the values obtained in this study (not shown).

We believe that the transfection efficiencies obtained with the current nonviral formulations represents a step forward in design of effective carriers, but they will require additional improvements. Most notably, the transfection efficiencies needs to be improved to the levels obtained with the retroviruses. Having transfection efficiencies in excess of 80% in the case of retroviruses allows one to utilize the transfected cells directly for clinical application. With nonviral carriers described in this study, it might be necessary to resort to cell purification after transfection, so as to concentrate the modified cells and avoid grafting unmodified BMSC. Alternatively, we envision to incorporate additional functionalities into the most successful carrier PLL-PA with the purpose of improving the transfection efficiency. Mechanisms to address nuclear targeting[49] or endosomal escape[50] might be incorporated to the lipophilic polymers if these mechanisms are critical barriers to intracellular trafficking of the pDNA/polymer complexes.

## 3.5. Conclusions

PA was used to impart a lipophilic character to the cationic polymers PEI and PLL, which are commonly used in their native forms for nonviral delivery of therapeutic genes. The polymers with PA substitution displayed slightly lower binding efficiency to pDNA, presumably because of interference of the bulky alkyl chains with the ionic interactions between the anionic pDNA and the cationic polymers. Whereas PEI modification with PA did not improve the DNA delivery into BMSC, PLL-substituted with ~15 PA/PLL provided a significantly higher delivery of exogenous pDNA into the cells. This was attributed to better cellular binding and internalization of PA-substituted PLL, as quantitated by the

flow cytometry. The improved delivery of pEGFP by PLL-PA was readily manifested in EGFP expression by BMSC; PLL-PA provided transfection efficiencies that were equivalent to an adenoviral vector, and significantly higher than the commercially available cationic lipids. We conclude that PA-substituted PLL is a promising reagent for transfection of the clinically relevant BMSCs.

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# 4. RELATIONSHIP BETWEEN THE EXTENT OF LIPID SUBSTITUTION ON POLY(L-LYSINE) AND THE DNA DELIVERY EFFICIENCY

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## 4.1. Introduction

Effective delivery of exogenous DNA into clinically relevant human cells, such as bone marrow stromal cells (BMSCs), is a major challenge in current gene therapy protocols. Viral and nonviral carriers are being pursued for this purpose. Because of the inherent mechanisms evolved to transfect mammalian cells, viral carriers have displayed more effective transgene delivery. Their clinical application, however, has been limited because of significant safety concerns, such as the host immune response mounted against them and the possibility of oncogenic transformations on target cells[1-4]. On the other hand, non-viral carriers, in particular cationic polymers, offer several advantages including low immunogenicity, the capacity to deliver large DNA payloads, stability and ease in scale-up. Cationic polymers facilitate the passage of DNA molecules through a cell membrane after condensing the DNA molecules into nanoparticles via cooperative electrostatic interactions. Poly(L-lysine) (PLL) and poly(ethylenimine) (PEI) have been initially used for DNA delivery[5,6]. Their general efficacy and utility, however, have been hampered by their relative high toxicity (in the case of PEI) and low transfection efficiency (in the case of PLL), when compared to the viral carriers.

Because significant components of cellular membranes are lipid-derived, one approach to improve the performance of polymer-based non-viral carriers is to incorporate hydrophobic moieties into the carriers[7-11]. These moieties are expected to function as membrane-anchoring moieties, enhancing the membrane compatibility of the polymeric carriers, and facilitating the membrane crossing of genetic cargo into the cells[12]. On the basis of this expectation, we recently designed[13] an amphiphilic lipopolymer by substituting the endogenous palmitic acid to PLL. Unlike unnatural hydrophobic molecules, endogenous lipids were preferred because they might ultimately be metabolized by mechanisms inherent in mammalian cells. The developed lipopolymer was shown to be biocompatible with the highly sensitive BMSC[13] and to be as effective as PEI, a relatively

effective but toxic polymeric gene carrier. It is not known if palmitic acid was the ideal lipid for PLL substitution. This study was accordingly designed to explore the feasibility of substituting PLL with several endogenous lipids of variable chain lengths, ranging from 8 to 18 lipid carbon chains. It was our intent to identify most suitable lipid(s), if any, for gene delivery. A relationship between gene delivery efficiency and the extent of lipid substitution on the carriers was additionally investigated. Our results did not identify a unique lipid for most effective DNA delivery, but rather indicated a general relationship between the extent of lipid substitution, and intracellular plasmid delivery and transgene expression in BMSC.

## 4.2. Material and Methods

### 4.2.1. Materials

Poly(L-lysine) hydrobromides (PLL-HBr; $M_{wvis}$ 25500, $M_{wvis}$ and 4000 Da), triethylamine (TEA), anhydrous dimethylsulfoxide (DMSO), anhydrous *N*,*N'*-dimethylformamide (DMF), caprylic acid (C8; 98%), myristic acid (C14; 99-100%), palmitic acid N-hydroxysuccinimide ester (C16; 98%), stearic acid (C18;95%), oleic acid (C18:1 9Z; 99%), linoleic acid (C18:2 9Z,12Z; 99%), *N*-hydroxysuccinimide ester (NHS), and dicyclohexylurea (DCC), Hanks' Balanced Salt Solution (HBSS), 5% (w/v) 2,4,6-trinitrobenzosulfonic-acid (TNBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and trypsin/EDTA were purchased from SIGMA (St. Louis, MO). Deuterated chloroform ($CDCl_3$) and water ($D_2O$) used as proton NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). Anhydrous ethyl ether and dry ethyl acetate were purchased from Fisher Scientific (Fairlawn, NJ). Dulbecco's Modified Eagle Medium (DMEM; high glucose with L-glutamine), Penicillin (10,000 U/mL), Streptomycin (10000 μg/mL) and Lipofectamine-2000 were purchased from Invitrogen (Carlsbad, CA). Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville,

GA). A succinimide ester of Cy5.5 (Cy5.5-NHS) was purchased from AMERSHAM (St. Laurence, QC) and used for labeling of plasmid DNA following manufacturer's protocol. A 4.7 kb-plasmid incorporating an enhanced green fluorescent protein (pEGFP-$N_2$) and a kanamycin resistance gene was obtained from BD Biosciences. The plasmid was replicated in kanamycin resistant DH5alpha E.coli strain grown in Luria-Bertani medium. After purification with a Qiagen Plasmid Giga Kit (Mississauga, ON), the plasmid concentration and purity were determined by UV spectroscopy. The plasmid was dissolved in ddH2O at 0.4 mg/mL for use in this study.

### 4.2.2. Synthesis and Characterization of the lipopolymers

The lipopolymers were synthesized by N-acylation of PLLs with NHS esters of the chosen lipid. The NHS esters (NHS-X, where X= caprylic, myristic, palmitic, stearic, oleic and linoleic acid) were synthesized in-house by mixing equal molar amounts of lipids, NHS and DCC in dry ethyl acetate. Briefly, the desired amount of lipids and NHS were dissolved in 3 mL of ethyl acetate, and then DCC in 1 mL ethyl acetate was added dropwise to lipid/NHS mixtures. The reaction was allowed to proceed overnight at room temperature under $N_2$, the urea byproduct was then filtered and the remaining NHS ester was concentrated with a rotary evaporator. The desired products were recrystallized from absolute ethanol at 4 $^{o}$C and the purity of the obtained product was confirmed by thin later chromatography[14].

The lipid:lysine mol ratios during the PLL substitution reactions were 0.16 and 0.66 and experimental details were according to a published method from our lab[13]. The lipid substitution ratios were determined by $^{1}$H NMR (Bruker 300 AM) after dissolving the polymers in $D_2O$ and using the characteristics hydrogen shift of lipids (~0.8 ppm; -C<u>H</u>3) and PLL (~4.3 ppm; –NH-C<u>H</u>-CO). Where indicated, the lipid methylene content was determined by multiplying the number of lipids substituted on the polymer (value determined by $^{1}$H NMR) and the number of

carbons present in each lipid chain. Further characterization of the modified PLL was attempted by mass spectrometry, but this was impeded by the aggregate formation of the polymers. After dissolving the polymers in $H_2O$, TNBS assay[15] was used to determine the free amine content of the polymers for subsequent studies.

### 4.2.3. BMSC Isolation and Expansion

BMSCs were isolated from the femurs of Sprague-Dawley rats as described in Ref. 16. The cells were suspended in a basic medium (DMEM supplemented with 10% FBS, 50 mg/mL ascorbic acid, 100 U/mL penicillin, and 100 μg/mL of streptomycin), seeded in a single 75 $cm^2$ flask (FALCON) for initial expansion and incubated in a humidified atmosphere of 95/5% air/CO2 at 37 oC. After medium change on day 3, the cells were either expanded on 75 $cm^2$ flasks (1:4 dilution), or seeded in multiwell plates (6-, 12- or 24-well plates from Corning) for the specific studies conducted (see Figure legends for specific configurations). The cells from passages 2 to-4 were only used for this study.

### 4.2.4. Cellular Uptake of Plasmid DNA

pEGFP-$N_2$ was labeled with Cy5.5-NHS as previously described[13]. For the uptake study, the BMSCs were seeded in 12-well plates and allowed to attach for 24 hrs before the uptake study. The labeled DNA was complexed with the desired polymer concentrations in the presence of 150 mM NaCl. After 30 min of incubation, the polyplexes were added to BMSC grown in DMEM with (10%) or without (0%) FBS. The final concentration of plasmid was 1.2 μg/mL and the polymers were 10, 3 and 1 μg/mL. After incubation for 24 hrs at 37 $^{\circ}C$, the cells were washed with HBSS, trypsinized, and cellular uptake was detected by BD Dickenson FACScalibur flow cytometry (FL4 channel, 635 nm laser, 5000 events/sample). The flow cytometer was calibrated for each run to obtain a background level of ~1% for control samples (i.e., untreated cells).

### 4.2.5. Transfection Studies

The polymer/pEGFP polyplexes were prepared by mixing the desired concentration of pEGFP and polymers in 150 mM NaCl. After 30 min, the polyplexes were added to BMSC in 6-well plates containing 1 and 10% FBS to give a final concentration of 1 μg/mL plasmid and 10 and 3 μg/mL polymers or Lipofectamine-2000. After 24 hrs at 37 $^{\circ}C$, either the cells were trypsinized for assessment of the EGFP expression by BD Dickenson FACScalibur flow cytometer (FL1 channel, 488 nm argon laser, 5000 events/sample) or the medium was replaced with fresh basic medium (DMEM with 10% FBS) for longer time cultures. The flow cytometer was calibrated for each run to obtain a background level of the EGFP expression of ~1% for control samples (i.e., untreated cells).

### 4.2.6. Cytotoxicity

Evaluation of cell viability was performed by the MTT assay. BMSCs were seeded on 48-well flat-bottomed microplates. The cells were incubated with 20 μL of the desired polymer concentration and 500 μL of basic medium (10% FBS) for 22 hrs at 37 $^{\circ}C$. The cell culture medium was then replace (400 μL), and 100 μL of MTT (5 mg/mL) was added per well. After 2 hrs, the medium was removed, 500 μL of DMSO was added to each well, and the plate was incubated in the dark for 5 min at room temperature so the crystals formed can be dissolved. The absorbance was read at 570 nm using a microplate reader. The negative control was assigned to 100% cell viability, and the remaining samples were normalized to this value[17].

### 4.2.7. Data Analysis

Where indicated, all results are summarized as mean ± standard deviation (SD) of the indicated number of replicates. Variations between the group means were analyzed by the Student's *t* test ($p < 0.05$). The significance ($p < 0.05$) of

correlations between the variables was tested by calculating the Pearson Product-Moment Correlation Coefficient (**r**) for sample size *N*, and relating the coefficient **r** to *t* distribution by $t = \mathbf{r} / [(1 - \mathbf{r}^2)/(N - 2)]^{1/2}$.

## 4.3. Results

### 4.3.1. Characterization of Lipopolymers

Cationic lipopolymers were synthesized by modifying low (4 kDa) and high (25 kDa) molecular weight PLL with N-hydroxysuccinimide (NHS) esters of several lipids (**Figure 4.1**).

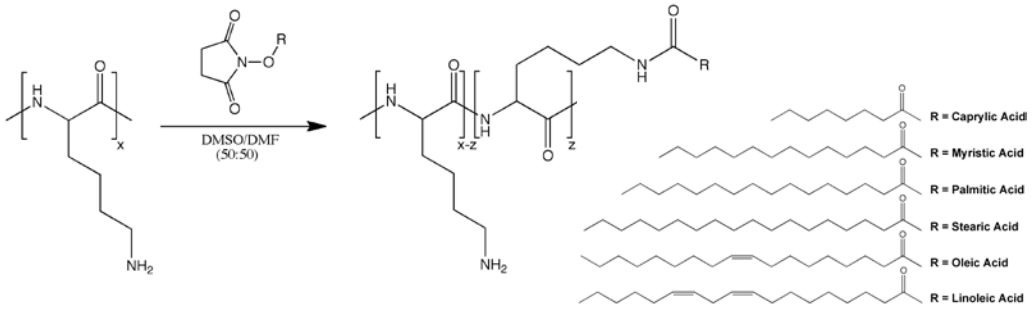


**Figure 4.1.** Synthesis of Lipid-substituted PLL by N-Acylation with NHS-Activated Lipids. R = caprylic acid, myristic acid, palmitic acid, oleic acid and linoleic acid.

The average degrees of substitutions ($^1$H NMR) obtained on PLLs after the reaction were summarized in Table 4.1 and **Figure 4.2**. With 4 kDa PLL, the number of lipids per PLL ranged form 0.9 to 4.1, whereas, with 25 kDa PLL, the number of lipids per PLL ranged from 2.3 to 10.0. No obvious correlations were evident between the chain length of the lipid and the number of lipid substituted per PLL. A higher lipid substitution was expected at higher lipid/lysine feed ratios; however, this was not the case for the lipopolymers prepared from the 4 kDa PLL (**Figure 4.2**). This expectation was realized for most lipids in the case of 25 kDa PLL, except oleic acid (C18:1 in **Figure 4.2**).

**Table 4.1.** Characteristics of the Polymer Library prepared from 25 and 4 kDa PLL at lipid/lysine feed ratios of 0.16 and 0.66.

| Polymer MW [kDa] | lipid | no. of carbons in the lipid chain | feed ratios (lipid/lysine) | Z[b] | lipid methylene content[c] |
|---|---|---|---|---|---|
| 25 | Caprylic acid | 8 | 0.16 | 3.9 | 31.2 |
| | | | 0.66 | 6.9 | 55.2 |
| 25 | Myristic acic | 14 | 0.16 | 8.7 | 121.8 |
| | | | 0.66 | 9.9 | 138.6 |
| 25 | Palmitic acid | 16 | 0.16 | 7.4 | 118.4 |
| | | | 0.66 | 9.8 | 156.8 |
| 25 | Stearic acid | 18 | 0.16 | 4.9 | 88.2 |
| | | | 0.66 | 10 | 180 |
| 25 | Oleic acid | 18:1[a] | 0.16 | 6.7 | 120.6 |
| | | | 0.66 | 6.2 | 111.6 |
| 25 | Linoleic acid | 18:2[a] | 0.16 | 2.3 | 41.4 |
| | | | 0.66 | 4.5 | 81 |
| 4 | Caprylic acid | 8 | 0.16 | 3 | 24 |
| | | | 0.66 | 1.9 | 15.2 |
| 4 | Myristic acid | 14 | 0.16 | 3.2 | 44.8 |
| | | | 0.66 | 3.6 | 50.4 |
| 4 | Palmitic acid | 16 | 0.16 | 4.1 | 65.6 |
| | | | 0.66 | 3.8 | 60.8 |
| 4 | Stearic acid | 18 | 0.16 | 2.4 | 43.2 |
| | | | 0.66 | 2.4 | 43.2 |
| 4 | Oleic acid | 18:1[a] | 0.16 | 2.3 | 41.4 |
| | | | 0.66 | 1.9 | 34.2 |
| 4 | Linoleic acid | 18:2[a] | 0.16 | 2.1 | 37.8 |
| | | | 0.66 | 0.9 | 16.2 |

[a]Number of carbons in the lipid chain:number of unsaturation. [b]Substitution (number of lipids per PLL) determined by $^{1}$H NMR. [c]Lipid methylene content = Z x number of carbons in the lipid chain.

Because polymers with variable number of lipids/PLL were obtained, the extent of lipid substitution was expressed by calculating the lipid methylene content in each polymer (= number of lipids/PLL obtained from 1H NMR multiplied by the number of carbons present in corresponding lipid chains; **Table 4-1** and **Figure 4.2B**). Polymers substituted with myristic, palmitic and stearic acid generally gave the highest lipid methylene content among the lipopolymers.

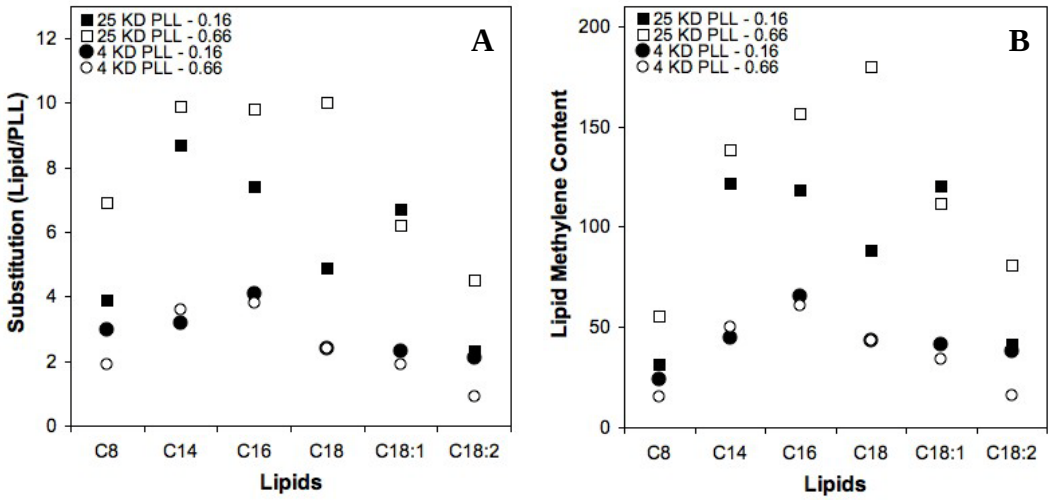


**Figure 4.2.** Number of lipids (A) and lipid methylene content (B) per PLL after substitution reactions. The data are summarized for PLLs of 25 kDa (squares) and 4 kDa (circles). The lipid/lysine feed ratio was either 0.16 (close symbols) or 0.66 (open symbles). Note that the larger PLL had generally higher substitutions, but no clear relationship between the lipid chain length and the extent of substitution was evident.

The ability of the synthesized polymers to condense DNA molecules was evaluated by agarose gel electrophoresis[13]. By using pEGFP, all polymers were able to effectively bind and condense the plasmid DNA in a concentration-dependent manner (data not shown). The concentration for effective condensation, typically given by IC50 (i.e., polymer/plasmid mass ratio to obtain 50% complex formation as assessed by agarose gel electrophoresis)[13], ranged between 0.3 and 0.5, and no clear effect of lipid substitution was evident on this particular parameter.

The cytotoxicity of the polymers was evaluated on BMSCs at polymer concentrations of 10 and 3 μg/mL (**Figure 4.3**). These concentrations were chosen based on our previous studies that determined concentrations for an effective plasmid delivery. The results indicated that the cytotoxicity of the lipopolymers derived from 4 kDa PLL was negligible in the chosen concentration range (**Figure 4.3A**). Most lipopolymers derived from 25 kDa PLL did not display cytotoxicity as well, except two polymers obtained from myristic and palmitic acid substitution at a lipid/lysine ratio of 0.16 (**Figure 4.3C**).

Note that polymers derived from 4 kDa PLL did not display any toxicity. Two lipopolymers derived from 25 kDa PLL (myristic and palmitic acid derived from lipid/lysine ratio of 0.16) displayed toxicity at 10 μg/mL but there was no obvious correlation between the lipid methylene content and cytotoxicity. The MTT absorbance from untreated cells is indicated with a gray triangle (**Figure 4.3C**). It was not clear why these two polymers displayed a cytotoxic effect because (i) similar polymers with higher myristic and palmitic acid substitutions (from feed ratio of 0.6) did not give such a cytotoxicity and (ii) no clear relations between the extent of lipid methylene content and cytotoxicity was evident (**Figure 4.3C**). At this point, we are unable to account for the observed cytotoxicity.

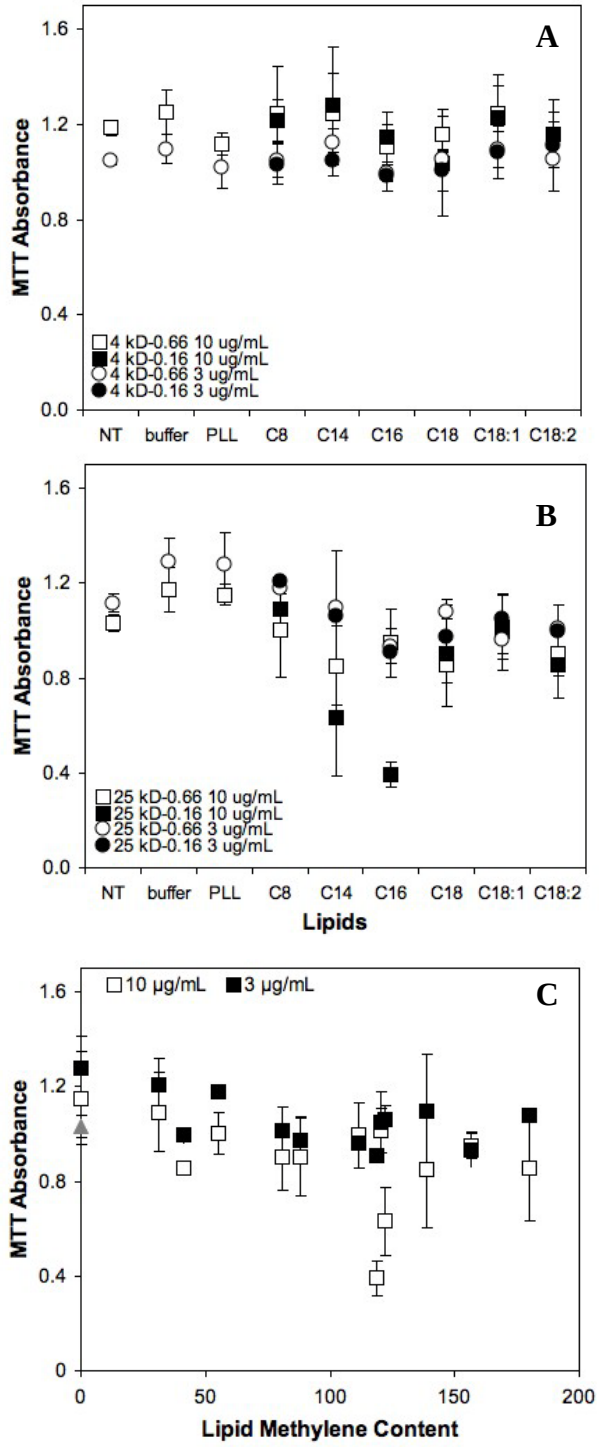


**Figure 4.3.** Cytotoxicity of polymers on BMSCs at polymer concetration of 10 and 3 µg/mL. The results are summarized for polymers derived from 4 kDa PLL (A) and 25 kDa PLL (B) at lipid/lysine ratios of 0.16 and 0.66. (C) Cytotoxicity of polymers derived from 25 kDa as a fuction of the lipid methylene content.

### 4.3.2. Cellular Delivery of Plasmid DNA

All native and lipopolymers were complexed with Cy5.5-labeled pEGFP at polymer/pEGFP ratios of 0.83, 2.5 and 8.33, all in excess of $IC_{50}$ values determined from gel electrophoresis. The final polymer concentrations in contact with cells were 1, 3 and 10 μg/mL, in line with our previous studies that identified concentrations necessary for effective plasmid delivery. The cellular delivery was assessed in a medium rich in FBS (10%) by using flow cytometry. With a relatively high concentration of polymers (10 μg/mL), the lipopolymers derived from 25 kDa PLL gave significantly higher uptake as compared to the polymers derived from 4 kDa PLL: > 70% of the cells displayed plasmid uptake in the case of 25 kDa polymers, whereas < 30% of cells displayed plasmid uptake with smaller PLL (**Figure 4.4A**). Given this significant difference in plasmid delivery, we decided not to pursue smaller PLLs and focus only on the polymers derived from 25 kDa PLL in further studies.

A subsequent plasmid delivery study was performed with 25 kDa PLL-derived lipopolymers at different polymer concentrations (10, 3 and 1 μg/mL). The results showed a positive correlation ($p < 0.002$, $< 0.05$, $< 0.008$ for 10, 3 and 1 μg/mL respectively) between the lipid methylene content and the plasmid delivery (**Figure 4.4B**). A 3-8 fold increase in plasmid delivery was obtained when compared to the unmodified 25 kDa PLL, which gave plasmid delivery to < 10% of the cells under the experimental conditions. Polymers with the highest substitution of myristic, palmitic and stearic acid (~10 lipids/PLL) gave the most effective pEGFP delivery. Incubating the cells with pEGFP in the absence of any carriers yielded < 2% Cy5.5-positive cells for all experiments, indicating the percentage of cells capable of plasmid uptake without the need of a carrier.

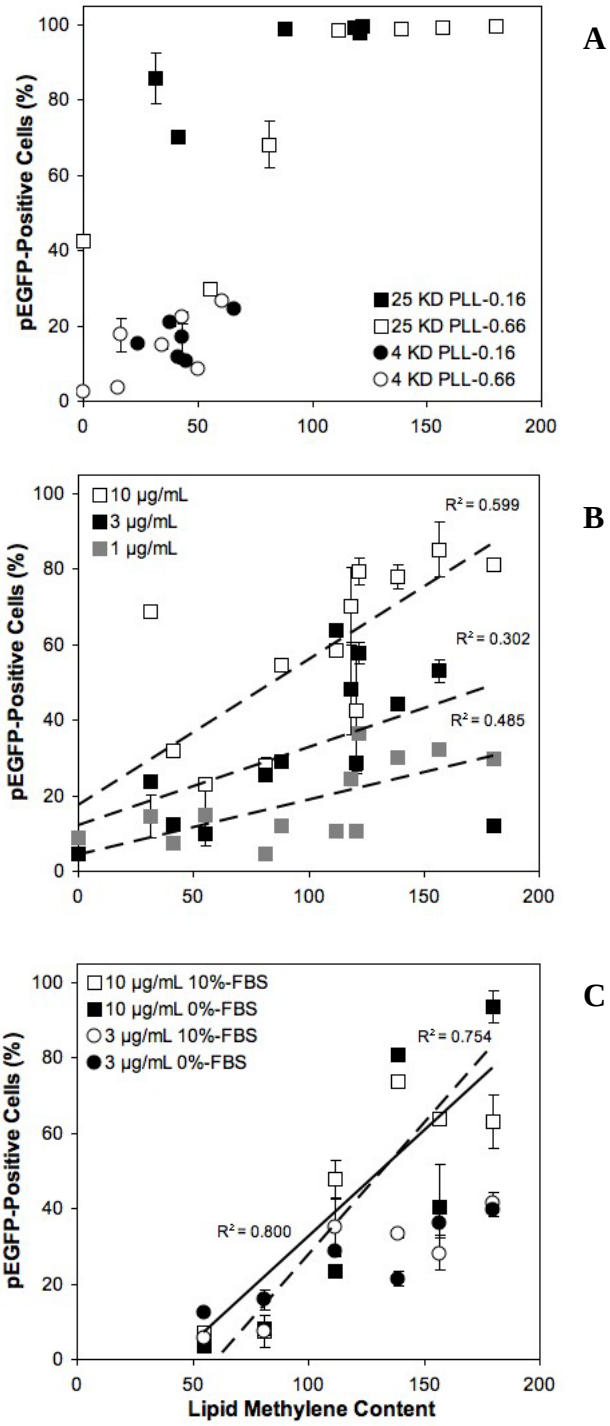


**Figure 4.4.** pEGFP delivery to BMSCs as a function of the lipid methylene content of PLLs. The cellular uptake was assessed after 24 hrs of incubation of complexes with cells. (A) Comparison of polymers derived from 4 and 25 kDa PLL. (B) pEGFP delivery by lipid-substituted 25 kDa PLL at 10, 3 and 1 µg/mL (in DMEM with 10% FBS). (C) Effect of serum (0 vs 10% FBS in DMEM) on the pEGFP delivery to BMSCs by selected lipid-substituted PLL (polymer:pEGFP = 10:1.2 and 3:1.2 µg/mL; polymers from a feed ratio of 0.66). pEGFP without any carrier yielded ~2% pEGFP-positive cells for all experiments. Linear regressions in Figure 4.4 C are for the high concetration of polymers.

Additionally, we studied the effect on pEGFP delivery in the presence and absence of serum proteins (i.e., with 0 and 10 % FBS) in the cell culture medium. Polymers from the feed ratio of 0.66 and concentrations of 10 and 3 μg/mL were tested in this study. Serum proteins were found not to interfere with plasmid delivery by the lipopolymers (**Figure 4.4C**).

### 4.3.3. Transfection Efficiency

Transfection studies with lipopolymers were conducted along with the most commonly used commercial lipid formulation, Lipofectamine-2000. After 24 hrs exposure of cells to complexes in FBS-containing medium (10% FBS), we observed a correlation ($p < 0.01$, $< 0.008$ for 10 and 3 μg/mL 10%FBS respectively and $p < 0.002$ for 10 μg/mL 1% FBS) between the transfection efficiency and the extent of lipid methylene content at the two polymer concentrations tested (**Figure 4.5A**). As much as 20-25% of the cells were modified under these conditions with the most substituted PLL (~10 myristic, palmitic, stearic acid per PLL). Unmodified PLL gave ~4% modification under the same conditions. The transfection of the cells was also investigated in a medium with reduced serum content (oMEM with 1% FBS). A correlation between the transfection efficiency and lipid methylene content was again evident (not shown). Lipofectamine-2000 formulation gave 2-3% transfection after 1 day of exposure (data not shown).

Using a select set of lipid-substituted polymers (polymers from feed ratio of 0.66), transfection efficiency was evaluated on days 7 and 14 (**Figure 4.5B**). The correlation ($p < 0.0002$ and 0.02 for days 7 and 14, respectively) between the

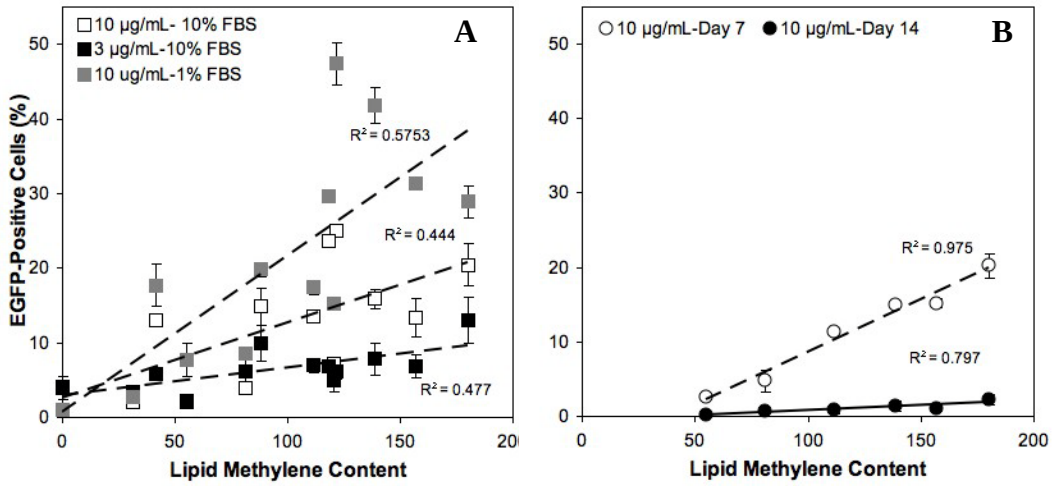


**Figure 4.5.** (A) Effect of the lipid methylene content of polymers on the EGFP expression on day 1. The plasmid concetration was 1 µg/mL, whereas the polymer concentrations were 3 and 10 µg/mL. (B) EGFP expression on days 7 and 14 at polymer/pEGFP concentrations of 10 and 1 µg/mL, respectively. Only polymers from a feed ratio of 0.66 were used for this study.

extent of lipid substitution and transfection efficiency was retained even after 7 days, however a significant drop in transfection efficiency was noted after 14 days. The EGFP expression with commonly used commercial transfection reagent Lipofectamine-2000 was ~1% on days 7 and 14 (data not shown).

## 4.4. Discussion

The development of effective non-viral gene delivery systems is crucial for successful gene therapy. To achieve this goal, we imparted lipophilic character to a relatively ineffective DNA carrier, PLL, by grafting hydrophobic moieties onto the polymer backbone, and evaluated its DNA delivery capability in BMSCs. Two different PLLs (4 and 25 kDa) were used to assess the influence of polymer MW on DNA delivery[17-19]. These PLL sizes were selected because they combine a relative good toxicological profile with some membrane activity and efficient condensation of DNA into stable particles[19]. Additionally, several endogenous lipids (myristic, palmitic, stearic, oleic and linoleic acids) were chosen for PLL substitution to avoid unnatural moieties in the carriers (except caprylic acid). Because PLL enables plasmid DNA condensation via interaction of its primary

amines with anionic phosphates groups of DNA, the reaction conditions were controlled to substitute < 10% of the PLL lysine, leaving sufficient free □-$NH_2$ to condense DNA while maintaining polymer aqueous solubility. By using two lipid/lysine feed ratios (0.16 and 0.66), we were able to synthesized a series of modified polymers with different degrees of lipid substitutions. The obtained lipopolymers did not always follow a predictable pattern of lipid substitution, and it is likely that improved reaction conditions need to be identified for better control of lipid substitution. Other solvents that can better solvate the reactants with widely differing properties (i.e., cationic polymer and hydrophobic lipids), or a scaled-up reaction (our current reaction called for 25 mg PLL, which is a relatively small amount), might be possible solutions. Because the resultant lipopolymers displayed a variable lipid substitution, we preferred to normalize the extent of lipid substitution based on lipid methylene content in each polymer. In this way, we explored correlations between the DNA delivery efficiency and the extent of modifications based on a common variable (i.e., lipid methylene content).

The condensation of DNA molecules for cellular internalization is a prerequisite for efficient gene delivery. In our case, all polymers were able to effectively bind and condense pEGFP even at the lowest polymer/pEGFP ratio used (1:1). Once plasmid DNA complexation was confirmed, the capability of lipid-substituted carriers for intracellular delivery of plasmid DNA was evaluated. Comparisons between carriers generated from PLLs of different MWs showed a greater uptake when high-MW lipopolymers were used. This difference was previously noted by others and was likely due to a higher density of ε -$NH_2$ present on high-MW PLL, increasing the number of DNA-polymer interactions necessary for a stable complex formation[19], which, in turn, increased the amount of genetic payload delivered through the plasma membrane. We also investigated the effect of polymer concentration on cellular uptake with lipopolymers derived from 25 kDa PLL; the plasmid delivery into the cells was proportional to polymer concentration and, more importantly, a correlation between the lipid methylene

content and plasmid delivery was evident at all concentrations tested. This indicates that there is a greater complex-membrane interaction when hydrophobic moieties are involved in complex formation, particularly for polymers with the highest content and carbon length of 14-18 (~10 lipids/PLL), which gave the most effective pEGFP delivery. These findings are in agreement with our previous publication and other research group that coupled PLL to hydrophobic agents to enhanced cellular uptake and transcription[7,9,13]. Although full pEGFP complexation was obtained even at the lowest polymer concentration (1 μg/mL) under our experiment conditions, an excess of polymer was still needed for more effective plasmid because increasing the polymer concentration from 1 to 10 μg/mL continued to improve the intracellular delivery, whereas unmodified PLL yield , 10% Cy5.5-positive cells under the experimental conditions.

Positively charged complexes are necessary for interaction with anionic cell surfaces. However, the cationic nature of the complexes may lead to additional unspecific interactions with negatively charged serum proteins. This could decrease cellular association with complexes, thus reducing or inhibiting endocytosis[19-21]. Cationic liposomes, for example, may display low DNA delivery efficiency because of strong interactions with serum proteins. Avenues to overcome serum inhibition involve increasing the charge ratio of lipid to DNA, and/or allowing sufficient maturation time for complexes before exposure to serum-containing medium for transfection[22,23]. Interactions of poly(amino acid)-based complexes with biological environment are often problematic, and PLL polyplexes are well-known to interact with body fluids and rapidly cleared from the systemic circulation[24,25]. Consequently, several strategies to address this problem have been used. One common strategy is based on the use of hydrophilic polymers, such as PEG[25-27] or HPMA[28], to create a hydrophilic coating that reduces interactions with serum proteins. More recently, Brown[10] *et al.*, designed a neutral gene delivery system based on palmitoyl-PEG-PLL that contained cholesterol, and the authors showed that this system augmented the half-lives of the complexes in the bloodstream as compared to complexes formed with PLL

alone. Kim[7] *et al.*, also observed a beneficial effect of low-density lipoprotein (LDL) in complexes formed with hydrophobized PLL (stearyl-PLL), and this beneficial effect was lost in a serum-free medium. Given the importance of serum interactions, we investigated the effect of serum on pEGFP delivery. Our experiments showed no apparent interference of serum proteins in cellular delivery of DNA by the lipid-substituted polymers. As was the case in the presence of FBS, plasmid delivery was proportional to the lipid methylene content in the absence of FBS (**Figure 4.4C**). This result suggests that the lipid-substituted PLLs could efficiently deliver DNA without the need for further modification of the carrier and/or polyplexes (e.g., lipid helpers, lipoproteins or hydrophilic coating).

Although the majority of the cells in culture can internalize the plasmid/carrier complexes, only a small fraction of the cells actually expresses the transgene. Generally, after internalization of the complex, plasmid/carrier complexes are transported through the endosomal/lysosomal pathway, leading to vesicle acidification to pH 5-6 by the action of an ATPase. A fraction of the complexes is degraded, but also some complexes are released from these compartments into the cytosol, so that it can be transported to the nucleus. In a previous publication[29], we were able to locate pEGFP/palmitic-PLL in the cytoplasm as well as in the nucleus on BMSCs after 24 hrs. This showed that the designed amphiphilic carriers not only protected the DNA from enzymatic degradation but also delivered the genetic payload into the nucleus. We expect that the use of other lipids on PLL, i.e., the ones describe on this paper, might have a similar response. Because of the large numbers of the carriers, however, this issue was not explored in this study, and additional studies are planned to explore the details of complex trafficking as a function of substituted lipid on the carriers.

Consistent with cellular uptake results, EGFP expression was proportional to the extent of lipid methylene content of the polymers. Lipid-substituted polymers with a lipid length between 16 and 18 gave the highest transfection levels; 20-

25% of the cells were modified under these conditions, while native PLL only gave ~4% and Lipofectamine-2000 gave 1-3% modification under the same conditions, indicating the significant potential of the designed polymers for plasmid delivery. Interestingly, cells under reduced serum conditions gave up to 2-fold higher extent of modification than the cells under normal serum conditions (10% FBS). Unlike plasmid internalization, it is possible that serum proteins might interfere with intracellular complex dissociation necessary for EGFP expression, leading to a lower level of EGFP expression. Future studies are planned to explore the reasons of better gene expression under reduced serum conditions. The transgene expression was maintained up to 7 days, and correlations between the extent of lipid substitution and transfection efficiency was observed during this time period. However, a significant drop in transgene expression was noted after 14 days, which is consistent with the transient nature of non-viral delivery in general[30].

Minimal or no cytotoxicity of a synthetic carrier is an important requirement for gene delivery systems. The use of gene carries that mimic naturally-occurring molecules (e.g., lipoproteins) could significantly reduce the toxicity of delivery systems. PLL has been known to be toxic on mammalian cells[31]; however, attaching hydrophobic molecules to polymeric backbone seems to decrease the cytotoxicity, when compared to unmodified polymers[13,32,33]. The high positive charge density of gene carriers is generally considered to be the reason for cytotoxicity[31,34], and shielding these charges with hydrophilic polymers or lipoproteins has been a productive strategy. Kim[7] *et al.,* observed a reduction of the toxicity of stearyl-PLL when low-density lipoprotein (LDL) was combined with this carrier, but the beneficial effect of stearic acid substitution, if any, was not studied in this polymer. Brown[10] *et al.,* and Pan[35] *et al.,* also used lipid-modified PLLs (palmitoyl-PLL-PEG and palmitoyl-PLL in a 'nanoemulsion' formulation, respectively) and showed a decrease of toxicity as compared to unmodified PLL. The toxicity of these carriers without PEG grafting or without the nanoemulsion formulation was not evaluated, so that the benefit of lipid

modification in reducing toxicity could not be assessed. In our study, the cytotoxicity of the polymers at concentrations effective for intracellular plasmid delivery to BMSCs was independent of the hydrophobic moiety used as well as, for the majority of the cases, to the extent of lipid substitution. These results suggest the possibility of improving plasmid delivery by the lipopolymers without adversely affecting cellular physiology. We recognize that the cytotoxicity of polycationic gene carriers is dependent on the cell system chosen. For example, our studies[36] using a different clinically relevant cellular system, skin fibroblasts, showed some toxicity with the polymers, in particular for those that gave effective pEGFP delivery (e.g., myristic acid-substituted PLL). Similarly, Choksakulnimitr[34] *et al.*, studied the degree of toxicity of polycationic carriers in brain microvessel endothelial cells, macrophages and hepatocytes. They observed that macrophages had a higher sensitivity to polymers than the hepatocytes and endothelial cells. We focus on highly sensitive BMSCs, since they are clinically useful, unlike other commonly used immortal cells. The BMSC might be more tolerant to our gene carriers than other cell types (e.g. skin fibroblasts), and this observation highlights the importance of evaluating toxicity of gene carriers in desired target cell phenotypes.

## 4.5. Conclusions

The results of this study indicated that several endogenous lipids are suitable for incorporation into PLL to serve as effective DNA carriers. Carriers with high MW and high lipid methylene content increased cellular uptake, generating significant gene expression with relatively low toxicity on clinically relevant BMSCs. Myristic, palmitic and stearic acid-substituted polymers gave the most effective DNA delivery, but this was likely because of high substitution ratios obtained with these lipids (~10 lipids/PLL). We noted for the first time a general relationship between the extent of lipid substitution and gene delivery efficiency. Even though other lipids were not effective, we speculate that they could be as effective as the myristic, palmitic and stearic acids if they are sufficiently

substituted on PLL, given that the established correlations were equally valid based on both the number of lipids and number of lipid methylenes substituted per polymer. We conclude that the described amphiphilic polymers should be effective for *ex vivo* modification of clinically relevant cells and may be further engineered with targeting moieties for modification of cells *in vivo*.

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# 5. Physicochemical properties, cellular trafficking and transgene expression with low molecular weight PEI modified with *trans* fat and essential fatty acids

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## 5.1. Introduction

Synthetic gene carriers take advantage of the chemical properties of DNA-binding compounds to form DNA complexes (or particles) that can readily be adsorbed onto cell surfaces and become internalized. Among the well-known gene carries are high (e.g., 25 kDa) molecular weight (MW) cationic polyethylenimines (PEI). The strong transfection efficiency of PEIs arises from the different pKa values of the primary, secondary and tertiary amines in the polymer backbone, which gives polymer a strong ability to bind to DNA molecules while retaining an effective buffering capacity against endosomal pH decrease, thus allowing the complexes to escape from the endosomal vesicles before enzymatic attacks[1]. However, the use of high MW PEI has been hindered by the polymer's relatively high toxicity due to its highly cationic nature. One way to reduce the toxic effect of polymers is to employ low MW PEI and conjugate the polymers' amine groups with specific ligands that can enhance the effectiveness of the polymer[2,3]. Ligands that can grant specificity to cell-surface molecules can create carriers with higher binding capacity to cell surface, thereby requiring lower amounts for effective gene transfer and decreasing the cytotoxicity of the carriers. Lipids have been grafted onto the carriers as well[4,5] that would enhance non-specific interaction with cellular membranes and/or intracellular trafficking. Although lipid substitution is known to improve carrier efficiency, the mechanism by which these polymers are internalized and subsequently transfect the target cells is poorly understood.

Lipid modification of proteins are routinely carried out intracellularly with a variety of lipids, such as myristic (C14), palmitic (C16), farnesyl (C15) or geranyl (C20) acids. These modifications affect how the protein interacts with the lipid-rich cellular membranes, allowing different modes of trafficking between the membrane compartments[6]. It has been possible to anchor the proteins onto the membranes as a result of such modification, or flip proteins in-and-out of membranous vesicles with reversible attachment of lipidic residues onto the proteins. Therefore, modification of carriers by conjugating fatty acids (lipids)

that can mimic this interaction has been actively pursued. Incani[7] *et al.,* have imparted lipophilic character to the relatively ineffective DNA carrier poly-L-lysine (PLL) by grafting endogenous lipids (myristic C14, palmitic C16, stearic C18, oleic C18:1 and linoleic C18:2 acids) onto the polymer backbone. The carriers with high MW and high lipid content increased cellular uptake in rat-bone marrow stromal cells (rat-BMSC). Despite the high internalization, however, only a small fraction of the cells actually expressed the transgene. The PLL substituted with C16 and C18 lipids gave the highest transgene levels compared to native PLL and commercial transfection agent Lipofectamine™ 2000. In line with these results, Abbasi[8] *et al.,* showed that the lipid-substituted polymers where able to protect the DNA payload against nuclease degradation and efficiently deliver plasmid DNA into human skin fibroblasts, particularly with C14 and C18 modified PLL. It is important to note that efficient gene delivery was obtained with high lipid substitution and especially with lipids longer than C8, suggesting that the length of the lipid chain is an important factor in the design of efficient gene delivery carriers. Neamnark[9] *et al.,* also modified low MW (2 kDa) PEI with the same series of lipids and observed an enhanced plasmid DNA delivery into 293T and rat-BMSC cells compared to the inefficient carrier 2 kDa PEI. However, significant gene transfection was evident only with 293T cells, and their transfection ability was equivalent to highly effective 25 kDa PEI without the toxic effect associated with the latter polymer. No particular lipid emerged as ideal substituents for transgene expression for this system, although linoleic acid (C18:2) appeared to be superior to other lipid substituents. The unique feature of the linoleic acid was its long carbon chain (the longest among the explored lipids) and the presence of the unsaturated double bond in the lipid chain. In general, as little as 1 lipid substitution per PEI was effective in transforming the ineffective 2 kDa PEI into an effective carrier.

This study further explored the desirable structural features of lipid substituents in order to enhance its transfection ability of low MW PEI. Since the longest lipid previously employed had 18 carbons, we synthesized new carriers by conjugating

fatty acids of longer chain lengths and with multiple unsaturated double bonds to the backbone of PEI. By using elaidic acid (ELA; C18:1T), α-linolenic acid (LLA; C22:3), behenic acid (BA; C22:0), erucic acid (EA; C22:1), and docosahexaenoic acid (DHA; C22:6), we generated a series of lipopolymers from the low MW PEI. Unlike the high MW PEIs, low MW native PEI has better cytotoxicity profiles, but they have shown to be ineffective as gene carriers. Thus, modifications on this type of polymer are desired to develop effective gene carriers without adversely affecting the toxicity profile of the carriers. In addition to exploring the influence of lipid chain length and number of double bonds on lipid substituents, we investigated the internalization mechanism and intracellular trafficking of the plasmid/carrier complexes by using established endocytic drug inhibitors, namely genistein, chlorpromazine and methyl-β-cyclodextrin (MβCD). These studies were conducted by using two cell types, the easy-to-transfect 293T cells and clinically-relevant human bone marrow stromal cells (h-BMSC).

## 5.2. Material and Methods

### 5.2.1. Materials

Branched 2 kDa PEI ($M_n$ 1.8 kDa and $M_w$ 2 kDa) and 25 kDa PEI ($M_n$ 10 kDa and $M_w$ 25 kDa), fluorescein isothiocyanate (FITC), chloroquine, L-ascorbic acid, sodium acetate, chlorpromazine, genistein, methyl-β-cyclodextrin (MβCD), anhydrous dichloromethane (DCM), triethylamine (TEA), Hank's Balanced Salt Solution (HBSS), dimethylsulfoxide (DMSO), 37% formaldehyde and deuterated water ($D_2O$) were purchased from SIGMA (St. Louis, MO). Acetic acid, ethanol and ethyl ether were purchased from Fisher Scientific. Behenoyl chloride (BA), 4,7,10,13,16,19 docosahexanoyl chloride (DHA), elaidoyl chloride (ELA), erucoyl chloride (EA), and α-linolenoyl chloride (LLA) were obtained from Nu-Chek Prep, Inc. (MN, USA). Dulbecco's Modified Eagle Medium (DMEM; high glucose with l-glutamine), penicillin (1000 U/mL), trypsin/EDTA, streptomycin (10 mg/mL), UltraPure™ Water (DNAse and RNAse free), and Hoechst 33258

were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). SYBR Green I was obtained form Invitrogen (CA, USA). Cy5 Label IT® Tracker™ Intracellular Nucleic Acid Localization Kid was obtained from Mirus Bio (Madison, WI) and used for labeling of plasmid DNA following manufacturer's protocol. The plasmids gWIZ (blank plasmid with CMV promoter but lacking a functional gene) and gWIZ-GFP (AcGFP expressing plasmid under the CMV promoter) were purchased from Aldevron (Fargo, ND).

### 5.2.2. Synthesis and Characterization of Lipopolymers

The PEI used for lipid substitution was freeze-dried to remove its water content. The lipopolymers were synthesized by *N*-acylation of 2 kDa PEI with lipids chlorides (**Figure 5.1**). The synthesis procedure was described in a previous work[10]. Briefly, 100 mg of PEI was dissolved in 5 mL of DCM under $N_2$ at room temperature. After addition of 160 μL of TEA, the desired fatty acid dissolved in 5 mL of DCM was gradually added to a PEI solution over a 30 min period. The solution was stirred for 12 hrs under $N_2$ and the reaction was quenched in an ice bath. The product was then concentrated in vacuo and washed (×3) with excess of ethyl ether. The residue was dried overnight under vacuum at room temperature. The lipid:PEI amine ratios were systematically varied during the synthesis procedure. Lipopolymers were analyzed by $^1$H NMR (Bruker 300 MHz; Billerica, MA) in $D_2O$. Proton chemical shifts specific for lipids (δ ~0.8 ppm; -C$\underline{H}_3$) and PEI (δ ~2.5-2.8 ppm; -HN-C$\underline{H}_2$-C$\underline{H}_2$-NH-) were integrated and normalized to the number of H's in each peak, and then used to obtain the extent of lipid modification on the polymers.

### 5.2.3. DNA binding and Complex Dissociation

Plasmid DNA binding ability of the lipopolymers was assessed using SYBR Green I dye exclusion assay, where the dye displays specific binding for double-

stranded DNA. Briefly, gWIZ-GFP solutions (2 μg/mL) were prepared in 150 mM NaCl and 1.5 to 96 μL of polymer solutions (5 μg/mL) was added to give a final volume of 50 μL (in triplicates). After 30 min of incubation at room temperature, 200 μL of SYBR Green I dissolved in TAE buffer (400 mM Tris-acetate/10 mM EDTA diluted ×1) were added to each sample. Quantification of free DNA was read on 96-well plate ($\lambda_{EX}$, 485 nm; $\lambda_{EM,}$ 527 nm). Percentage binding was calculated from the fluorescence density values (*F*) as 100% × {*F*(DNA only) – *F*(specific polymer/DNA weigh ratio) ÷ *F*(DNA only) – *F*(background)}. The sigmoidal binding curves were generated by plotting % bound DNA vs polymer/DNA ratios, and the $BC_{50}$ value was calculated corresponding to polymer/DNA ratios giving 50 % of the plasmid binding.

Electrophoretic Mobility Shift Assay (EMSA) was used to determine the dissociation of plasmid DNA complexes with lipopolymers. For this study, complexes at DNA/polymer 1:5 (μg:μg) were formed in 10 μL of 150 NaCl. After 30 min of incubation, 10 μL of basic medium (with 10% FBS) was added to the samples and incubated for additional 1 hr. Heparin sulfate was then added at a final concentration range of 0.05-1 mg/mL, and samples were further incubated for 10 min at 37 $^{\circ}$C. Samples were then loaded to the agarose gel (0.8%) containing 1 μg/mL of ethidium bromide in TEA buffer (400 mM Tris-Acetate/10 mM EDTA diluted ×1). The agarose gel was run at 130 V for 20 min, and DNA bands were visualized under UV light (Alpha Innotech; San Leandro, CA). Percentage of DNA dissociation was calculated from the fluorescence density values (*F*) as 100% × {*F*(specific heparin concentration) – *F*(background) ÷ *F*(DNA only) – *F*(background)}. % Complex dissociation was plotted as a function of the heparin concentration.

### 5.2.4. Size and Zeta Potential Measurements

Diameters and electrophoretic mobilities (ζ-potential) of the particles formed by complexing lipopolymers with plasmid DNA were determined by Zetasizer, Nano

ZS (Malvern Instruments, Worcestershire, UK). Samples were analyzed at 25 °C using medium viscosity for water of 0.89 cp and for NaCl of 1.14 cp[11]. Complexes were prepared at plasmid DNA/polymer mass ratios of 1:2, 1:5 and 1:10 in 1 mL of 150 mM NaCl (using gWIZ as the plasmid) and incubated for 30 min prior to analysis (DLS) or in 40 μL of 150 mM NaCl (incubated for 30 min) and then diluted in pure water (incubated 30 min) for ζ-potential analysis. Measured sizes and ζ-potentials were presented as the averaged value of 12 and 10 runs respectively.

### 5.2.5. Cell Culture

The 293T cells were maintained at 37°C on 75 $cm^2$ flasks with high-glucose DMEM containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS under a humidified atmosphere of 95/5% air/$CO_2$. At 80-90% confluence, the cells were trypsinized with trypsin-EDTA and seeded in multiwell plates for analysis (see below), or sub-cultured on 75 $cm^2$ flasks for passage typically at 1:10 dilution. The human bone marrow stromal cells (h-BMSC) were also passaged on 75 $cm^2$ flasks by using high-glucose DMEM with 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL ascorbic acid and 10% FBS. The cells were typically typsinized and seeded in multiwell plates for analysis or split 1:4 for routine passage.

### 5.2.6. Uptake of Plasmid DNA

The cellular uptake of plasmid DNA was assessed by using FITC-labeled plasmid. The FITC-labeled plasmid was obtained by reacting 20 μL gWIZ (5 μg/mL diluted in 130 μL of pure water) with 30 μL of FITC (100 mM in DMSO) in an orbital shaker at 37 °C for 3 hrs. The labeled plasmid was precipitated with 30 μL of 3 M sodium acetate (pH 5.2) and 825 μL of ethanol (95%), incubated at -20 °C for 15 min, centrifuged at 13800 rpm and washed with ethanol (×2). The precipitated plasmid was dissolved in pure water at 0.4 mg/mL. Complexes with

FITC-labeled gWIZ were prepared in 20 μL of 150 mM NaCl to give a final DNA/polymer mass ratio of 1:5 (μg:μg) per well. After 30 min of incubation, the complexes were added to 293 and h-BMSC grown in 24-well plates with 500 μL of basic medium (in triplicate). The complexes were incubated for 24 h at 37 °C after which medium was replaced. After further 24 hrs, the cells were washed with HBSS, trypsinized and suspended in HBSS with 3.7% formaldehyde. The uptake of FITC-labeled gWIZ was detected by flow cytometry (Beckman Coulter QUANTA SC) using FL1 channel (3000-5000 events/sample). The results were expressed as either (i) percentage of cells displaying FITC-labeled DNA uptake or (ii) mean fluorescence of the cells positive for FITC-labeled DNA. The instrument was calibrated with control samples (i.e., cells exposed to FITC-gWIZ without polymer) to yield 1-2% FITC-positive cells. As an additional control, cells incubated with complexes prepared from unlabeled gWIZ were used to investigate any induction of cellular autofluorescence as a result of complex exposure to the cells.

### 5.2.7. In Vitro Cell Transfection

The efficiency of lipopolymer complexes for gene delivery was determined by using a plasmid carrying the reporter gene Green Fluorescent Protein (gWIZ-GFP). The complexes were prepared at different DNA/polymer weight ratios under physiological salt concentration (150 mM) and used to transfect 293 and h-BMSC. The preparation of complexes was described in Section 5.2.6, except gWIZ-GFP was used as the plasmid DNA instead of FITC-labeled gWIZ. The complexes were incubated with the cells for 24 hrs at 37 °C. Subsequently, the transfection medium was replaced with fresh medium and the cells were further incubated for desired time periods. At the time of assessment, the cells were trypsinized, suspended in HBSS with 3.7% formaldehyde and GFP expression was quantified by flow cytometry (Beckman Coulter QUANTA SC) using FL1 channel (3000-5000 events/sample). The percentage of GFP-positive cells were that were treated with gWIZ-GFP alone (i.e., without polymer) was set at 1-2 %.

To investigate the effect of the endosomolytic agent chloroquine on transfection efficiency, the transfection medium was supplemented with 10, 25 and 50 μM of chloroquine for 30 min followed by incubation of cells with DNA/polymer complexes at 1:5 weight ratio. Culture medium was replaced after 24 hrs of exposure to complexes and chloroquine. Cells were washed with HBSS and quantification of the extent of cellular transfection was performed by flow cytometry at 48 hrs post-incubation.

### 5.2.8. Confocal Microscopy

Confocal microscopy was used to assess intracellular trafficking of plasmid DNA in h-BMSC. The gWIZ-GFP was labeled with Cy5 according to the manufacturer's instructions. A sample of 1 μg/mL of labeled and unlabeled DNA was complexed with 5 μg/mL of polymer in 100 μL of NaCl for 30 min. Complexes were further incubated in 900 μL of OPTIMEM 1% for 20 min. The h-BMSC seeded in 6 well-plate were incubated with the transfected medium for 6 and 24 hrs, and then the cells were fixed with 3.7% formalin in HBSS for 30 min, followed by staining of the cell nuclei with 1 mL of Hoechst 33528 (300 ng/mL in HBSS) for 30 min (×2). The cells were washed several times with HBSS and analyzed by a confocal microscope (Zeiss LSM 510 Lacer Scanning Confocal Microscope) at different time points. Instrumental settings for Cy5 and Hoechst 33528 excitations were 633 and 405 nm, respectively. Untreated cells were used as negative control and all areas were analyzed using Z sectioning.

### 5.2.9. Cytotoxicity

Toxicity of the complexes was evaluated by the MTT assay. Briefly, 293T and h-BMSC were seeded in 48-well flat-bottomed multiwell plates with 500 μL of medium. Complexes were prepared as described before and the cells were incubated with the complexes and/or drugs for 24 hrs at 37 $^{\circ}$C, prior to the addition of 100 μL of MTT (5 mg/mL in HBSS) per well. After further incubation

(2 hrs), the medium was removed and 500 μL of DMSO was added to each well. The plate was incubated in the dark for 5 min at room temperature and the optical density was measured at 570 nm. The reference control (untreated cells) was assigned to 100% cell viability, and the treated cells were normalized to this reference.

## 5.3. Results and Discussion

### 5.3.1. Synthesis of Lipopolymers

A series of lipid substitutions was attempted based on different lipid:PEI feeding ratios ranging from 0.1 to 4.0. However, only the lipid:PEI mol ratio of 1.26 yielded significant lipid substitutions (**Table 5.1**). Despite our expectation to obtain similar polymer modifications at the same lipid:polymer reaction ratio for different lipids, no apparent correlation between the initial feed ratio and the degree of substitution was found. It was also not clear why higher feed ratios did not yield more lipid substitutions. One can speculate that the limited modification of the polymer was due to stearic effect caused by the longer lipids used in this study. Our previous studies with relatively shorter lipids (C8 to C18 with minimal unsaturation in the lipid backbone) gave relatively predictable lipid substitution ratios as a function of lipid:PEI feed ratio in the reaction medium[9]. Whereas substitution with shorter lipids yielded as much as ~20% amine modification, the reaction conditions for this study yielded polymers with <6% of amine modification, leaving ample free amino groups to condense plasmid DNA while maintaining aqueous solubility. Polymer substituted with saturated BA or highly unsaturated DHA gave the lowest lipid substitutions whereas lipid substitution with EA gave the highest polymer modification.

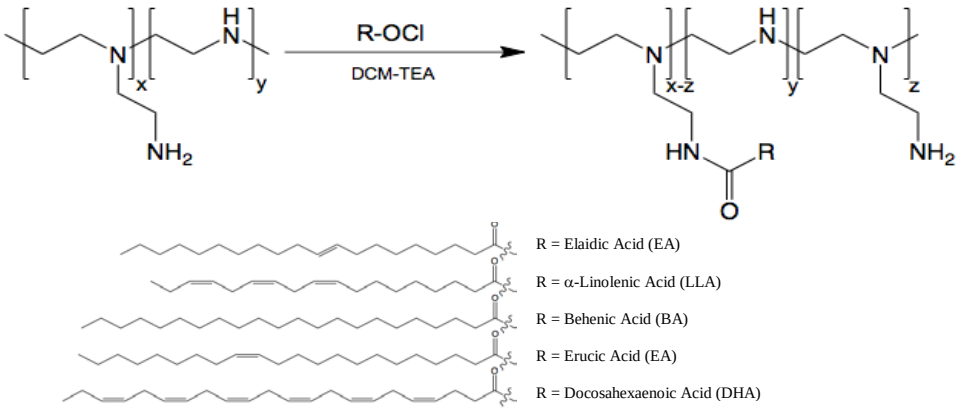



**Figure 5.1.** Reaction scheme for synthesis of lipid-substituted PEI by N-Acylation with acid chlorides.

**Table 5.1.** Lipid substituted PEI2 prepared at the lipid/ethylenimine feed ratios of 1.26. [a]Number of lipids per PEI substituted was determined by $^{1}$H-NMR. [b]% substitution refers to the percentage of primary amines modified with corresponding lipid.

| Polymer | Substituent | Lipid:PEI[a] | %substitution[b] |
|---|---|---|---|
| PEI2-BA | Behenoyl chloride (C22:0) | 0.2 | 1.4 |
| PEI2-EA | Erucoyl chloride (C22:1) | 0.8 | 5.7 |
| PEI2-DHA | Docosahexanoyl chloride (C22:6) | 0.2 | 1.4 |
| PEI2-LLA | Linolenoyl chloride (C18:3) | 0.5 | 3.6 |
| PEI2-ELA | Elaidoyl chloride (C18:1T) | 0.6 | 4.3 |

## 5.3.2. Characterization of DNA/Lipopolymer Complexes

Plasmid DNA condensation into small-size particles and neutralization of the anionic charge of plasmid DNA are indispensable roles for any gene delivery system. Accordingly, plasmid DNA binding properties of the polymers were assessed with the SYBR I Green. The binding behavior observed was similar for all lipopolymers, evident by the overlapping sigmoidal curves of the percentage of bound DNA vs. polymer/DNA weight ratios (**Figure 5.2A**). The results showed that binding occurred almost independent of the extent of lipid substitution (in the investigated range), contrary to previous observations[9,11]. It must be noted though the extent of lipid substitution was relatively small in this study, compared to other studies considered[9,11]. Among the polymers synthesized in this study,

stronger binding was obtained with PEI2-BA (lowest $BC_{50}$) compared to native PEI2 and PEI2-EA (highest $BC_{50}$). The lipid substitutions on PEI appeared to lead to a higher tendency to form DNA complexes irrespective of the lipid and the number of substitution. In addition to electrostatic interactions, hydrophobic interactions also seem to be involved in DNA complexation and condensation. Thus, the incorporation of hydrophobic moieties might cause a cooperative binding transition of lipid-modified polymers as seen by others[12,13]. Regardless of the modification, both modified and native PEIs condensed the plasmid DNA similarly at the high mass ratios.

Plasmid DNA dissociation from the complexes was investigated next by addition of different concentrations of heparin to the complexes. No dissociation was observed at less than 0.1 mg/mL heparin (**Figure 5.2B**). Considering that the complexes were incubated in tissue culture medium (with serum) for this assay, the complexes were likely to be stable under cell culture conditions. PEI2-LLA had the highest resistance to dissociate, since only ~45% of the plasmid DNA dissociated at the highest heparin concentration used, followed by the PEI2-BA and PEI2-DHA where the dissociation was ~60% at heparin concentration below 0.5 mg/mL. The most heparin-sensitive complexes were obtained with the PEI2, where almost complete dissociation (80-100%) was observed at heparin concentration >0.3 mg/mL. It is important to note that even though all lipopolymers were able to bind plasmid DNA in a similar fashion (from **Figure 5.2A**), their dissociation pattern was significantly different. The binding pattern is likely to be predominantly affected by the amine groups and, having relatively equivalent number of amines (since the substitution ratios were relatively small), the lipopolymers behaved similarly to that of the native PEI2 from which they were derived. However, the role of lipids seems to be particularly evident in the dissociation process, the latter groups strengthening the stability of the complexes and making them resistant to dissociation by the highly anionic heparin. Polymers modified with lipids containing the smallest (single) double bonds resulted in complexes easier to dissociate compared to the highest (6) and moderated (3)

number of double bonds and to the saturated lipid. No particular correlation was evident between the ability of lipopolymers to dissociate vs. the degree of lipid substitution.

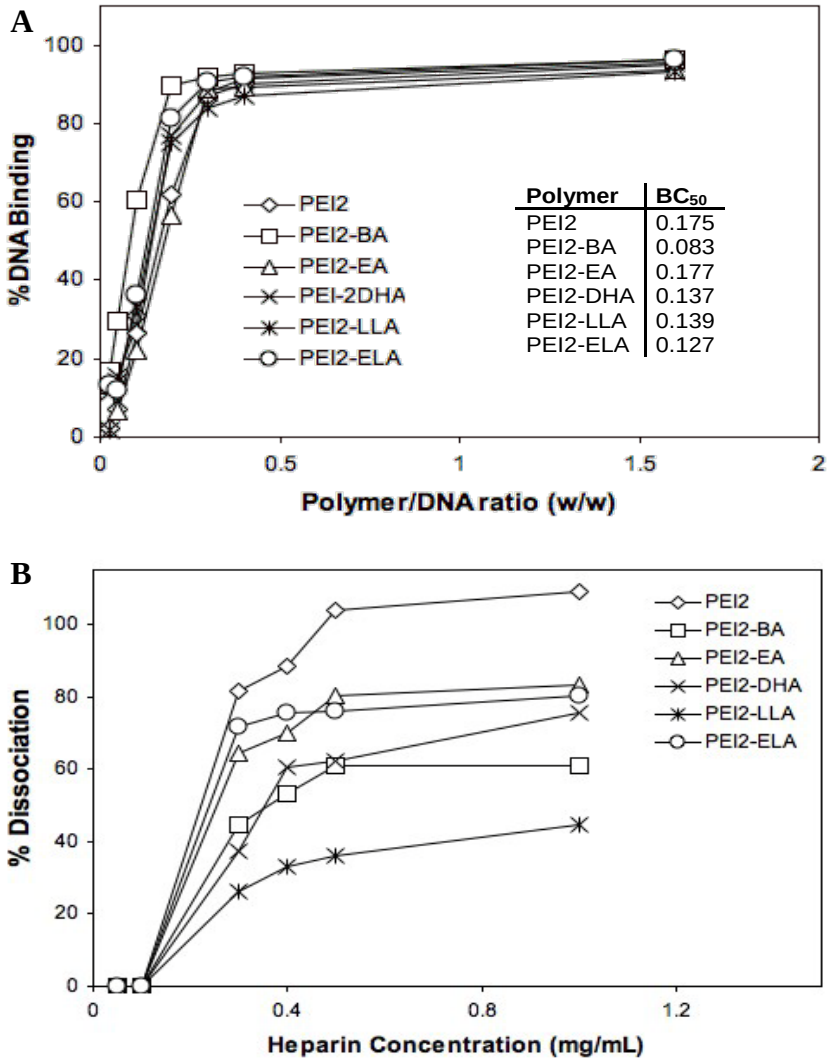



**Figure 5.2.** (A) Assessment of polymer/DNA complexation by SYBR Green I assay. % Binding as a function of polymer concentration was plotted and sigmoidal curve fits were performed. Polymer ratios required for 50% binding of DNA are listed in the table (insert). (B) Assessment of complex dissociation induced by different concentrations of heparin. Complexes formulated at weight ratio of 5 (DNA/polymer = 1:5 μg/μg) were analyzed by EMSA for this study.

The size of the complexes was examined as a function of plasmid DNA/polymer weight ratios. The MW of the PEI significantly affected the size of the complexes; whereas PEI2 resulted in ~400 nm particles, PEI25 resulted in ~150 nm particles

(**Figure 5.3A**). The latter was in line with our previous studies[14] as well as the studies reported in the literature[3]. The relative ratio of plasmid DNA to polymer did not seem to make a significant impact on the particle sizes for native PEIs. Lipid conjugation affected the hydrodynamic diameter of the complexes, where the particle sizes were increased (530 - 590 nm) as a result of lipid conjugation on the native PEI2. PEI2-BA notably formed bigger particle size (716-850 nm) among the lipopolymers tested. Others have reported an increase in particle size after alkylation with C12, C14 and C16 on PEI, particularly if the number of substitution was increased. However, acylation with the same lipids were less sensitive to such a change in size[13]. Moreover, if the DNA/polymer ratio was increased the particle size decreased in an independent study[13]. In contrast, acylation of PEI with shorter C2, C3 and C4 chains resulted in larger particles as the chain length and the degree of substitution increased[11]. These results differ from ours since our particles were not sensitive to the size of the lipid and the DNA/polymer ratio (except PEI2-ELA).

Negligible effect of the lipid substitution was observed on the ζ-potential of the particles. Native PEIs as well as the modified polymers showed similar ζ-potential values and a positive net surface charge independent of the DNA/polymer ratios employed for complex formation (**Figure 5.3B**). Particle surface charge was expected to decrease after lipid modification[15] since primary amine groups of the cationic PEI2 were modified in our polymer derivatization scheme. It is possible that the negligible effect of lipid substitution was due to the low degree of lipid modification of the polymers.

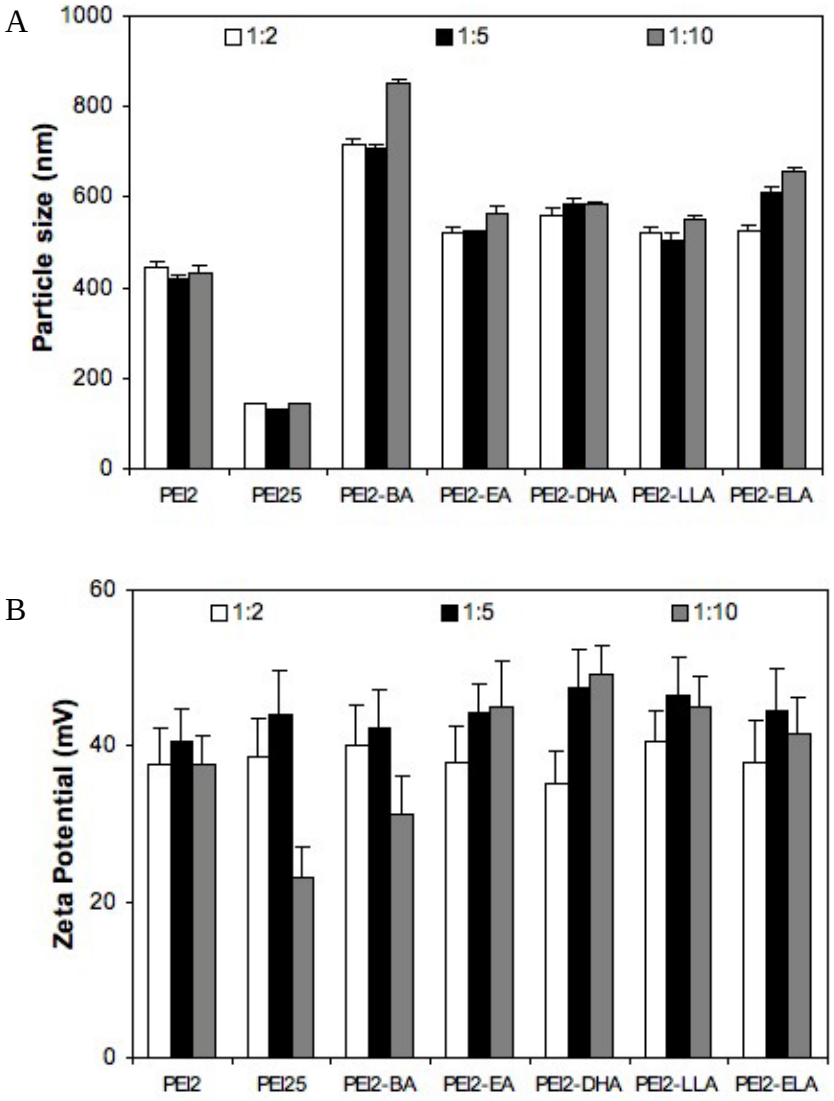



**Figure 5.3.** (A) Particle size and (B) ζ-Potencial of complexes determined at DNA/polymer ratios of 1:2, 1:5 and 1:10 (w:w). All measurements were performed in triplicates.

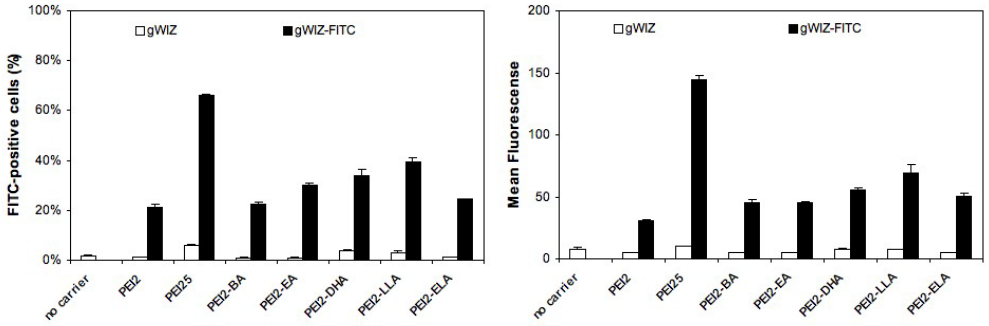
### 5.3.3. Plasmid DNA Delivery into Cells

Assessment of plasmid DNA delivery to the cells was investigated by using 293T cells and h-BMSC and with FITC-labeled gWIZ. Analysis were performed by using flow cytometry on day 2 (293T cells and h-BMSC) and day 3 (293T cells only) post-incubation with complexes, to allow cells to recover from complex treatment and to reduce "background" due to cellular autofluorescence[9]. Under the experimental conditions, only <2% and ~5% of the cells were positive for the

plasmid DNA after treatment with lipopolymers/gWIZ and PEI25/gWIZ complexes, where non-labeled gWIZ was used in these control complexes. This was indicative of minimal autofluorescence (background) in the experimental system. The cellular uptake with lipopolymers was similar in both cell types (compared **Figure 5.4A and 5.4B**), where PEI2-LLA and PEI2-DHA complexes showed up to 2-fold higher uptake as compared to native PEI2 complexes. This result indicated that the lipids even at relatively low substitutions on PEI coul enhance cellular uptake of plasmid DNA. The lipopolymers PEI2-BA, PEI2-EA and PEI2-ELA were relatively less effective for plasmid DNA delivery. With all polymers, the plasmid DNA was present to a lesser extent in 293T cells after 3 days of incubation (data not shown). The decrease in plasmid DNA-positive cells may be caused by the release of the complex into the medium, or by cell division resulting in dilution of the internalized plasmid[16].

Particles bearing a net cationic charge are believed to facilitate cellular uptake through the anionic cell membrane. However, the lipopolymers and the native PEIs displayed similar surface charges, so that this did not appear to be the reason for differences in cellular uptake (it must be noted that the ζ-potential was determined under buffer conditions, before addition to cell culture medium, which might alter the surface charges subsequently). The smallest particle size was formed with PEI25 and these particles showed the highest cellular uptake as compared to the rest of the lipopolymers. This was evident in both cell types, which are quite different morphologically and functionally, yet displayed similar levels of uptake for each lipopolymer. Whereas DHA and ELA level of substitutions differed most widely (see **Table 5.1**), the uptake was similar for these two lipopolymers, so that the levels of substitution *per se* did not seem to explain the uptake levels as well. Taking into account these considerations, it appears that the smaller PEI25 particles were better internalized but the uptake mechanism for the lipopolymer complexes might be more complicated with no single factor capable of fully explaining the obtained results fully[17,18].

A. 293T cells




B. h-BMSC

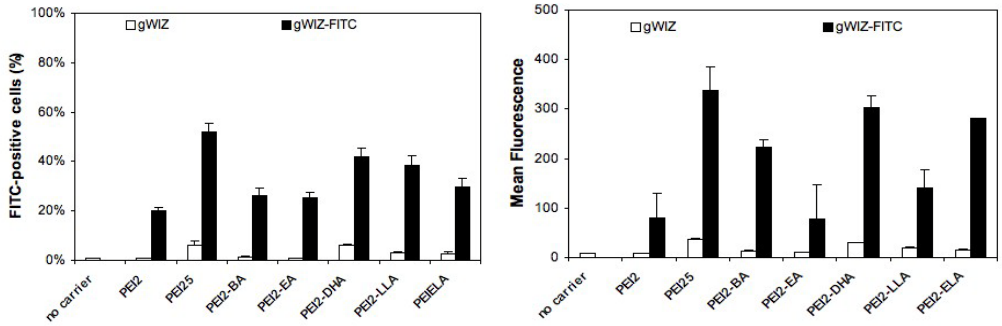



**Figure 5.4.** Uptake of plasmid DNA by (A) 293T cells and (B) h-BMSC at DNA/polymer ratio of 1:5 μg/mL. The uptake was assessed after 2 days of complex exposure to the cells. The cells were treated with either unlabeled gWIZ complexes or FITC-labeled gWIZ complexes.

### 5.3.4. Confocal Microscopy Analysis for Complex Uptake

The intracellular fate of the complexes was followed in h-BMSC. Although 293T cells were also analyzed initially, their large nuclear size with respect to a limited cytoplasmic size impeded accurate microscopic analysis of intracellular trafficking. Only complexes from PEI2, PEI25 and PEI2-LLA were used in this study to limit the scope of the microscopic analysis. The complexes were visible inside the h-BMSC cells at 6 hrs of post-treatment (**Figure 5.5**). Discrete complexes were observed in the periphery of the nuclei for PEI25, indicative of single complex entrapment in endosomes and peri-nuclear trafficking. After 24 hrs, nuclear localization was still evident for this polymer, but intracellular fluorescence began to appear in larger compartments suggesting that numerous

complexes may be entrapped within single endosomes as a result of endosomal fusion. In contrast, PEI2 and PEI2-LLA complexes were not visible as discrete complexes at 6 hrs of post-treatment. Aggregates were instead seen as big big red patches in the cytoplasm for these complexes and not near the nuclei (**Figure 5.5**). The relative sizes of the complexes observed under confocal microscopy were in agreement with the size of the particles measured with the zetasizer. The result was reminiscent of the previous observations with rat-BMSC treated with Lipofectamine™ 2000[19]. Complexes with this carrier were observed to form spaghetti-meatball like structures as described by Sternberg[20] *et al.* Furthermore, the microscopic results also agreed with the findings of Rejman[18] *et al.*, where particles of ~500 nm were largely localized at the periphery of the cell nucleus and smaller particles (~200 nm) were accumulated in the perinuclear region. After 24 hrs, fluorescence remained confined to these patches for PEI2 and PEI2-LLA, though brightness and quantity had diminished. This effect may be attributed to cellular exclusion of the complexes or to dilution following cell division as seen by other groups using different cell line and carriers[16,21].

To determine if the plasmid DNA was localized inside the nucleus, a series of z-stack images were captured at both time points. The data indicated that the DNA complexes were not localized inside the nucleus of h-BMSC, instead they were mostly spread throughout the cell cytoplasm and, in some cases, and the fluorescence was visible around or on top of the nucleus (not shown). This suggests that the nuclear uptake of the plasmid DNA was a significant impediment in the case of h-BMSC.

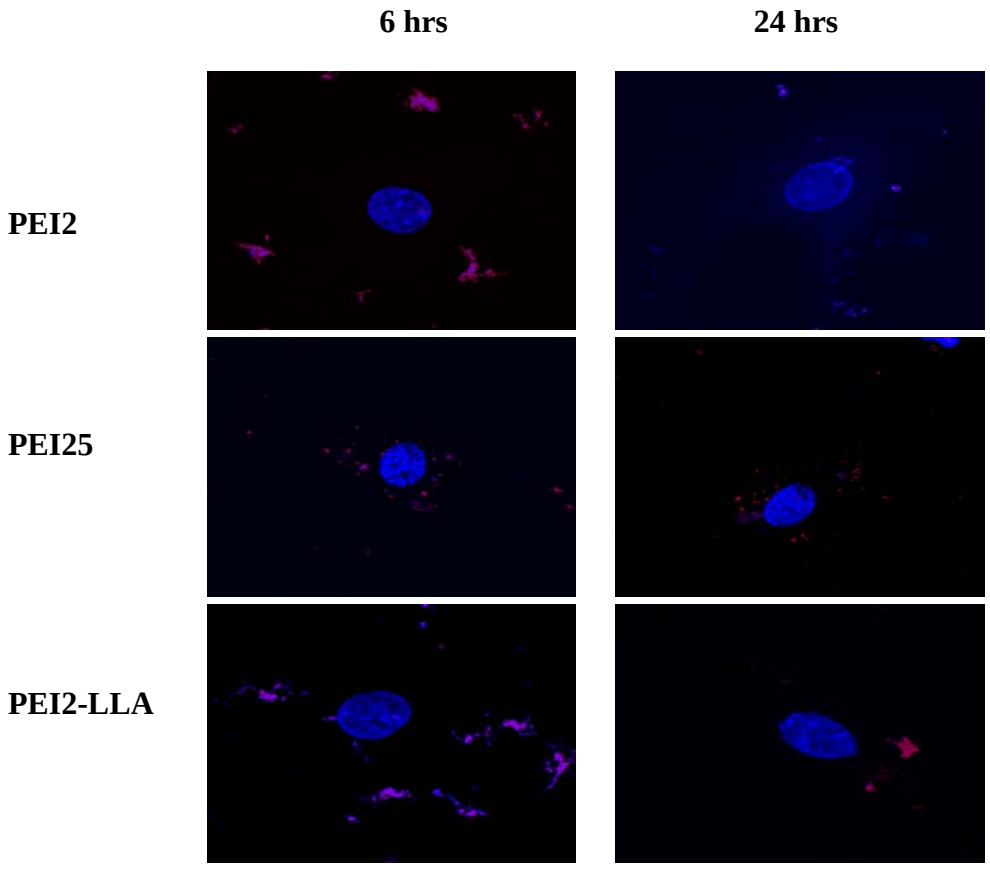



**Figure 5.5.** Confocal images of h-BMSC treated with DNA complexes of PEI2, PEI25 and PEI2-LLA (DNA/polymer ratio of 1:5 (μg/μg)). The cells were analyzed at 6 hrs and 24 hrs post-treatment. (A) Complex internalization was observed with Cy5-labeled gWIZ-GFP (red) and (B) GFP expression was observed only with complexes of PEI25 (bright green). Nuclei were stained with Hoechst (blue).

### 5.3.5. In Vitro Cell Transfection and Cell Viability

An appropriate balance between the lipophilicity and hydrophilicity is necessary for carriers to cross the plasma membrane of the cells. Substituting lipids is expected to increase the lipophilicity of hydrophilic polymers, increasing their ability to cross cell membranes and enhancing the transfection efficiencies. Modifications of PEI-based polymers have been attempted before with acyl or alkyl aliphatic lipids of variable chain lengths. Alkylation of PEI2 with C12 and C16 enhanced its transfection efficiency to ~30% and ~50% in COS-7 cells respectively of that of PEI25 (PEI2 was not effective) without compromising cell

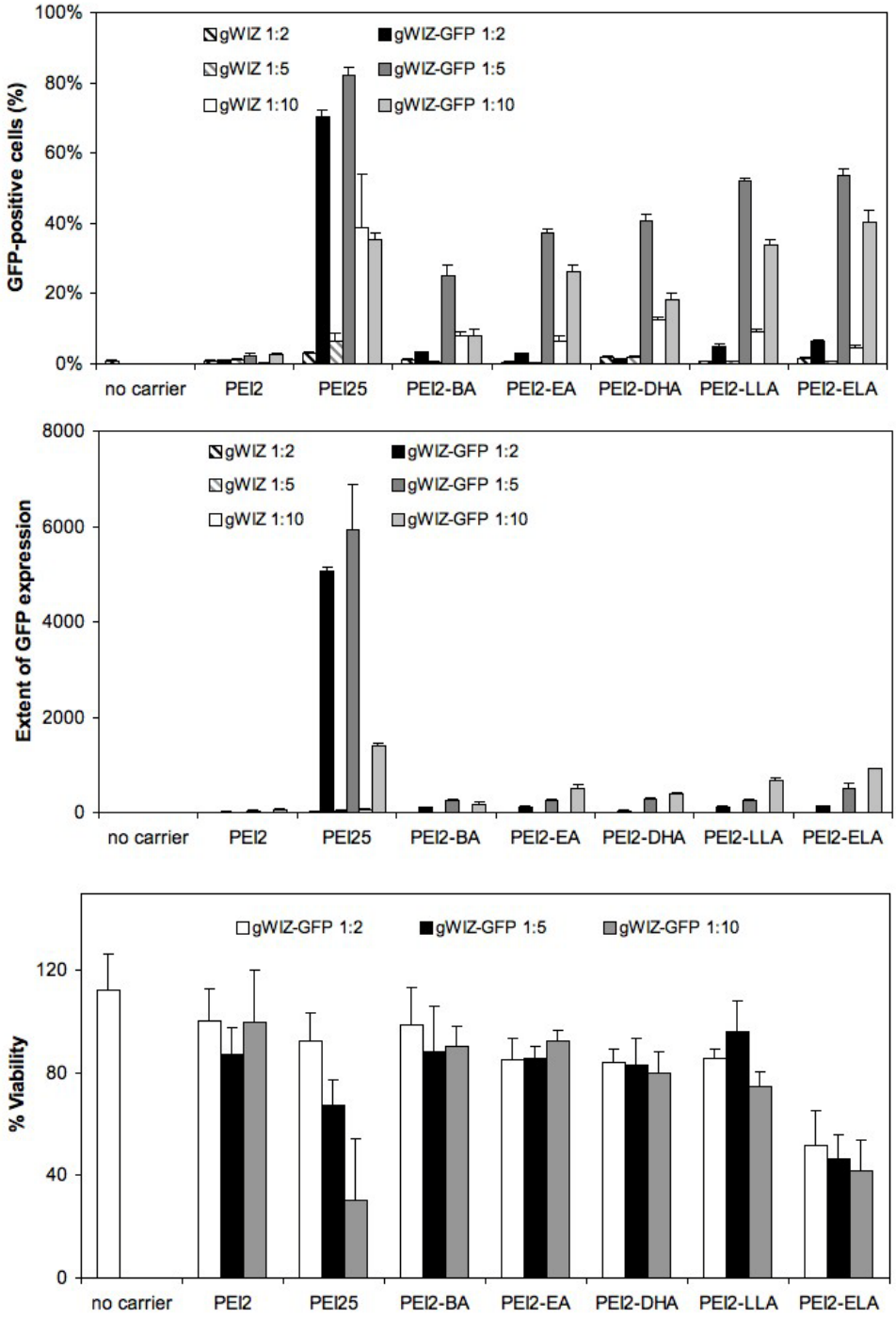



**Figure 5.6.** Transfection efficiency of lipopolymers at different plasmid/polymer ratios (w/w). (A) Percentage of GFP-positive cells. (B) Mean fluorescence of the GFP-positive cell population. The 293T cells were incubated for 24 hrs with complexes, after which medium was replaced and the cells were analyzed by flow cytometry 24 hrs later. (C) Cytotoxicity by MTT assay after 24 hrs of complex treatment. The cell viability was expressed as percentage of untreated cells (no carrier), mean + SD of triplicate wells.

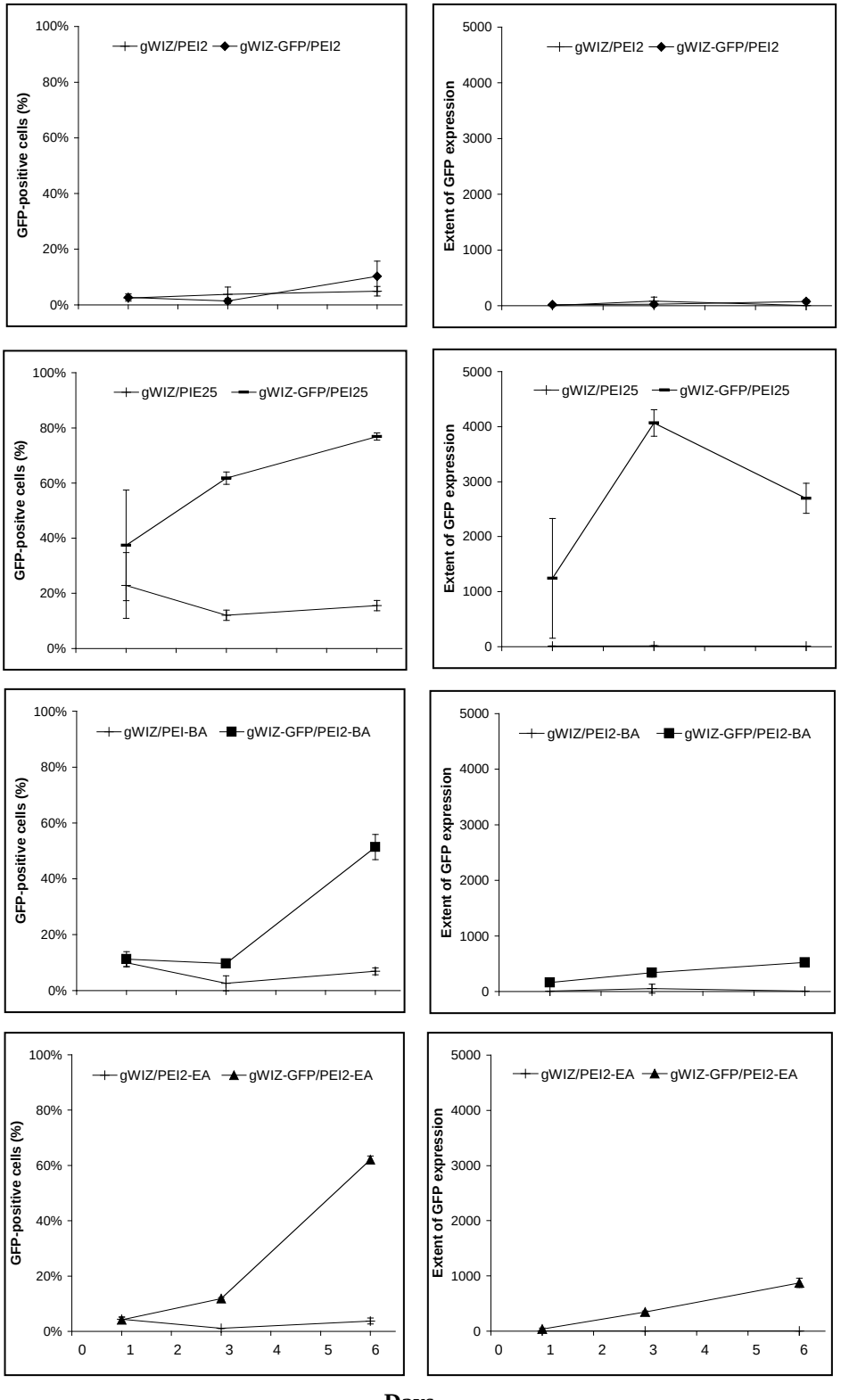
viability[22]. Kim[23] *et al*., also improved the transfection efficiency of PEI2 in 293 cells by coupling acyl C14 to PEI2. However, the resultant lipopolymers were more toxic than the native PEI2. Thomas[22] *et al*., observed that the increased efficiency was related to the increase in the number of lipid substituted in PEI. Other groups also observed similar results; increasing acetylation on PEI25 (up to 57%) enhanced the transfection efficiency as compared to the native PEI25 in MDA-MB-231, HEK293 and C2C12 cells without affecting cell viability. Further acetylation, despite increasing the plasmid uptake, resulted in lower level of gene expression. This was attributed to decreased buffering capacity of the polymers, inhibiting endosomal release. Cellular proteins or endosomal membranes could have also dissociated the polyplexes more easily in this case, leaving unprotected plasmid DNA in the cytosol[15,24]. Increasing the length of the acyl group (from C2 to C4) and the degree of acylation on PEG-PEI750 also improved transfection efficiency in COS-1 cells[11]. Particularly, 30% of modification proved to be the most efficient carrier, but higher modifications (e.g., 40-50%) decreased the gene delivery properties as before. Although these substitutions decreased the pKa, buffering capacity and cationic nature of the carrier, they increased the lipophilicity for cell membrane interactions and inhibited aggregation in some cases needed for optimal particle delivery. Additionally, cytotoxicity experiments indicated that the cells were not affected by exposure to the complexes even after 48 hrs.

In contrast, our previous studies that employed a larger number of lipids[9] showed no clear effect of the number of lipid substituted on PEI2 (with C8, C14, C16, C18, C18:1 and C18:2 lipids) on the obtained transfection efficiency. Longer lipids enhanced the transfection efficiency as compared to the C8 and, particularly linoleic acid (C18:2) substitution was most beneficial in 293T cells. This enhancement could be attributed to the presence of double bonds on this lipid, which was thought to increase mobility for membrane-crossing molecules by reducing the crystallinity and phase transition temperature of the membrane[25]. Despite the high cellular uptake of plasmid observed in rat BMSC with C16- and

C18:2-substituted PEI2 (which was equivalent to uptake in 293T cells), these lipopolymers lead to ineffective gene expression in rat BMSC. This result suggests that the efficiency of these carriers did not only depend on the particle size or the net charge of complex, but also the nature of the internalizing cell type[26].

The transfection ability of the current lipopolymers was tested in 293T cells at different DNA/polymer weight ratios. As expected, incubating gWIZ-GFP plasmid alone (i.e., without a carrier) with the cells did not give any distinct fluorescence (**Figure 5.6**). GFP-positive cells were observed with the complexes of all lipopolymers, but not the native PEI2 (i.e., 1-2% at various DNA/polymer ratios). The maximum transfection was obtained at the DNA/polymer ratio of 1:5; up to ~50% of cells were positive on day 3 for lipopolymers with marginal background for control gWIZ complexes (i.e., <1.8% for lipopolymers and ~6% for PEI25; **Figure 5.6A**). Increasing the DNA/polymer ratios, despite not affecting the physical properties of the complexes in an obvious way, actually resulted in a substantial decrease in GFP-positive cells with all lipopolymers. Significant cell autofluorescence by the blank gWIZ/polymer complexes was also manifested at high DNA/polymer ratio, particularly for PEI2-BA, PEI2-DHA and PEI25. However, the mean fluorescence of the cells population showed that this effect was minimal. The PEI25 was more effective than the lipopoymers at 1:2 and 1:5 DNA/polymer ratios, where >60% GFP-positive cells (**Figure 5.6A**) and a mean GFP fluorescence of 6-7 fold higher (**Figure 5.6B**) were obtained. However, at the higher ratio of 1:10, the autofluorescence was quite high (most noticeable in the percentage of GFP-positive cells) and was likely due to increased toxicity of the complexes at this ratio (see **Figure 5.6C**).

The duration of gene expression was next investigated at the optimum DNA/polymer ratio of 1:5. The 293T (**Figure 5.7**) and h-BMSC (**Figure 5.8**) were treated with the complexes and GFP expression was analyzed on day 1, 3 and 6. On day 1, no distinguishable difference between cells treated with


GFP-positive cells (%)
Extent of GFP expression
gWIZ/PEI2
gWIZ-GFP/PEI2
gWIZ/PIE25
gWIZ-GFP/PEI25
gWIZ/PEI25
gWIZ/PEI-BA
gWIZ-GFP/PEI2-BA
gWIZ/PEI2-BA
gWIZ/PEI2-EA
gWIZ-GFP/PEI2-EA
Days

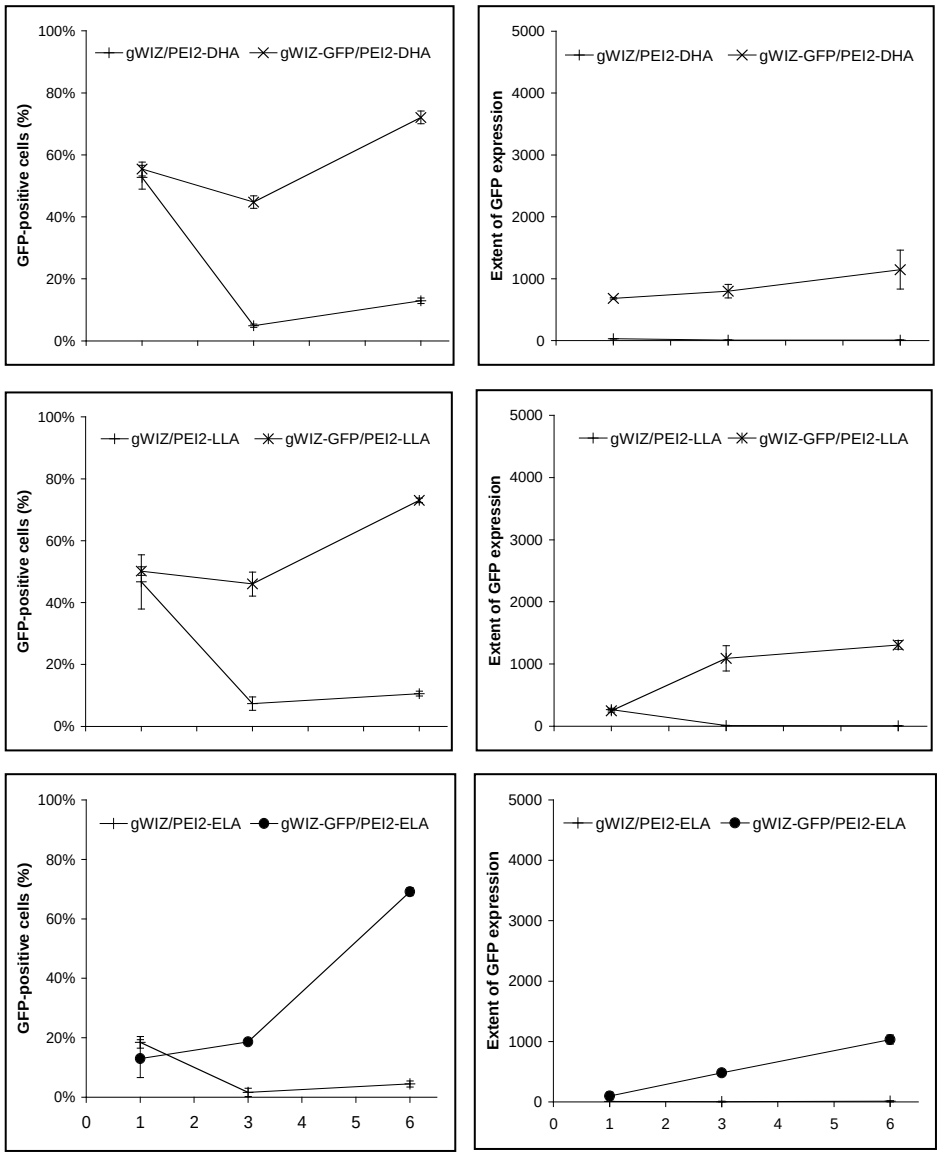



**Figure 5.7.** Time-dependent transfection effect with lipopolymers in 293T cells. Percentage of GFP-positive cells (left column), mean fluorescence of GPF-positive cell population (middle column) and cell concentration of samples (right column) was assessed by flow cytometry. Complexes at DNA/polymer ratio of 1:5 μg/mL were prepared and incubated with the cells for 24 hrs. Culture medium was replaced and cells were further incubated for 3 and 6 days.

gWIZ/polymer and gWIZ-GFP/polymer complexes was observed in both cell lines. Further incubation led to a reduction of autofluorescence background (as evident by a decreased %GFP-positive cell count) and significant gene expression was observed in 293T cells only. As before, PEI2 was not effective in this experiment and PEI25 gave >60% GFP-positive cells where the mean fluorescence of the cells peaked on day 3. Most lipopolymers attained the same level of GFP-positive cells by day 6, in particular with highly unsaturated *cis* DHA, LLA and ELA *trans* double bond lipids (72, 73 and 69% respectively; **Figure 5.7**). However, the mean fluorescence of the cells treated with the lipopolymers were ~4-fold lower at day 6 compared to the cells treated with PEI25 complexes. Since the lipopolymers developed are of larger size it is possible that the increase on transfection efficiency was related to complex sedimentation as described before for native PEI[27]. Alternatively slower dissociation of the internalized complexes might have led to more gradual expression of the GFP transgene.

A similar experiment was conducted with h-BMSC, but only PEI2-LLA was evaluated as a prototypical lipopolymer, given tits successful performance, in 293T cells. In contrast to 293T cells, no difference was observed between the cells treated with gWIZ/polymer and gWIZ-GFP/polymer complexes at any time point (**Figure 5.8**). This was the case even with PEI25 that was clearly effective in 293T cells. The %GFP-positive cells were generally low in h-BMSC (<20%) and similar for cells treated with both types of complexes. The mean GFP fluorescence in h-BMSC was only a small fraction of the 293T cells: ~100 au for h-BMSC vs. ~4000 au for 293T cells. These results clearly show that certain biological barriers exist in h-BMSC that prevents active transgene expression. These results were also in line with confocal microscopic observations where little plasmid, if any, was observed in the nucleus. Occasionally GFP positive h-BMSC was observed (**Appendix B**) but only rarely (i.e., ~1% of population).

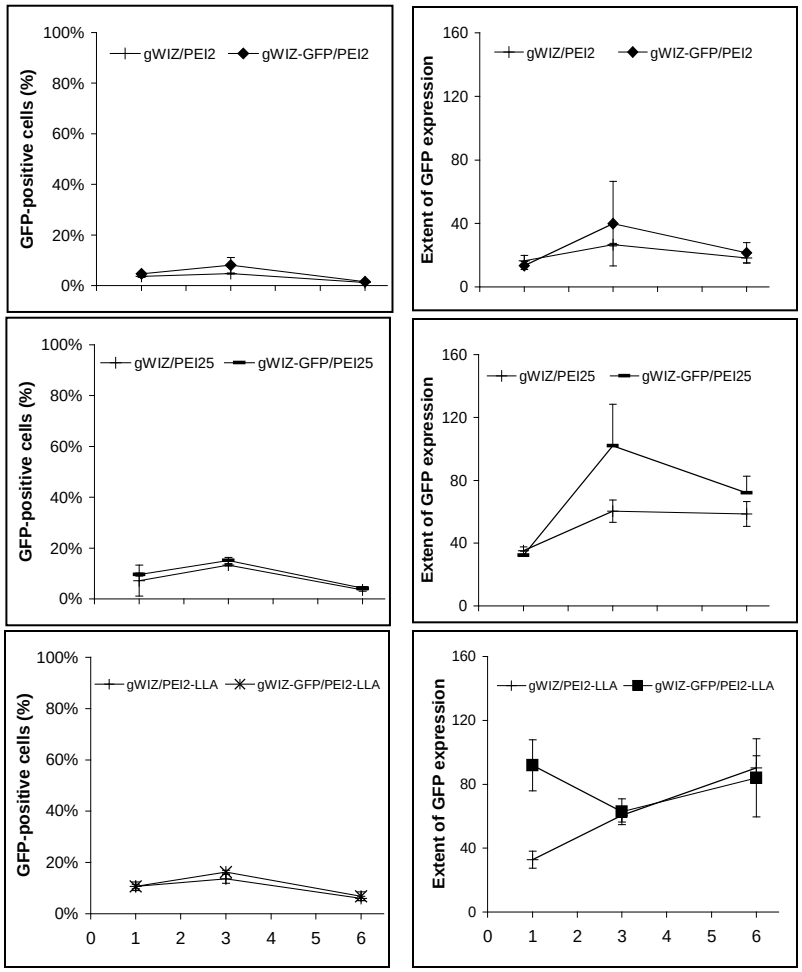



**Figure 5.8.** Time-dependent transfection of h-BMSC with PEI2, PEI25 and PEI2-LLA complexes. Percentage of GFP-positive cells (left column), mean fluorescence of GPF-positive cell population (middle column) and cell concentration (right column) was assessed by flow cytometry. Complexes at DNA/polymer ratio of 1:5 μg/mL were prepared and incubated with the cells for 24 hrs. Culture medium was replaced and cells were further incubated for 3 and 6 days.

Despite of the high cellular uptake observed in both 293T cells and h-BMSC, only a small percentage of h-BMSC expressed the reporter gene. Cells internalize complexes through a variety of pathways. It is thought that complexes of DNA/PEI complexes are taken up by the cells via endocytosis[28,29]. However, the pathway and mechanism of the complex transit or the freed DNA from the cytoplasm into the nucleus is unknown. Bieber[30] *et al.*, demonstrated that complexes with PEI carrier are entrapped in the lysosomes. Moreover, the limiting step in the transfection of PaTu 8902 cells was the transfer of complexes

from the lysosome to the nucleus, rather than the endocytotic uptake or transcription of DNA complexes. Apparently, high MW PEI complexes exhibited sufficient local membrane damage to release the complexes into the cytoplasm compared to low MW PEI. Cells damaged in this way accumulated high concentrations PEI complexes in the cytoplasm as well as in the nucleus. This could possibly explain the low transfection values observed with treated h-BMSC. The complexes with low MW PEIs could not rapidly escape the lysosomal entrapment and avoid possible enzymatic degradation, impeding the complex transit and eventually gene expression. This suggests that the ability of the carriers to employ the appropriate intracellular pathways in order to reach the nucleus is dependent upon the cell type as seen by others[31].

### 5.3.6. Transfection and Cytotoxicity with Chemical Modulators of Endocytic Pathway

Endocytosis has been established as the main mechanism for internalization of polymer complexes into the cell. The most common pathways include clathrin- and caveolin-mediated endocytosis, cholesterol-mediated lipid rafts, phagocytosis and macropinocytosis. These pathways differ in the size and fate of the internalized particles as well as the composition of the vesicle coat, if any[26]. Chemical drugs that inhibit various steps in the endocytosis-mediated uptake and intracellular trafficking, have been the main tool used for characterizing the entry pathways of the gene/carrier complexes. However, the specificity of these drugs is not always certain and their functionality is mostly dependent on the type of cell employed as well as the dose of the drug employed, so that the results should be interpreted with caution[21,32].

Chloroquine was initially employed in this study to evaluate if the endosomal release of the complexes was an impediment to achieve robust gene expression. This was considered a significant issue especially for h-BMSC, which did not exhibit any transgene expression despite significant plasmid DNA uptake. This

drug is not only known to facilitate endosomal release of complexes by causing osmotic swelling of endosomes, but also (i) to protect DNA from intracellular nuclease degradation and (ii) to interact electrostatically with DNA, helping to dissociate it from the carrier[33]. The transfection with plasmid alone and with PEI2 complexes was not affected by chloroquine in 293T cells (**Figure 5.9A**). A positive effect of chloroquine was observed in 293T cells at the highest drug concentration (50 μM) for only PEI2-BA and PEI2-ELA lipopolymers; both the GFP-positive cells and mean fluorescence of the cell population was increased in these cases (**Figure 5.9A**). The chloroquine helped the lipopolymers modified with saturated BA and unsaturated ELA *trans* double bond lipids, which are believed to perturb membranes to a lower extent as compared to *cis* double bond lipids[25]. It appears that endosomal release might be limiting with the carriers incorporating these lipids, but not limiting for the other lipids. However, the effective chloroquine concentration (50 μM) reduced the cell numbers in culture (as assessed by flow cytometry), indicating some toxicity due to the drug itself. Lower chloroquine concentrations (10 and 25 μM) seemed to be beneficiary with the h-BMSC, where all polymers exhibited a small improvement of gene expression (**Figure 5.9B**) without compromising the cell number (note that toxicity was again evident in h-BMSC with 50 μM chloroquine concentration).

A. 293 cells B. h-BMSC

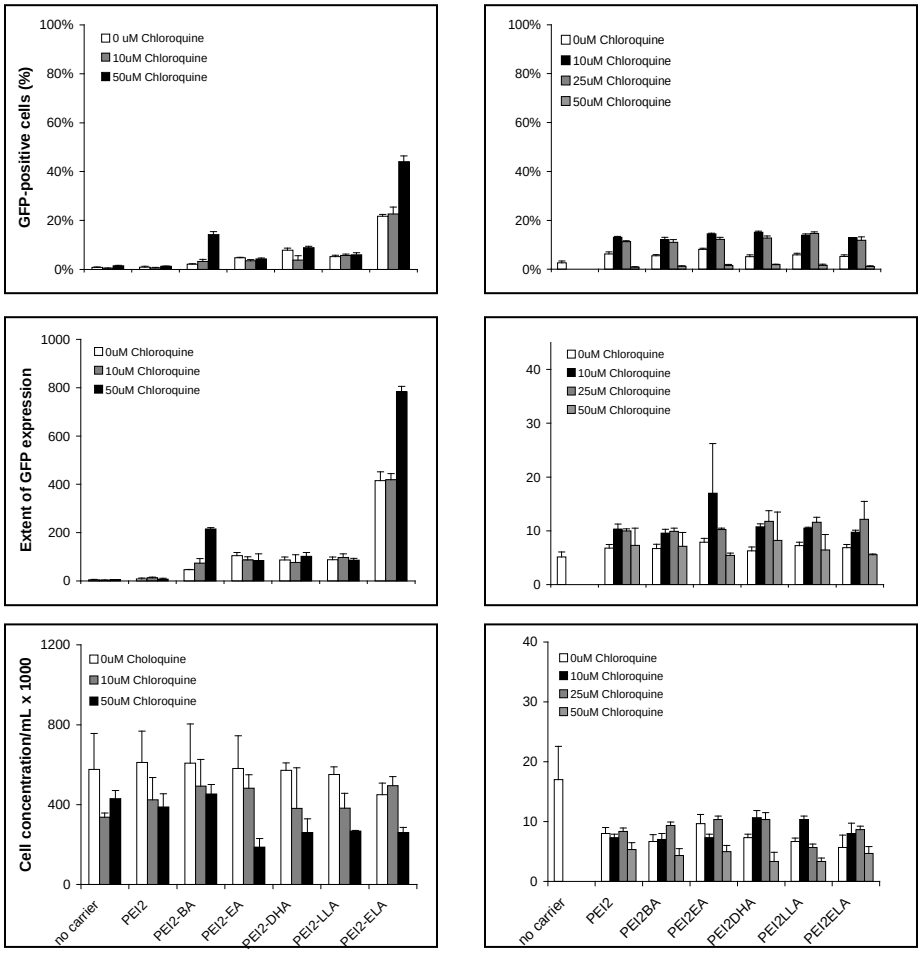



**Figure 5.9.** Effect of chloroquine on transfection efficiency with (A) 293T cells and (B) h-BMS cells, transfected at DNA/polymer ratio of 1:5 (1:5 μg/mL). Cells were incubated with the drug at the indicated concentrations for 30 min after which complexes were added and incubated for 24 hrs further. Culture medium was replaced and cells were further incubated for additional 24 hrs. Percentage of GFP-positive cells, mean fluorescence of GFP-positive cell population and cell concentration per well was assessed by flow cytometry.

The increase obtained with chloroquine, however, was relatively marginal (especially with mean GFP expression) so that the chloroquine treated h-BMSC did not attain similar levels of transfection as PEI25 treated 293T cells.

Rejman[18] *et al.*, reported that complex internalization by clathrin-dependent endocytosis is limited to <200 nm particles (i.e., size range obtained with PEI25 complexes) and caveolin-dependent endocytosis prevails for bigger particles of

200-500 nm (i.e., size range obtained with lipopolymer complexes). To better elucidate the endocytic pathway of the lipopolymer complexes, three chemical inhibitors of specific endocytic processes (genistein, chlorpromazine and MβCD) were employed to determine if discrete pathways was beneficial in successful transfection. The 293T cells were pre-incubated with the chemical inhibitors and the gene expression was subsequently evaluated (since transfection was not successful in h-BMSC, the assay was not performed with that cell line). The carriers chosen were PEI2, PEI25 and PEI2-LLA, which gave the highest cellular uptake and gene expression among the lipopolymers. These drugs have shown to cause morphological effects and toxicity even with short incubation times (e.g., ≥ 2 h)[32]. To reduce such effects and in particular any possible autofluorescence, we quantified GFP expression 24 h after removing the inhibitors and complexes from the cells.

A cytotoxicity study was first conducted to optimize the concentrations of chemical modulators; the cell viability was investigated by the MTT assay after 24 h exposure to the modulators (**Figure 5.10**). Even though the drugs concentration used for these experiments were far lower (i.e., genistein < 200 μM, chlorpromazine < 10 μg/mL and MβCD < 10-50 mM) than the concentrations used for other cell lines[26,27,32], a cytotoxic effect was observed in our hands. Cells

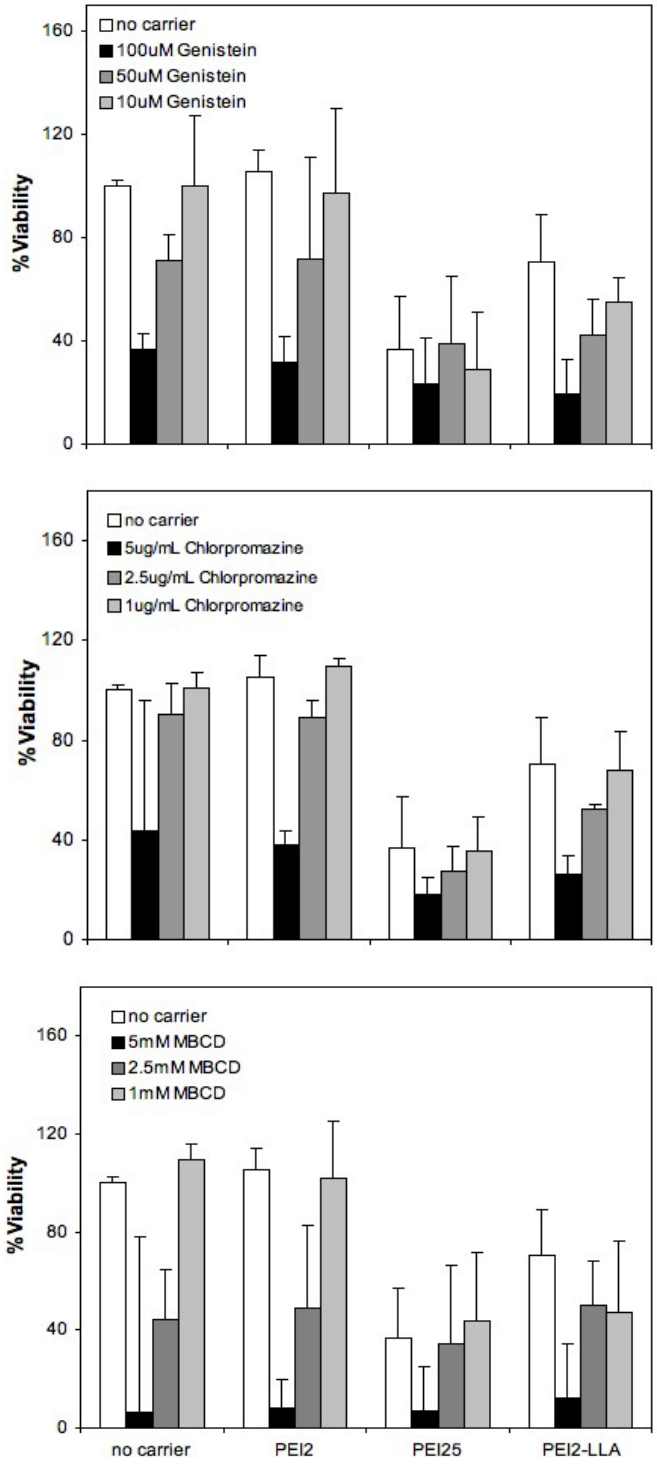



**Figure 5.10.** Cytotoxicity of 293T cells incubated for 90 min with endocytosis inhibitors prior to treatment with complexes (DNA/polymer ratio of 1:5 μg/mL). MTT assay was assessed 24 hrs post-complex incubation and cell viability was expressed as a percentage of untreated cells (no carrier), mean + SD of triplicate wells.

treated with the highest drug concentration, 100 and 50 μM of Genistein, 5 μg/mL of Chlorpromazine and 5, 2.5 mM of MβCD showed high cytotoxicity. The combination of the complexes with inhibitors increased this effect, an observation previously noted for polymeric gene delivery systems[26]. This effect was most evident on cells treated with MβCD/polymers; even at the lowest MβCD of 1 mM, all drug/polymer incubation reduced cell viability. Herein, similar results were observed with genistein/PEI2 or PEI25 treatment. Cells treated with chlorpromazine at 2.5 and 1 μg/mL showed a reduced toxicity even in the presence of polymers, particularly for PEI2 and PEI2-LLA.

Despite the observed cytotoxicity effect observed at these drugs concentrations, the effect of the drugs on transfection efficiency suppression was evaluated. Treatment with 10-100 μM genistein, an inhibitor of caveolin-dependent endocytosis, did not result in a reduction in transfection at any concentration for PEI2 and PEI2-LLA. Theses observations contradict Rejman's results, where particles between 200-500 nm were suggested to undergo caveolin-dependent endocytosis. Only PEI25 was marginally affected (i.e., mean fluorescence was reduced without changes in %GFP-positive cells, **Figure 5.11**). The clathrin-dependent endocytosis inhibitor chlorpromazine reduced the GFP-positive cells at 5 μg/mL for PEI 25 complexes. This suggests that clathrin-dependent endocytosis was involved only with PEI25-mediated transfection process, as expected given the size of the complex. However, it is important to mention that the cell concentration was reduced with this drug treatment. The manifested cell toxicity was not likely the reason for increased GFP expression, since the higher concentrations of the drugs led to increased toxicities without influencing the GFP expression (**Figure 5.11**). It is possible that extending the incubation time might have allowed the cells to better recover from the treatment and better reveal the effect of the chlorpromazine.

Treatment with MβCD, an inhibitor of cholesterol-dependent endocytosis, reduced gene expression in a dose-dependent fashion. This compound forms

water-soluble inclusion complexes with cholesterol, causing the removal of cholesterol from the cholesterol-phospholipids-glycolipid rich lipid rafts in plasma membrane[34,35]. A remarkable reduction in GFP-positive cells was observed at 5 mM MβCD for PEI25 (~54%), while a much smaller reduction (7%) was observed for PEI2-LLA and for both carriers cell viability was compromised. This suggests that the endocytosis of PEI25 and, to a lesser extent, PEI2-LLA appeared to be dependent on the integrity of the lipid rafts. The importance of lipid rafts for PEI25 mediated transfection was noted before[36]. It appears that the PEI2-LLA entry into the cells was not as much dependent of lipid rafts, possibly due to increased hydrophobicity the complexes that may facilitate plasma membrane penetration of complexes. However it is important to mention that the possibility of inhibited gene expression observed with MβCD was because of the cytotoxicity effect instead of a specific inhibitory effect as observed before with other cell lines[32].

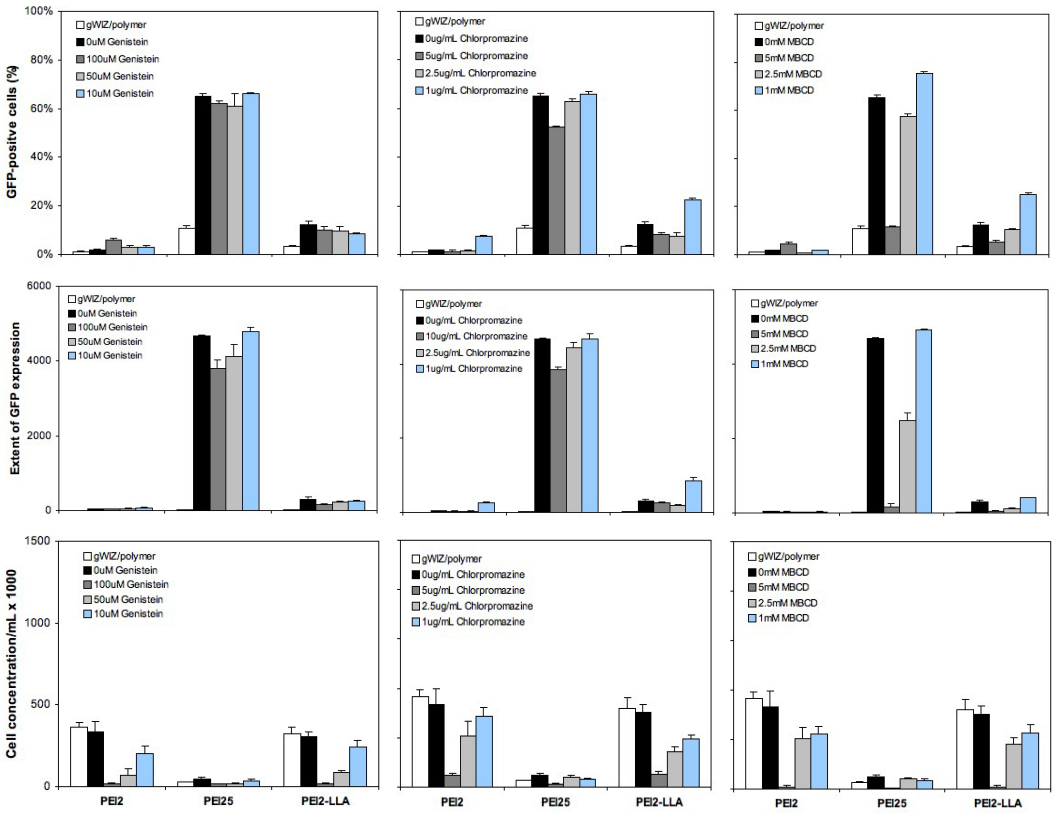



**Figure 5.11.** Effect of endocytosis inhibitors genistein (left column), chlorpromazine (middle column) and MßCD (right column) on transfection. The 293T cells were incubated with the indicated drugs for 90 min prior to complex uptake, then the complexes (DNA/polymer ratio of 1:5 μg/mL) were added and incubated for 24 hrs. Culture medium was replaced and cells were further incubated for additional 24 hrs. Percentage of GFP-positive cells, mean fluorescence of GFP-positive cell population and cell concentration per well was assessed by flow cytometry.

## 5.4. Conclusions

We have successfully modified low MW (2 kDa) PEI with fatty acids of long (>18C) acyl saturated and unsaturated chains. Gene delivery and expression by these lipopolymers was found to be dependent on the cell type: only 293T cells were able to process the complexes in an appropriate fashion, leading to high levels of transfection whereas h-BMSC did not. This was the case even though the cellular uptake of plasmid DNA in h-BMSC was as high as the 293T cells. Increasing the polymer content in complexes did not affect the physical properties of the complexes (i.e., size and ζ-potential), but compromised the cell viability significantly. The highest transfection efficiency was found with highly

unsaturated (3-6 double bonds) lipid-substituted PEI2. Internalization studies indicated that effective transfection followed both clathrin and cholesterol-mediated endocytosis in 293T cells, and lipid-substituted PEI2 did not rely on lipid rafts on cell membrane as much as the PEI25 for effective transfection. Our results suggest that the uptake pathway mediating successful gene expression depends on the cell line and type of PEI. We conclude that the chosen fatty acids were not as advantageous as the shorter fatty acids (C14to C18) previously used for PEI2 substitution. It is possible that increasing the substitution ratio on the polymers might lead to further beneficial effects, but better control of the chemical grafting scheme is needed to this end.

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# 6. General Conclusions and Future Directions

The widespread application of gene therapy depends on development of suitable methods for gene delivery. In fact, the greatest obstacle in the field revolves around the engineering of appropriate gene carriers[1]. Current carriers battle different problems: for example, lack of cell targeting *in vivo*, inefficient long-term expression, and low transfection rates[2]. Additional considerations include the capacity of the vector to package genes of sufficient size and the vector's immunogenicity. Therefore, an ideal gene therapy delivery system would be injectable, targetable to specific sites *in vivo*, regulatable (i.e., control expression levels of target genes at will), and able to maintain long-term gene expression, and finally, be immunologically compatible[1]. Delivery systems currently used for gene therapy purposes can be broadly divided into viral and nonviral vectors.

The focus of this thesis was on the design of nonviral carriers, in particular cationic polymers, which have been used for gene delivery since the late 1980s[3]. Cationic polymers such as PLL and PEI contain high densities of primary amines, which are protonated at neutral pH. This high density of positive charges allows the cationic polymers to form stable complexes with genetic payloads (e.g., plasmid DNA). The cationic polymers self-assemble with DNA to generate condensed structures of variable sizes capable of entering the cell. These polymers vary widely in their structures, which range from linear to highly branched molecules and influence their complexation with nucleic acids and their transfection efficiency. In addition to providing positive charges for DNA complexation, the primary amines also serve as functional groups to chemically modify the polymers with ligands (i.e., lipids) that can enhance the outcome of one or more of the steps in the transfection process.

Modifications of cationic polymers with lipid groups have been employed to facilitate cellular delivery, given the lipophilic nature of cell membranes. To design effective gene carriers that will modify primary BMSC *in vitro* (an

important cell phenotype for clinical gene therapy), PEI and PLL were substituted with PA via amide linkages. Depending on the reaction conditions, PEI and PLL were substituted with 2.2-5.2 and 13.4-6.2 PA per polymer chain, respectively (**Chapter 3**). The PA substituted polymers displayed slightly lower binding efficiency compared to native polymers towards a plasmid containing pEGFP-N2 in an agarose gel-binding assay. The cell binding of PLL-PA, but not PEI-PA, was particularly enhanced, resulting in a high percentage of cells displaying significant polymer uptake. Cytotoxicity of PEI-PA was not altered compared to native PEI, while PA substituents on PLL reduced the toxicity of the polymer. pEGFP delivery into the BMSC was also significantly increased with the PLL-PA (vs. PLL), but not PEI-PA (vs. PEI). The transfection efficiency of PLL-PA was significantly higher (~5-fold) than the unmodified polymer. Notably, PLL-PA mediated more transgene expression than native PEI liposomal carriers (Fugene$^{TM}$ and Lipofectamine-2000$^{TM}$) or an adenoviral carrier (GFP-Av) one day after transfection. However, further incubation time (7 days) lead to an equivalent level of transfection for all the carriers used. Adding PA to PEI did not improve transfection capabilities of the carrier, suggesting that the success of the lipidation approach is dependent on the carrier to be modified. Therefore, we conclude that PA substitution on PLL provides an effective carrier for transfection of primary cells derived from the bone marrow[4].

Given the success obtained on PLL-PA, we further modified PLL with several endogenous lipids of variable chain lengths (lipid carbon chain ranging from 8 to 18 saturated and unsaturated) in order to identify other possible lipids that can enhance gene delivery properties of the native carrier. We imparted an amphiphilic property to PLL by substituting ~10% of the ε-$NH_2$ with lipids. Lipid-modified PLL of high molecular weight (~25 vs. 4 kDa) were found to be more effective in delivering plasmid DNA intracellularly in BMSC (**Chapter 4**). For lipid-substituted 25 kDa PLL, a correlation between the extent of lipid substitution (no. of lipid substituted times no. of carbon in the lipid chain) and the pEGFP-N2 delivery efficiency was observed. Additionally, transgene expression

by BMSC significantly increased (20-25%) when amphiphilic PLLs were used for plasmid delivery as compared to native PLL and the commercial transfection agent Lipofectamine-2000$^{TM}$. The transfection efficiency of the polymers was positively correlated with the extent of lipid substitution. The amphiphilic polymers were able to modify the cells up to 7 days after transfection, after which the expression was decreased to background levels within a week. We noted that C14-, C16- and C18-substituted PLL gave the most effective DNA delivery, but this was likely because of the high substitution ratios obtained with these lipids (~10 lipids/PLL). We speculate that other lipids can be as effective if they are substituted at equivalent levels. We conclude that lipid-substituted PLL can be used effectively as non-viral carrier for DNA and the extent of lipid substitution was important for effective gene delivery[5].

It is important to note that the studies carried out in **Chapter 3** and **Chapter 4** could be further improved with a better experimental design. The transfection experiments were performed with pEGFP/polymer complexes and normalized against cells treated with saline solution or pEGFP only. They did not include a transfection group with blank plasmid and its carrier complexes. A blank plasmid is one that mimics EGFP plasmid with the same frame but without the nucleotides that encode for GFP. Adding these 'blank' complexes to the studies would have provided a better comparison between the fluorescence level of the GFP-expressing cells and the background, since treating the cells with blank complexes were subsequently shown to lead to an autofluorescence signal without actual EGFP expression. The approach described above was implemented in the studies described in **Chapter 5,** where transfection of 293T and BMSC with a mock plasmid gWIZ and its counterpart gWIZ-GFP was combined with the carriers and compared against each other.

Given the successful transgene expression obtained with low molecular weigh (MW) PEI modified with the same series of lipids described above[6], the last research chapter of this work (**Chapter 5**) was directed towards the modification

of 2 kDa PEI with longer lipids; saturated fatty acid (BA, C22), trans fat (ELA, C18:1T) and essential fatty acids (EA, C22:1; DHA, C22:6 and LLA, C18:3). Transfection efficiency with these carriers proved to be cell dependent. Only 293T cells were able to express the GFP, but not BMSC. The PEI modified with highly unsaturated lipids resulted (LLA and DHA) in efficient carriers, which where able to increase transgene expression overtime (6 days). However, the level of transfection obtained from the carriers was less efficient than the highly efficient 25 kDa PEI. As expected, the latter displayed significant toxicity but the lipopolymers were more favourable in this respect. Furthermore, the perturbation of the mechanisms of endoytosis using specific drugs led to propose the possible endocytic pathway that lipid-substituted carriers might take in order to efficiently transfect 293T cells. Complexes of PEI25 utilized the cholesterol-mediated and clathrin-mediated endocytosis as reported by others[7,8], whereas PEI2-LLA complexes did not follow any endocytosis pathway and the complexes possibly penetrated the plasma membrane directly.

Throughout the chapters of this thesis, we show that lipid modification of PLL and PEI is an efficient route for constructing carriers that could potentially be used as gene delivery carriers. However, there is still room for improvement, since these carriers do not ensure site-specific gene expression in the cells. The described carriers lack moieties that could give targetability to specific cell types, internalization follow by endosomal escape and nuclear transport. The incorporation of additional functionalities (i.e., receptor-binding domains, endosomolytic or nuclear targeting moieties) into the polymer can create specific interactions with the cell surface, target a particular internalization pathway, provide endosomal escape or improve nuclear localization. Although we have previously conjugated a small peptide RGD (arginine-glycine-aspartic acid peptide) to native PEI (branched 25 kDa) without successfully enhancing gene expression in primary cells (BMSC)[9], we have not explored the ability of other moieties and the new derived polymers. Conjugation of folate[10,11], transferring[12,], epidermal growth factor[13,14], nuclear localization sequences (NLS)[15,16] to PLL and

PEI are among the possibilities for this purpose.

In **Chapter 5** we observed that despite of the high cellular uptake of the complexes, transgene expression was minimal in h-BMSC. The derivates of PEI carriers seem to remain in the endocytic vesicles of the cells limiting their ability to deliver their nuclear active cargo. Therefore, functionalization of these carriers with membrane lytic peptides could enable efficient release of the DNA payloads into the cytoplasm. For this purpose, conjugation of HGP (15-amino acid peptide derived from HIV gp41)[17] peptide to PEI were recently shown to enhance transgene expression compared to native PEI. Moreover, conjugations with melittin[18] have shown to endow nuclear homing activity as well as endosomolytic action enhancing transfection efficiency in a broad range of cell lines (tumor cells, primary hepatocytes and human umbilical vein endothelial cells). Another strategy to facilitate DNA release is to include reducible crosslinkers like disulphide linkages in the polymer chain. Disulphide bonds are quickly cleaved inside the cells by the reducing enzymes such as glutathione reductase or sulphydryl species, leading to a rapid dissociation of the DNA/polymer complex, hence releasing the DNA. This method has been applied on PLL[18] and PEI[19,20], and resulted in improved gene carrier properties as well as diminished cytotoxicity when compared to native polymers. Furthermore, conjugation of PLL and PEI with labile thioester linkages could be an additional alternative for the construct of effective gene release. Thioester are common intermediates in many biosynthetic reactions. They are involved in the synthesis of all esters, including the formation and degradation of fatty acids, peptides, sterols and others[21]. Lipid attachment through amide linkage (N-acylation) results in stable linkages, in contrast attachment through thioester linkages (S-acylation) are meta-stable and usually display a half-life of hours[21]. Therefore, we believe that the introduction of the lipids described before (**Chapter 4 and 5**) through a degradable thioester linkage (C-S-CO-C) instead of amide linkage (C-NH-CO-C) would generate carriers (i.e., PLL and PEI) with an easier and faster release property from the cytosolic compartments enhancing their gene delivery efficacies. This reasoning

could be used for the native or modified polymers described in this work as they could improve their delivery properties if specificity is gained, hence taking them from the bench work to an in vivo application.

A final consideration is the proliferation rate of target cells, which can play a major role in whether the complexes gain access to the nucleus. In general, during cell division nuclear envelope breaks down and reforms allowing the complexes or freed DNA to diffuse into the daughter's cell nucleus and eventually transcription takes place. This process occurs very slowly in primary cells than transformed cell lines[22], which may account for the lower transfectability of the former as seen with h-BMSC compared to 293T cells (**Chapter 5)**. Therefore, to maximize the efficacy of the carriers, the robust internalization pathway should be considered during the carrier design process and mechanisms facilitating nuclear entry in non-dividing (or slowly dividing) cells should be incorporated into the carriers.

## 6.1. References


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# Appendix A

This section is intended to update the literature on hydrophobic modifications of cationic polymers, in particular PEI. A brief discussion of the progress of lipid-modified PEI, such as gene encapsulation, adsorption to cell membrane, serum inhibition, gene dissociation, cytotoxicity, and gene or tissue-targeting on recent studies (2009-2010) was addressed. The structure-function relationships discussed here provided important information and insight for improvement of these exciting gene carriers. As described in this work, most studies have shown that incorporation of hydrophobic chains can improve gene delivery efficiency, mainly explained by (i) hydrophobic interaction conferred to the resulting amphiphilic polycation derivatives and (ii) enhanced cellular uptake by the hydrophobic chains via the lipophilic cell membrane.

In 2009, Aravindan L[1] *et al*. found that increasing acylation of 25 kDa PEI using acetic (C2) or propionic (C3) anhydride reduced the plasmid DNA/polymer interaction, surface charge and buffer capacities because of the reduce number of amines available for protonation and binding. However, reduction in the concentration of amine groups helped to facilitate complex dissociation inside cells, which increased gene transfection compared to native PEI. Particular ~70% amine modifications in PEI by C3 formed small particle size of ~130 nm with a positive net surface charge (compared to ~107 nm with unmodified PEI). This carrier showed better membrane interaction, optimal binding strength with plasmid DNA, more hemacompatible, less cytotoxic and was a superior transfection agents with HEK 293 cells compared to C2-modified and native PEI polymers. Hydrophobic chains also improved the delivery properties of low MW (800 Da) PEI. PEI modified with alkyl acrylates of 6 and 12 carbons improved complex stability and exhibited good binding affinity to siRNA, particular C6-modified PEI showed significant lytic properties compared to shorter C2- or larger C12-modified PEI[2]. C2-, C4- and C12-modified PEIs were quite unstable against aggregation and either precipitated during preparation or was not internalized because of the large size of the particles. Moreover, 10 residues of C6

on PEI were able to induce efficient knockdown bioactivity of transfected Neuro2A/EGFPLuc cell compared to lower degree of C6-modification, C2-, C4-, C12-modified PEI. It gave comparable results to commercial transfection agent Lipofectamine 2000. Importantly, the formulations caused significant knockdown even in the medium with high content of serum. However, C6-modified PEI decreased cell viability; therefore further formulations with other carriers such as lipids or C12-modified PEI were needed to reduce the cytotoxicity effect. In the same line, increasing the hydrophobic chain length beyond C6 on high MW (25 kDa) PEI reduced transfection efficiency and increased toxicity with CHO-K1 cells. Grafting 12.5% of 25 kDa with C12 formed larger and unstable complexes size of 1800 nm with plasmid DNA[3] to less hydrophobic side groups such as cellobiose (polyalcohol containing 12 carbons in backbone with hydroxyl groups) and low MW PEG.

In contrast, increasing the hydrophobic chain length to 18 carbons on 25 kDa PEI effectively delivers siRNA in B16 melanoma cells[4]. Both native and modified PEI formed complexes of ~110 nm and displayed positive net surface charge. Compared to native PEI, C18-modified PEI showed higher potency in STAT3 (signal transducer and activator of transcription 3) silencing accompanied by a significant induction of IL-6 secretion and a reduction of VEGF (vascular endothelial growth factor) production. Moreover, with C18-modified PEI complexes, the level of the cellular Caspase 3 activity (an indicator of apoptotic activity) was found to be 2.5 times higher than that of PEI complexes. Consistently, in vivo results indicated significant regression in tumor growth and tumor weight after siRNA/C18-modified PEI treatment as compared to the siRNA/PEI. This was accompanied with significant increase in IL-6 levels and Caspase 3 activity, and a significant decrease in VEGF level and STAT3 activity in the tumor tissue.

A different approach for PEI design was carried out by Oskuee[5] *et al*. The authors formed dendrimers with polycationic PEI cores surrounded by hydrophobic alkyl

shells of various lengths, each surrounded by an anionic outer shell of carboxylated moieties at the physiological pH. This group found that increasing the hydrophobic chain with alkylcarboxylate to 6 and 10 carbons and substituting less than 20% of the amines on 10 kDa PEI formed positive charge complexes with particle size ranging from 130 to 190 nm. Moreover, these carriers substantially increased transfection in Neuro2A mammalian cells up to 5-fold higher as compared to native 10 kDa PEI, which was comparable to 25 kDa PEI, with the greatest increases occurring with C6-modified PEI derivatives. Additionally, the alkyl chain length and density were shown to have no significant effect on toxicity. Such an increase in transfection efficiency and reduction in toxicity was attributed to the formation of an optimal hydrophobic shell and outer anionic shell and that decreasing or increasing the length and density of the alkyl chain, was going to cause a loss of dendrimeric architecture, resulting in negligible transfection activity as seen with C2- and C16-derivatives. In a later publication, the same group[6] found that siRNA delivery with high 25 kDa PEI modified with the carboxylates listed above was increased compared to the native PEI, and these results were independent of the hydrophobic chain length. Instead, the delivery properties and cytotoxicity of the modified polymer was dependent of the degree of substitution. The authors proposed that a certain reduction in cationic charge density led to reduced toxicity without compromising complex stability. Thus, higher concentrations of modified carrier were applicable for siRNA delivery without toxic side effects, enhancing endosomal escape and avoiding endolysosomal degradation.

Dehshahri[7] *et al*. improved the gene delivery properties of 10 kDa PEI by alkylating the primary amines with variable alkylcarboxylated chains of 2, 6, 10 and 16 carbons, followed by coupling to various oligoamines (ethylendiamine, diethylentriamine, spermidine and spermine) through amide linkages. The modified polymers bound to DNA and formed particles size in the range of 56-97 nm with a net positive charge allowing the complexes to enter the cell through non-chlathrin mediated endocytosis[8]. These carriers yielded optimal transfection

efficiencies which were much greater than those of native 10 kDa PEI, and comparable to the transfection efficiency of 25 kDa PEI with Neuro2A murine neuroblastoma cells. Moreover, the optimal alkyl chain length was found to be six carbons; however, a shorter alkyl chain (C2) at higher degrees of substitution (29% of primary amines) resulted in transfection efficiencies approaching those of C6 alkylation at lower degrees of substitution (9% and 17% of primary amines). The most effective oligoamine was found to be diethylentriamine, which gave the highest buffering capacity in the endosomal pH range and reduced cytotoxicity. The most probable reasons for the improved transfection efficiency were a more favorable hydrophobic–hydrophilic balance and greater buffering capacity in the endosomal pH range.

It is important to mention that the work describe above was only intended to update the most recent literature on the polymers used in this thesis, and it only focused on the insertion of hydrophobic moieties into cationic carriers and its influence on gene delivery performance. Formulations that involved polymer modification with other agents were not discussed.

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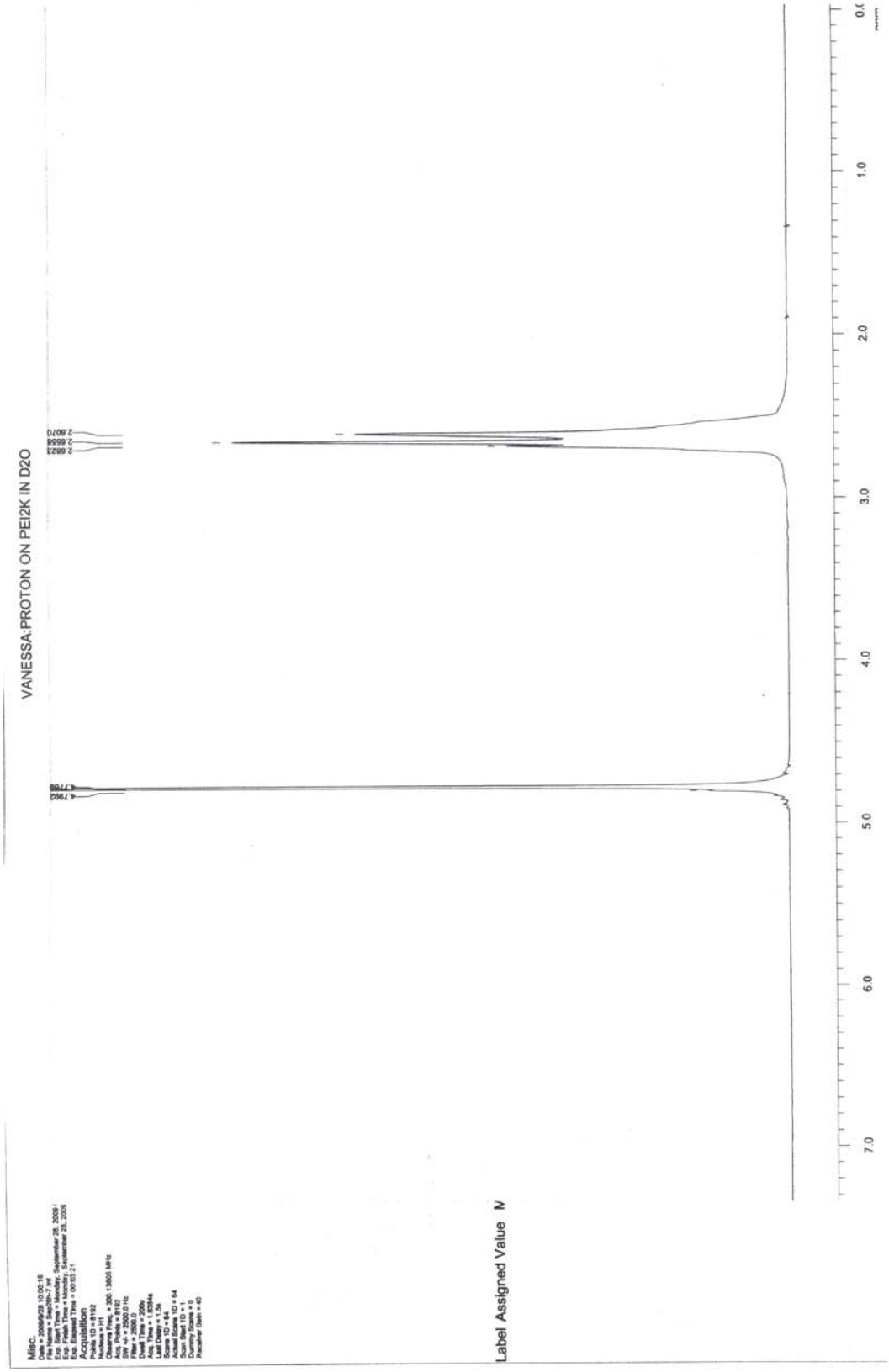
# Appendix B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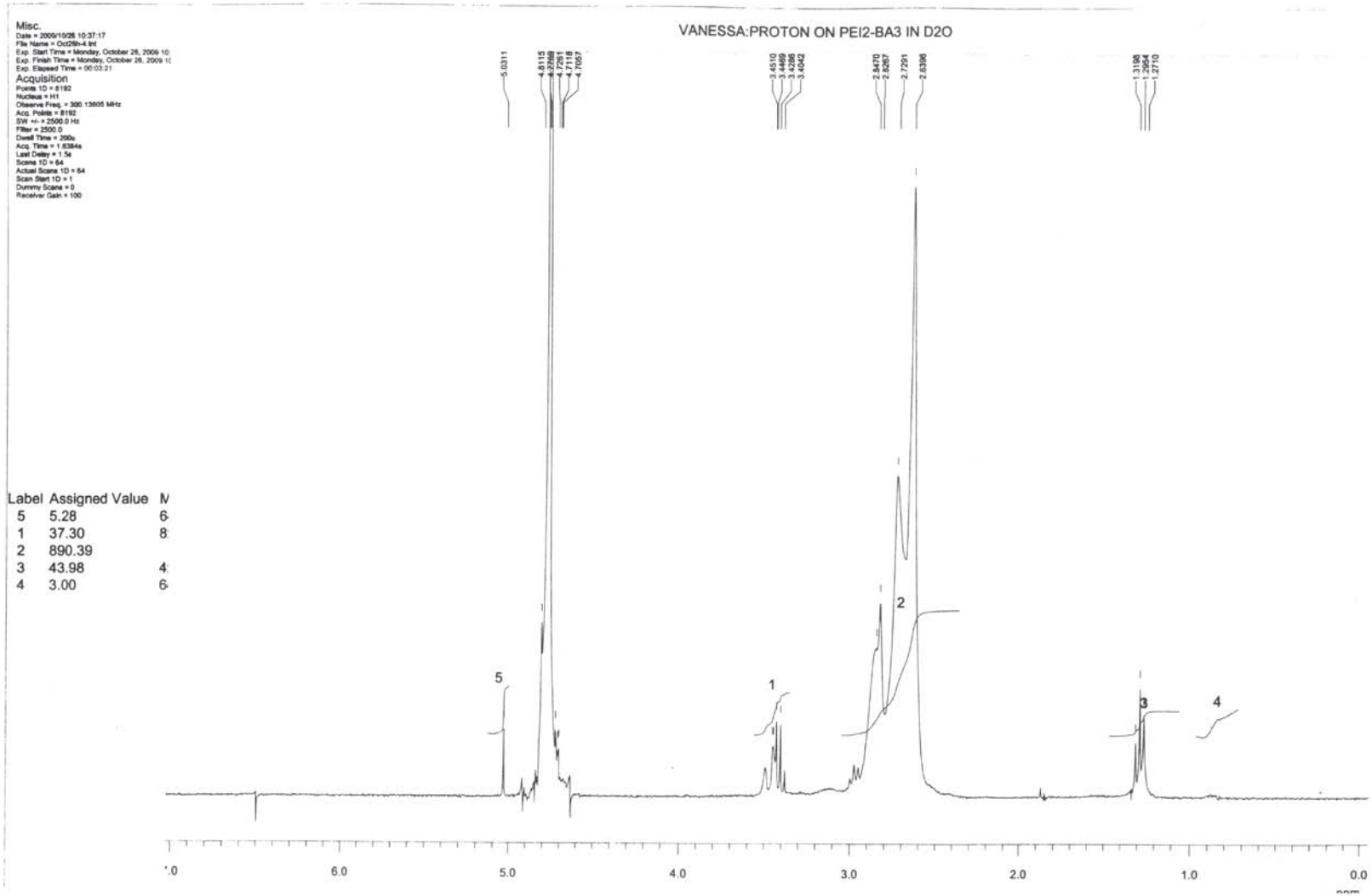
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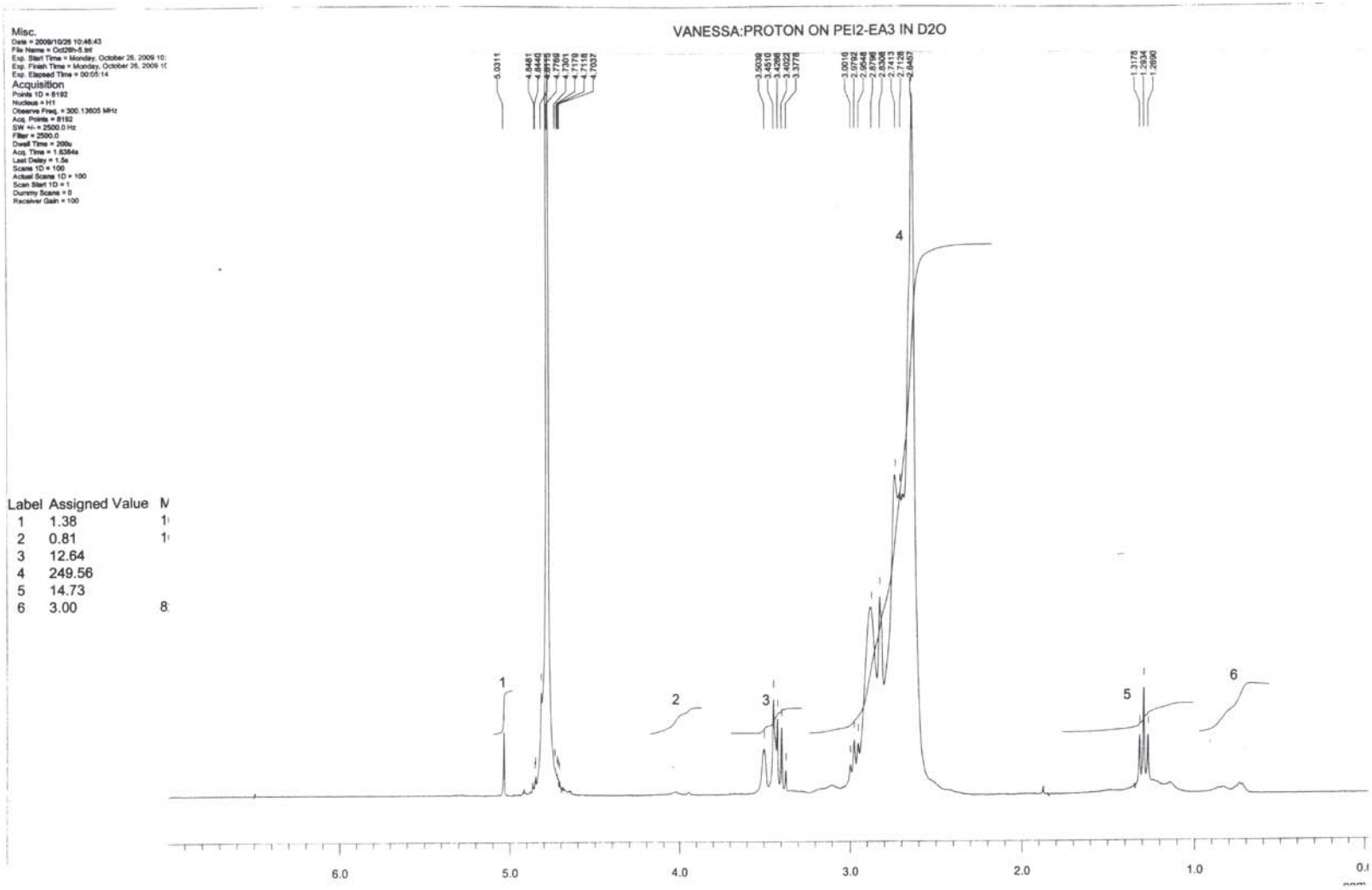
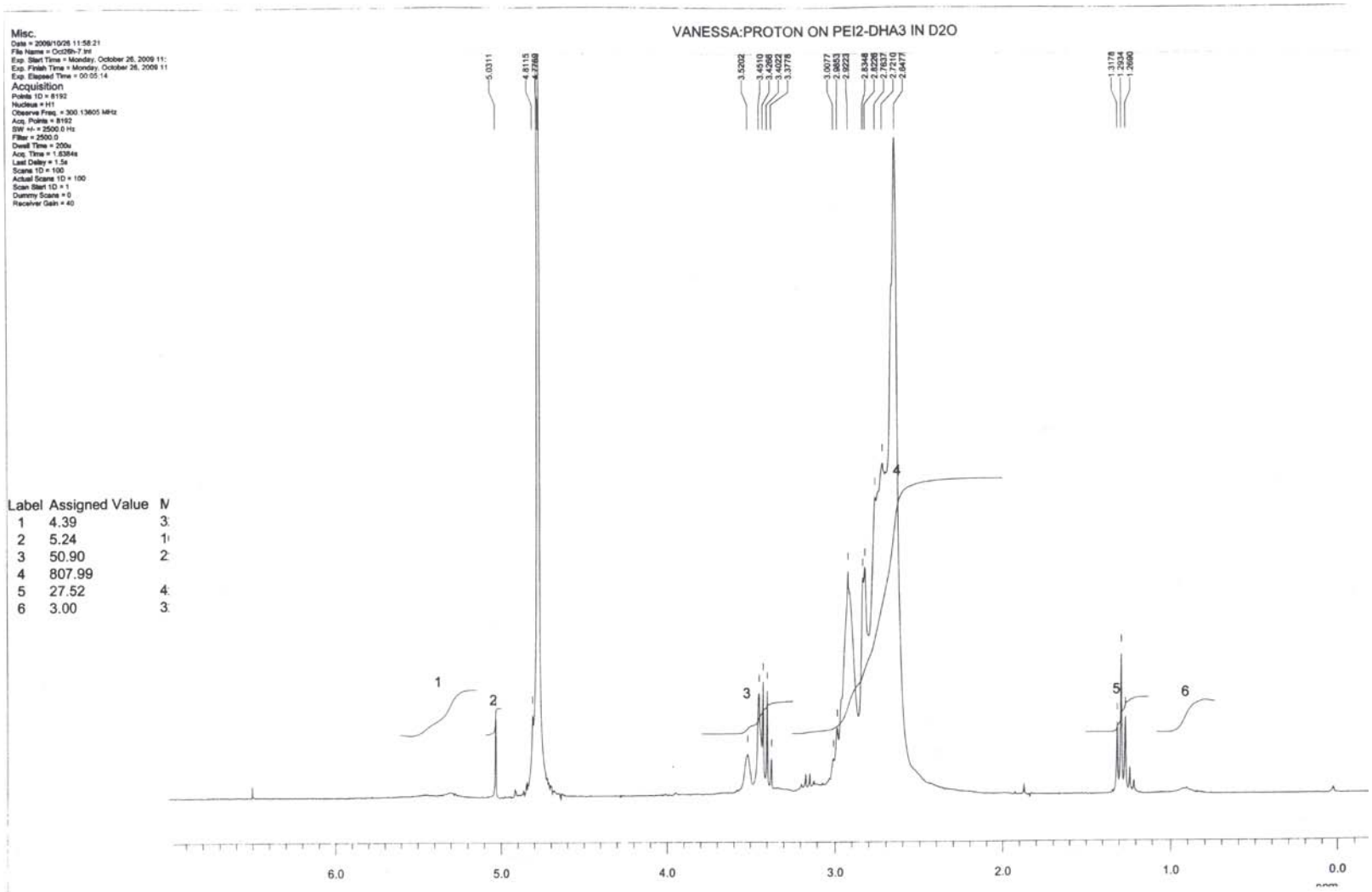
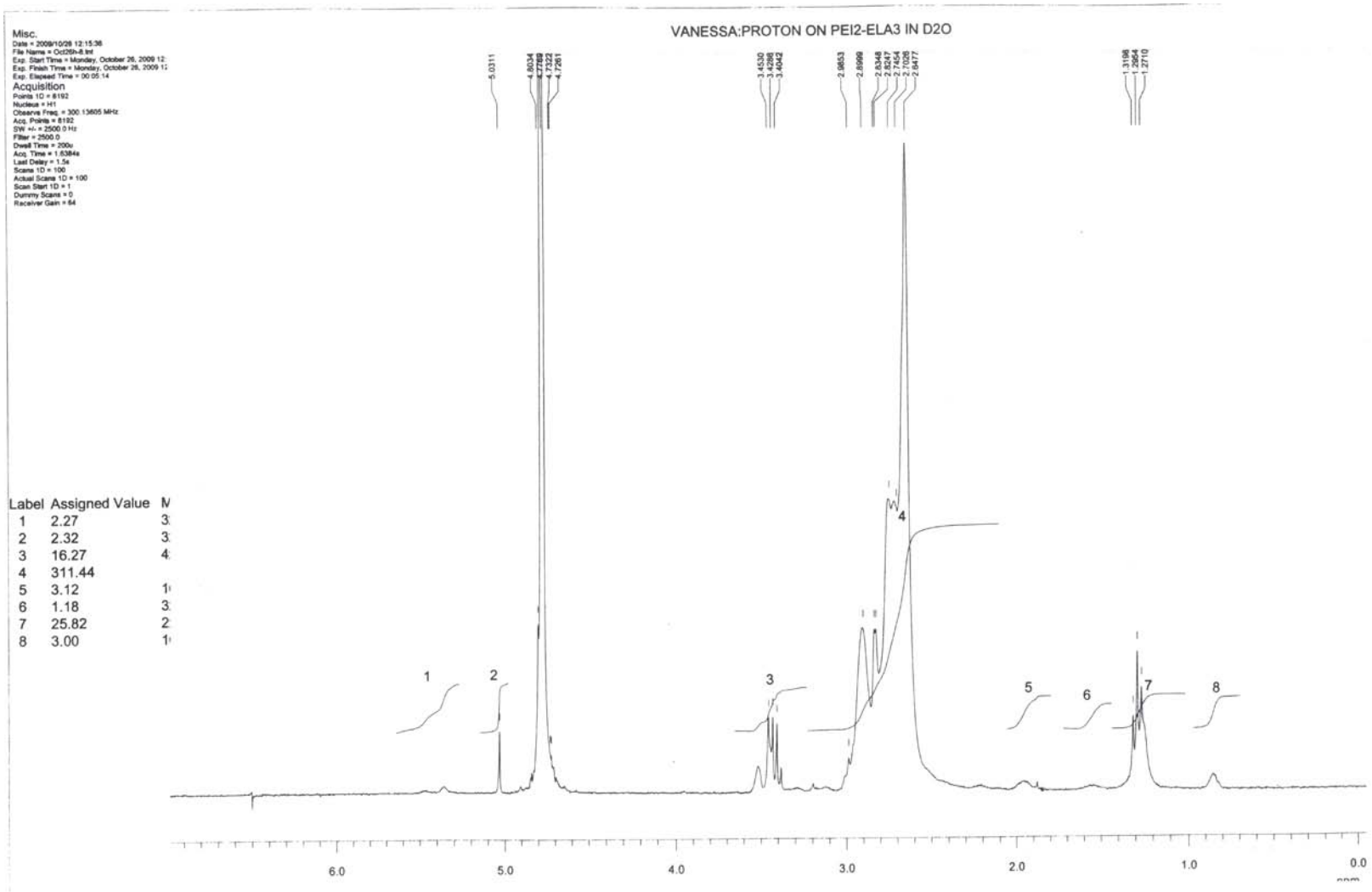

VANESSA:PROTON ON PEI2-EA3 IN D2O
Misc.
Acquisition
Label Assigned Value
1 1.38
2 0.81
3 12.64
4 249.56
5 14.73
6 3.00
1
2
3
4
5
6
6.0
5.0
4.0
3.0
2.0
1.0

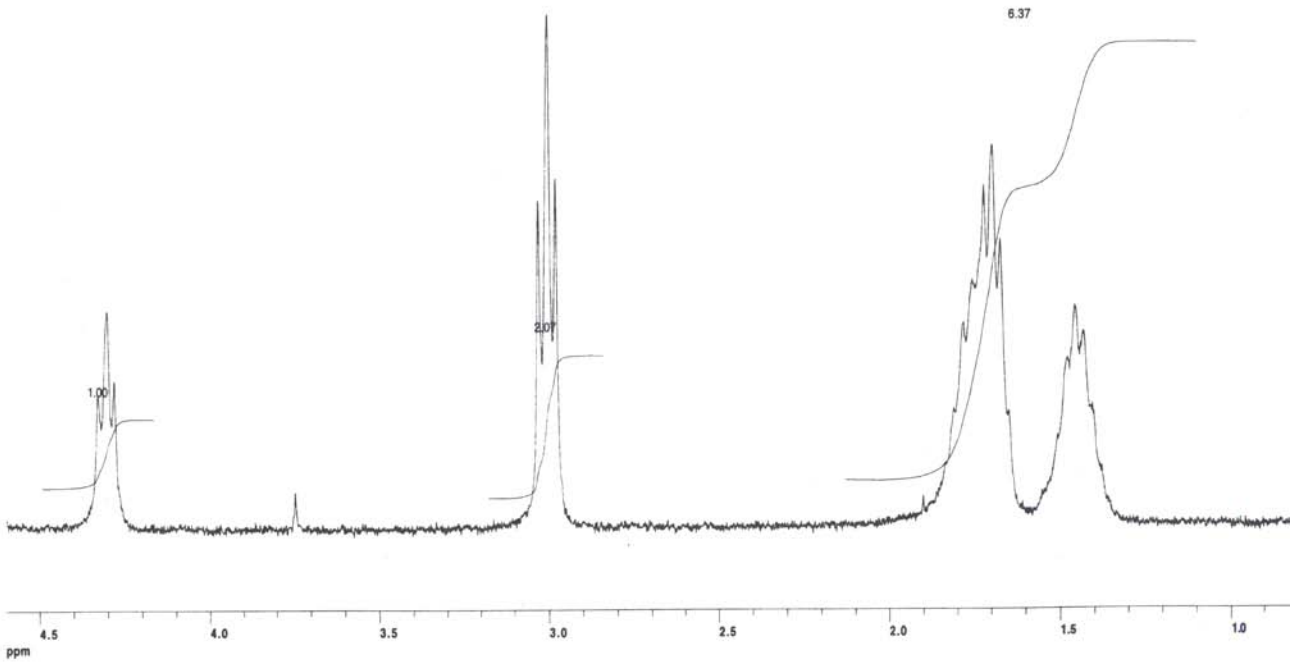

VANESSA:PROTON ON PEI2-DHA3 IN D2O
Misc.
Data = 2009/10/26 11:58:21
File Name = Oct26h-7.fid
Exp. Start Time = Monday, October 26, 2009 11:
Exp. Finish Time = Monday, October 26, 2009 11
Exp. Elapsed Time = 00:05:14
Acquisition
Points 1D = 8192
Nucleus = H1
Observe Freq. = 300.13605 MHz
Acq. Points = 8192
SW +/- = 2500.0 Hz
Filter = 2500.0
Dwell Time = 200u
Acq. Time = 1.6384s
Last Delay = 1.5s
Scans 1D = 100
Actual Scans 1D = 100
Scan Start 1D = 1
Dummy Scans = 0
Receiver Gain = 40
5.0311
4.6115
4.2769
3.5202
3.4610
3.4266
3.4022
3.3776
3.0077
2.8853
2.8223
2.8348
2.8226
2.7637
2.7210
2.6477
1.3178
1.2934
1.2690
Label Assigned Value
1 4.39
2 5.24
3 50.90
4 807.99
5 27.52
6 3.00
1
2
3
4
5
6
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ppm

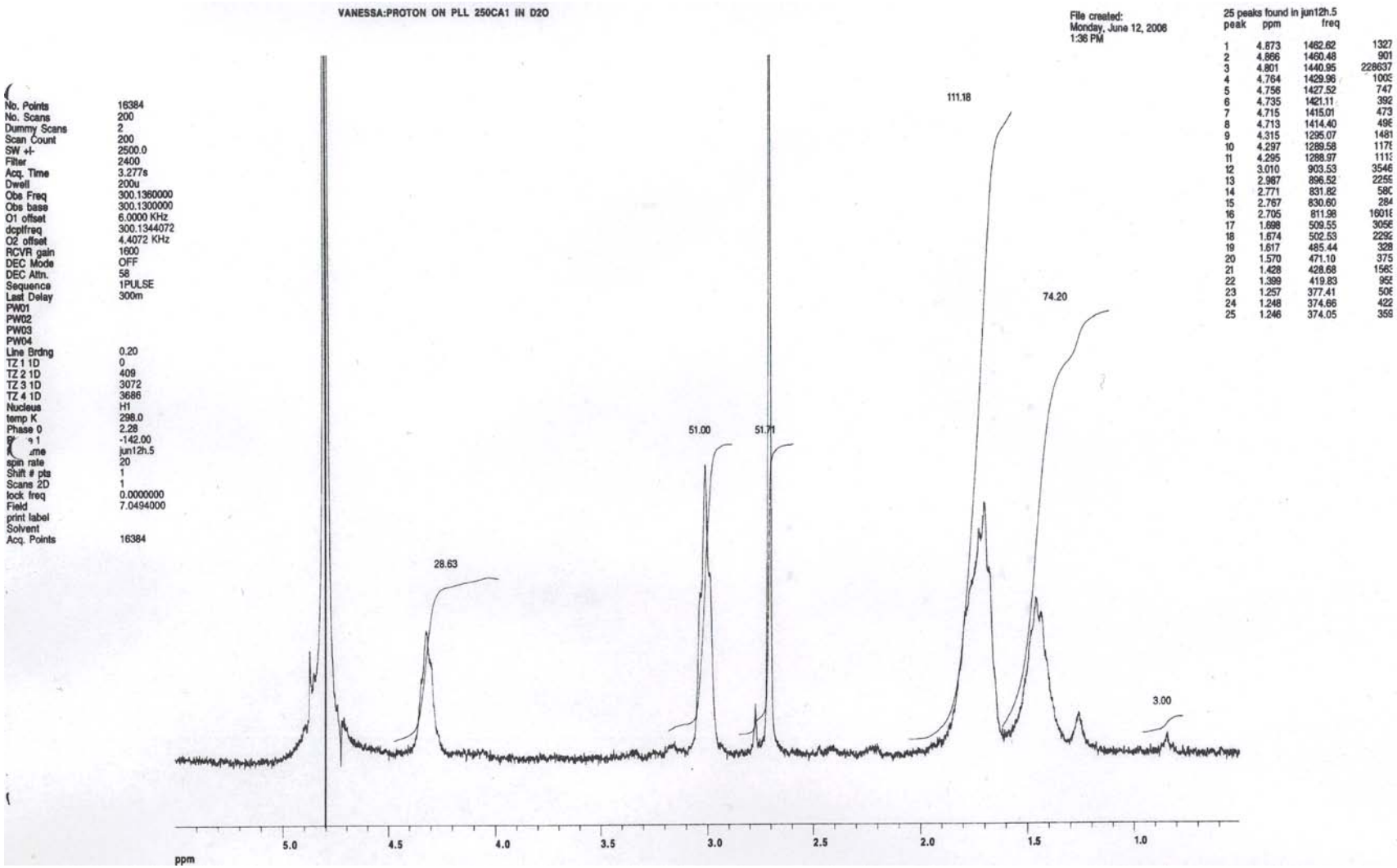

VANESSA:PROTON ON PEI2-ELA3 IN D2O
Misc.
Acquisition
Label Assigned Value
1 2.27
2 2.32
3 16.27
4 311.44
5 3.12
6 1.18
7 25.82
8 3.00
1
2
3
4
5
6
7
8
6.0
5.0
4.0
3.0
2.0
1.0
0.0

VENESSA:PROTON ON PLL IN D2O

File created:
Thursday, May 26, 2005
2:42 PM

| | |
|---|---|
| Points | 16384 |
| No. Scans | 400 |
| Dummy Scans | 2 |
| Scan Count | 140 |
| SW +/- | 2500.0 |
| Filter | 2400 |
| Acq. Time | 3.277s |
| Dwell | 200u |
| Obs Freq | 300.1360000 |
| Obs base | 300.1300000 |
| O1 offset | 6.0000 KHz |
| dcplfreq | 300.1344072 |
| O2 offset | 4.4072 KHz |
| RCVR gain | 800 |
| DEC Mode | OFF |
| DEC Attn. | 58 |
| Sequence | 1PULSE |
| Last Delay | 300m |
| PW01 | |
| PW02 | |
| PW03 | |
| PW04 | |
| Line Brdng | 0.20 |
| TZ 1 1D | 0 |
| TZ 2 1D | 409 |
| TZ 3 1D | 3072 |
| TZ 4 1D | 3686 |
| Nucleus | H1 |
| temp K | 0.0 |
| Phase 0 | 6.56 |
| Phase 1 | -142.00 |
| Name | may26h.9 |
| Spin rate | 20 |
| Shift # pts | 1 |
| Scans 2D | 1 |
| lock freq | 0.0000000 |
| Field | 7.0494000 |
| print label | |
| Solvent | |
| Acq. Points | 16384 |




VANESSA:PROTON ON PLL 250CA1 IN D2O
File created:
Monday, June 12, 2006
1:36 PM
No. Points 16384
No. Scans 200
Dummy Scans 2
Scan Count 200
SW +/- 2500.0
Filter 2400
Acq. Time 3.277s
Dwell 200u
Obs Freq 300.1360000
Obs base 300.1300000
O1 offset 6.0000 KHz
dcplfreq 300.1344072
O2 offset 4.4072 KHz
RCVR gain 1600
DEC Mode OFF
DEC Attn. 58
Sequence 1PULSE
Last Delay 300m
PW01
PW02
PW03
PW04
Line Brdng 0.20
TZ 1 1D 0
TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
Nucleus H1
temp K 298.0
Phase 0 2.28
P 1 -142.00
me jun12h.5
spin rate 20
Shift # pts 1
Scans 2D 1
lock freq 0.0000000
Field 7.0494000
print label
Solvent
Acq. Points 16384
25 peaks found in jun12h.5
peak ppm freq
1 4.873 1462.62 1327
2 4.866 1460.48 901
3 4.801 1440.95 228637
4 4.764 1429.96 100
5 4.756 1427.52 747
6 4.735 1421.11 392
7 4.715 1415.01 473
8 4.713 1414.40 49
9 4.315 1295.07 1481
10 4.297 1289.58 117
11 4.295 1288.97 111
12 3.010 903.53 3546
13 2.987 896.52 225
14 2.771 831.82 58
15 2.767 830.60 284
16 2.705 811.98 16011
17 1.698 509.55 3056
18 1.674 502.53 229
19 1.617 485.44 328
20 1.570 471.10 375
21 1.428 428.68 156
22 1.399 419.83 95
23 1.257 377.41 50
24 1.248 374.66 422
25 1.246 374.05 359
111.18
74.20
51.00
51.71
28.63
3.00
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm

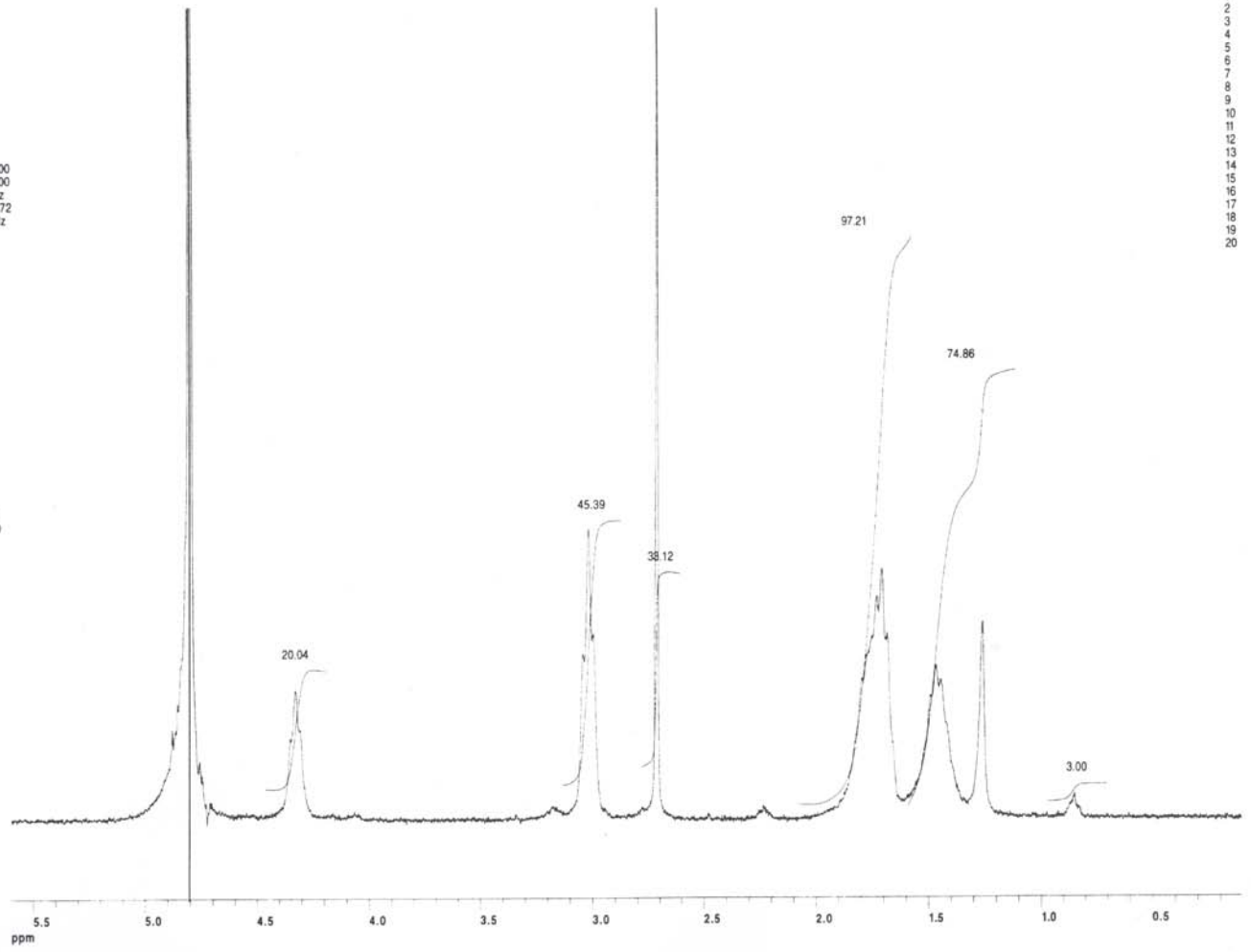
VANESSA:PROTON ON PLL250MA1 IN D2O

File created:
Monday, June 19, 2006
2:36 PM

| | |
|---|---|
| No. Points | 16384 |
| No. Scans | 200 |
| Dummy Scans | 2 |
| Scan Count | 100 |
| SW +/- | 2500.0 |
| Filter | 2400 |
| Acq. Time | 3.277s |
| Dwell | 200u |
| Obs Freq | 300.1360000 |
| Obs base | 300.1300000 |
| O1 offset | 6.0000 KHz |
| dcplfreq | 300.1344072 |
| O2 offset | 4.4072 KHz |
| RCVR gain | 400 |
| DEC Mode | OFF |
| DEC Attn. | 58 |
| Sequence | 1PULSE |
| Last Delay | 300m |
| PW01 | |
| PW02 | |
| PW03 | |
| PW04 | |
| Line Brdng | 0.20 |
| TZ 1 1D | 0 |
| TZ 2 1D | 409 |
| TZ 3 1D | 3072 |
| TZ 4 1D | 3686 |
| Nucleus | H1 |
| temp K | 298.0 |
| Phase 0 | -23.50 |
| Phase 1 | -152.00 |
| Name | jun19h.8 |
| spin rate | 20 |
| Shift # pts | 1 |
| Scans 2D | 1 |
| lock freq | 0.0000000 |
| Field | 7.0494000 |
| print label | |
| Solvent | |
| Acq. Points | 16384 |

20 peaks found in jun19h.8

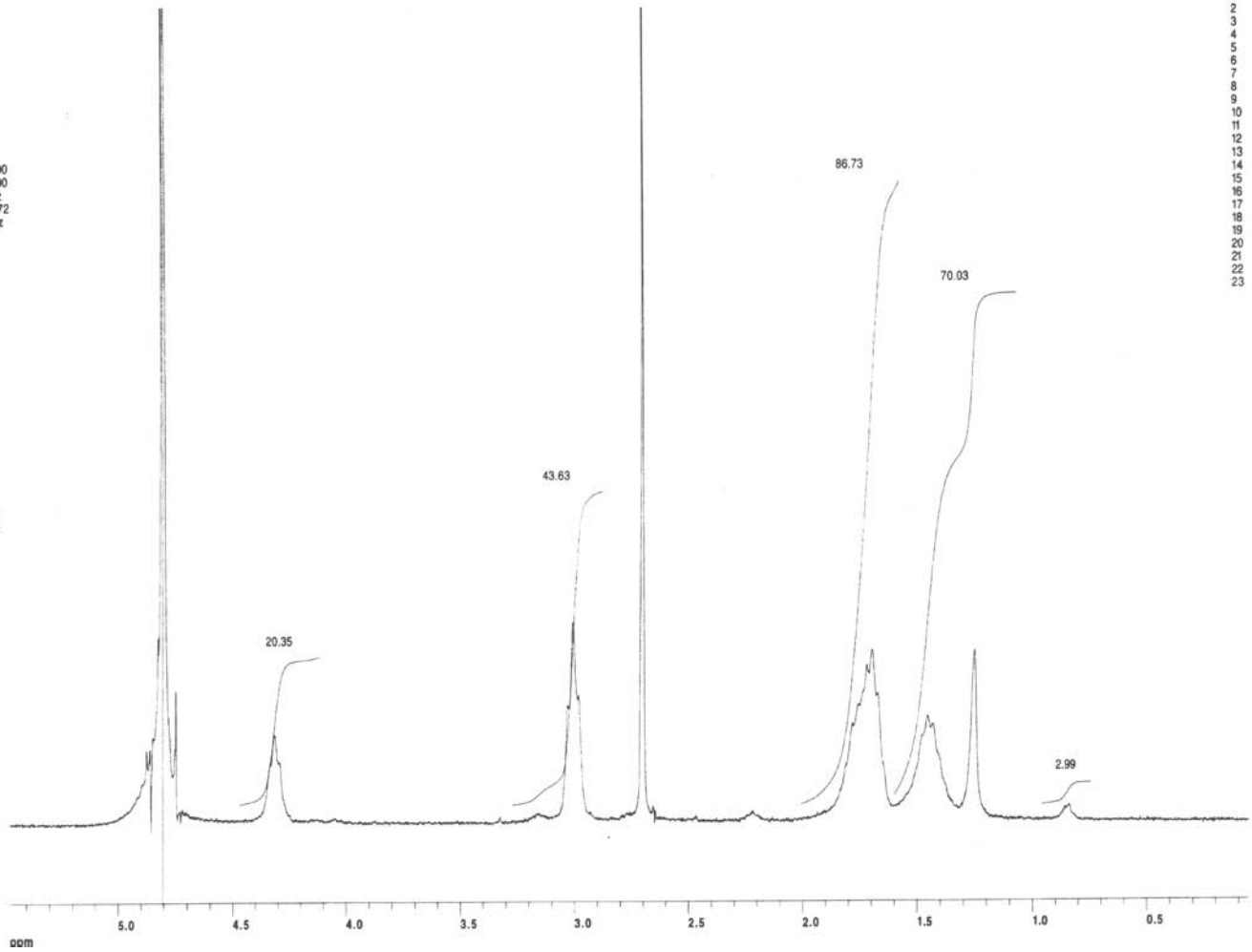
| peak | ppm | freq | |
|---|---|---|---|
| 1 | 4.879 | 1464.45 | 4508 |
| 2 | 4.855 | 1457.12 | 5696 |
| 3 | 4.802 | 1441.25 | 1116150 |
| 4 | 4.757 | 1427.83 | 2933 |
| 5 | 4.753 | 1426.61 | 2270 |
| 6 | 4.747 | 1424.77 | 2226 |
| 7 | 4.321 | 1296.91 | 5895 |
| 8 | 4.303 | 1291.41 | 4276 |
| 9 | 3.019 | 905.98 | 14617 |
| 10 | 2.994 | 898.65 | 9036 |
| 11 | 2.711 | 813.81 | 49402 |
| 12 | 1.727 | 518.40 | 10840 |
| 13 | 1.706 | 511.99 | 12494 |
| 14 | 1.680 | 504.36 | 9015 |
| 15 | 1.657 | 497.35 | 3772 |
| 16 | 1.465 | 439.67 | 7665 |
| 17 | 1.437 | 431.43 | 6595 |
| 18 | 1.410 | 423.19 | 4118 |
| 19 | 1.404 | 421.36 | 3387 |
| 20 | 1.258 | 377.72 | 9612 |



VANESSA:PROTON ON PLL250PA1 IN D2O

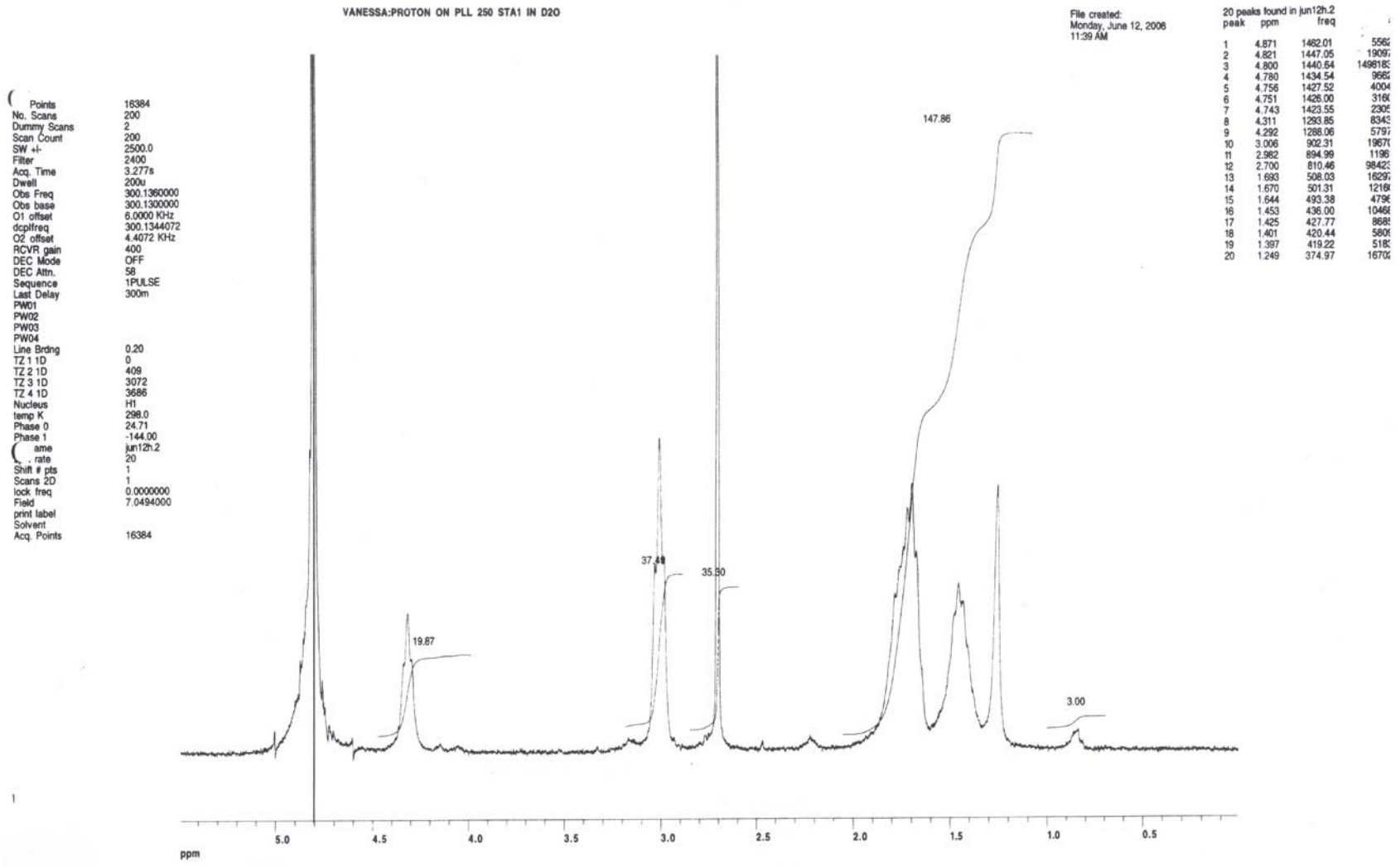
File created:
Tuesday, June 13, 2006
1:49 PM

| | |
|---|---|
| No. Points | 16384 |
| No. Scans | 200 |
| Dummy Scans | 2 |
| Scan Count | 200 |
| SW +/- | 2500.0 |
| Filter | 2400 |
| Acq. Time | 3.277s |
| Dwell | 200u |
| Obs Freq | 300.1360000 |
| Obs base | 300.1300000 |
| O1 offset | 6.0000 KHz |
| dcplfreq | 300.1344072 |
| O2 offset | 4.4072 KHz |
| RCVR gain | 640 |
| DEC Mode | OFF |
| DEC Attn. | 58 |
| Sequence | 1PULSE |
| Last Delay | 300m |
| PW01 | |
| PW02 | |
| PW03 | |
| PW04 | |
| Line Brdng | 0.20 |
| TZ 1 1D | 0 |
| TZ 2 1D | 409 |
| TZ 3 1D | 3072 |
| TZ 4 1D | 3686 |
| Nucleus | H1 |
| temp K | 298.0 |
| Phase 0 | 45.03 |
| Phase 1 | -155.00 |
| Name | jun13h.6 |
| spin rate | 20 |
| Shift # pts | 1 |
| Scans 2D | 1 |
| lock freq | 0.0000000 |
| Field | 7.0494000 |
| print label | |
| Solvent | |
| Acq. Points | 16384 |




23 peaks found in jun13h.6

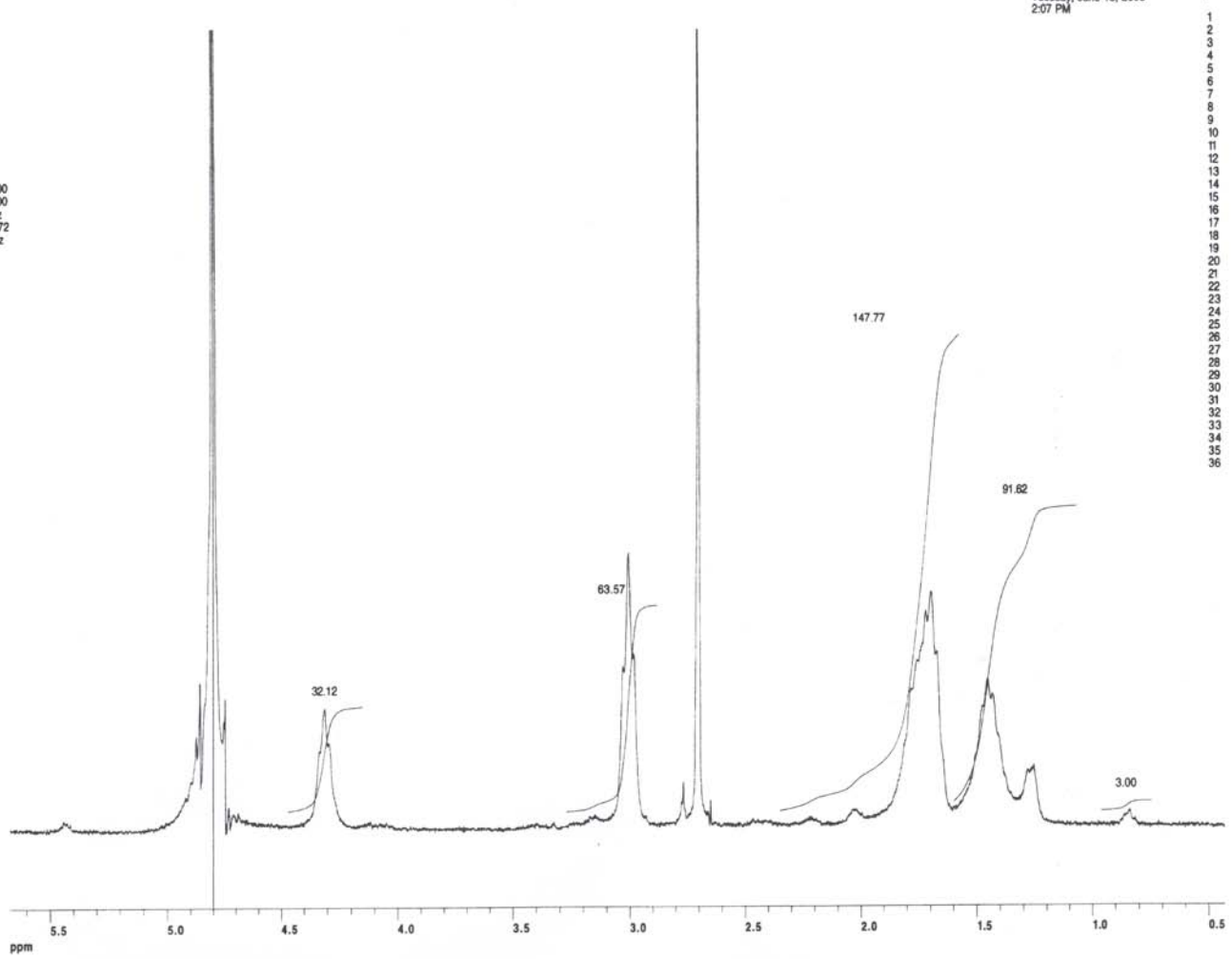
| peak | ppm | freq | |
|---|---|---|---|
| 1 | 4.873 | 1462.62 | 108 |
| 2 | 4.858 | 1458.04 | 113 |
| 3 | 4.846 | 1454.38 | 131 |
| 4 | 4.824 | 1447.97 | 2824 |
| 5 | 4.801 | 1440.95 | 275535 |
| 6 | 4.778 | 1433.93 | 159 |
| 7 | 4.747 | 1424.77 | 201 |
| 8 | 4.309 | 1293.24 | 129 |
| 9 | 4.288 | 1286.84 | 85 |
| 10 | 4.272 | 1282.26 | 34 |
| 11 | 3.003 | 901.40 | 298 |
| 12 | 2.980 | 894.38 | 187 |
| 13 | 2.698 | 809.85 | 1553 |
| 14 | 1.716 | 515.05 | 2364 |
| 15 | 1.692 | 507.72 | 2574 |
| 16 | 1.668 | 500.70 | 193 |
| 17 | 1.627 | 488.19 | 37 |
| 18 | 1.450 | 435.09 | 159 |
| 19 | 1.422 | 426.85 | 133 |
| 20 | 1.372 | 411.90 | 53 |
| 21 | 1.368 | 410.68 | 46 |
| 22 | 1.354 | 406.40 | 351 |
| 23 | 1.247 | 374.36 | 2597 |


VANESSA:PROTON ON PLL 250 STA1 IN D2O
File created:
Monday, June 12, 2006
11:39 AM
20 peaks found in jun12h.2
peak ppm freq
1 4.871 1462.01
2 4.821 1447.05
3 4.800 1440.64
4 4.780 1434.54
5 4.756 1427.52
6 4.751 1426.00
7 4.743 1423.55
8 4.311 1293.85
9 4.292 1288.06
10 3.006 902.31
11 2.982 894.99
12 2.700 810.46
13 1.693 508.03
14 1.670 501.31
15 1.644 493.38
16 1.453 436.00
17 1.425 427.77
18 1.401 420.44
19 1.397 419.22
20 1.249 374.97
Points 16384
No. Scans 200
Dummy Scans 2
Scan Count 200
SW +/- 2500.0
Filter 2400
Acq. Time 3.277s
Dwell 200u
Obs Freq 300.1360000
Obs base 300.1300000
O1 offset 6.0000 KHz
dcplfreq 300.1344072
O2 offset 4.4072 KHz
RCVR gain 400
DEC Mode OFF
DEC Attn. 58
Sequence 1PULSE
Last Delay 300m
PW01
PW02
PW03
PW04
Line Brdng 0.20
TZ 1 1D 0
TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
Nucleus H1
temp K 298.0
Phase 0 24.71
Phase 1 -144.00
ame jun12h.2
. rate 20
Shift # pts 1
Scans 2D 1
lock freq 0.0000000
Field 7.0494000
print label
Solvent
Acq. Points 16384
147.86
37.48
35.50
19.87
3.00
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ppm

VANESSA:PROTON ON PLL2500A1 IN D2O

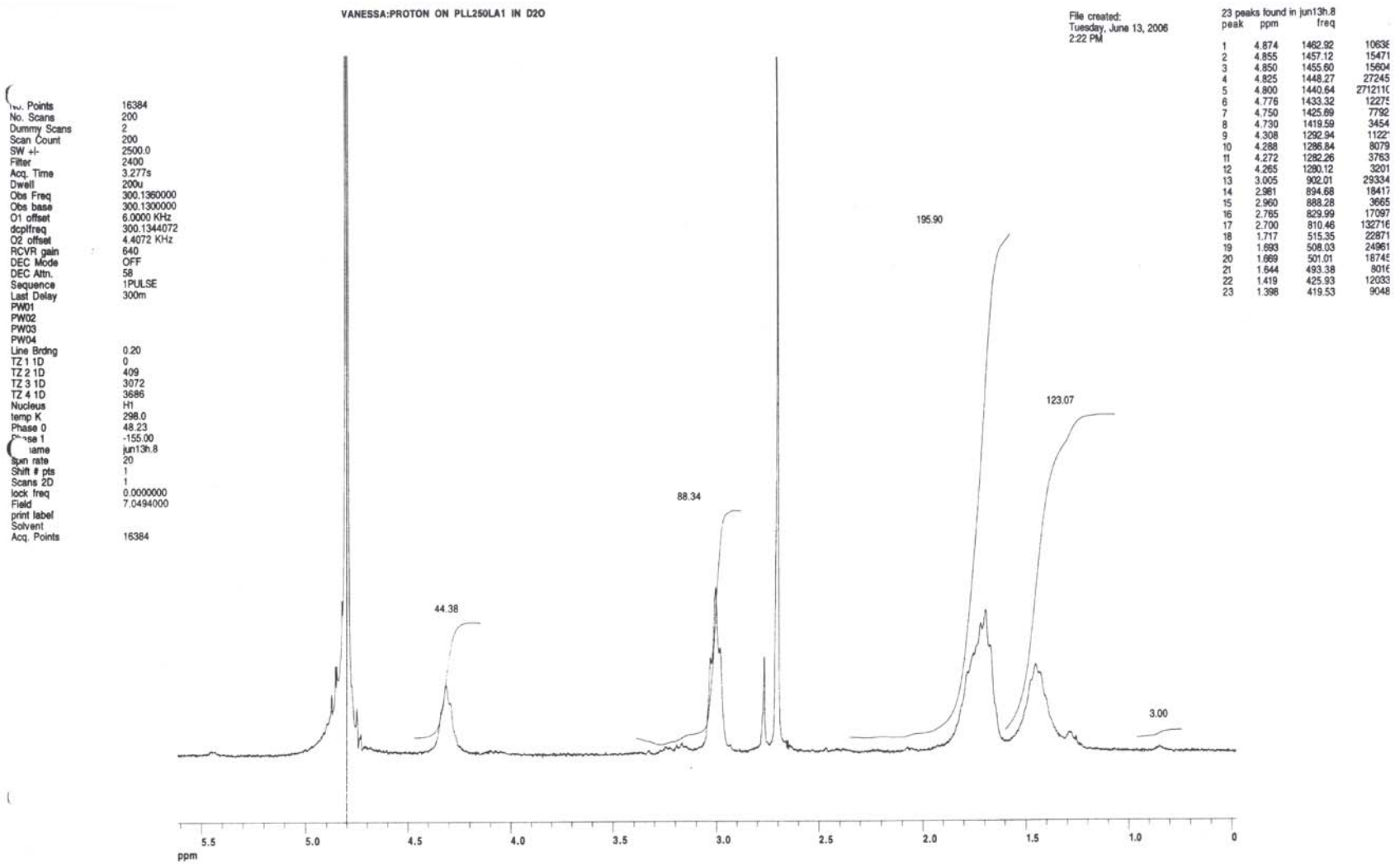
File created:
Tuesday, June 13, 2006
2:07 PM

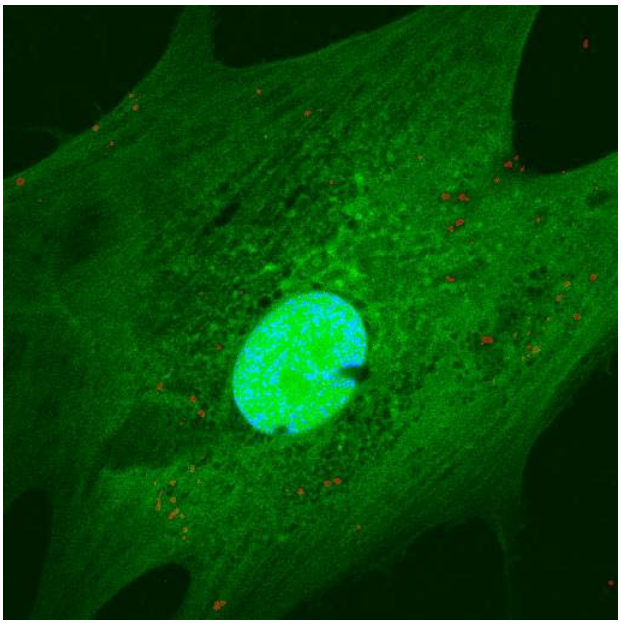
| | |
|---|---|
| ... Points | 16384 |
| No. Scans | 200 |
| Dummy Scans | 2 |
| Scan Count | 200 |
| SW +/- | 2500.0 |
| Filter | 2400 |
| Acq. Time | 3.277s |
| Dwell | 200u |
| Obs Freq | 300.1360000 |
| Obs base | 300.1300000 |
| O1 offset | 6.0000 KHz |
| dcplfreq | 300.1344072 |
| O2 offset | 4.4072 KHz |
| RCVR gain | 800 |
| DEC Mode | OFF |
| DEC Attn. | 58 |
| Sequence | 1PULSE |
| Last Delay | 300m |
| PW01 | |
| PW02 | |
| PW03 | |
| PW04 | |
| Line Brdng | 0.20 |
| TZ 1 1D | 0 |
| TZ 2 1D | 409 |
| TZ 3 1D | 3072 |
| TZ 4 1D | 3686 |
| Nucleus | H1 |
| temp K | 298.0 |
| Phase 0 | 13.81 |
| Phase 1 | -139.00 |
| ...ame | jun13h.7 |
| ...n rate | 20 |
| Shift # pts | 1 |
| Scans 2D | 1 |
| lock freq | 0.0000000 |
| Field | 7.0494000 |
| print label | |
| Solvent | |
| Acq. Points | 16384 |




36 peaks found in jun13h.7

| peak | ppm | freq | |
|---|---|---|---|
| 1 | 4.921 | 1476.96 | 515 |
| 2 | 4.874 | 1462.92 | 1453 |
| 3 | 4.857 | 1457.73 | 2274 |
| 4 | 4.801 | 1440.95 | 411674 |
| 5 | 4.769 | 1431.49 | 1580 |
| 6 | 4.752 | 1426.30 | 1675 |
| 7 | 4.747 | 1424.77 | 2030 |
| 8 | 4.731 | 1419.89 | 359 |
| 9 | 4.311 | 1293.85 | 1855 |
| 10 | 4.303 | 1291.41 | 1430 |
| 11 | 4.289 | 1287.14 | 1232 |
| 12 | 4.268 | 1281.04 | 472 |
| 13 | 4.260 | 1278.60 | 326 |
| 14 | 3.005 | 902.01 | 4272 |
| 15 | 2.980 | 894.38 | 2638 |
| 16 | 2.953 | 886.44 | 378 |
| 17 | 2.765 | 829.99 | 715 |
| 18 | 2.699 | 810.15 | 19444 |
| 19 | 2.648 | 794.89 | 432 |
| 20 | 1.718 | 515.66 | 3371 |
| 21 | 1.714 | 514.44 | 3262 |
| 22 | 1.692 | 507.72 | 3603 |
| 23 | 1.669 | 501.01 | 2727 |
| 24 | 1.642 | 492.77 | 1043 |
| 25 | 1.449 | 434.78 | 2232 |
| 26 | 1.425 | 427.77 | 1971 |
| 27 | 1.409 | 422.88 | 1467 |
| 28 | 1.402 | 420.75 | 1345 |
| 29 | 1.367 | 410.37 | 647 |
| 30 | 1.330 | 399.08 | 456 |
| 31 | 1.286 | 385.96 | 853 |
| 32 | 1.276 | 382.90 | 872 |
| 33 | 1.266 | 379.85 | 905 |
| 34 | 1.249 | 374.97 | 803 |
| 35 | 1.245 | 373.75 | 705 |
| 36 | 1.234 | 370.39 | 390 |


VANESSA:PROTON ON PLL250LA1 IN D2O
File created:
Tuesday, June 13, 2006
2:22 PM
No. Points 16384
No. Scans 200
Dummy Scans 2
Scan Count 200
SW +/- 2500.0
Filter 2400
Acq. Time 3.277s
Dwell 200u
Obs Freq 300.1360000
Obs base 300.1300000
O1 offset 6.0000 KHz
dcplfreq 300.1344072
O2 offset 4.4072 KHz
RCVR gain 640
DEC Mode OFF
DEC Attn. 58
Sequence 1PULSE
Last Delay 300m
PW01
PW02
PW03
PW04
Line Brdng 0.20
TZ 1 1D 0
TZ 2 1D 409
TZ 3 1D 3072
TZ 4 1D 3686
Nucleus H1
temp K 298.0
Phase 0 48.23
Phase 1 -155.00
Name jun13h.8
Spin rate 20
Shift # pts 1
Scans 2D 1
lock freq 0.0000000
Field 7.0494000
print label
Solvent
Acq. Points 16384
23 peaks found in jun13h.8
peak ppm freq
1 4.874 1462.92 10636
2 4.855 1457.12 15471
3 4.850 1455.60 15604
4 4.825 1448.27 27245
5 4.800 1440.64 271211
6 4.776 1433.32 12275
7 4.750 1425.69 7792
8 4.730 1419.59 3454
9 4.308 1292.94 1122
10 4.288 1286.84 8079
11 4.272 1282.26 3763
12 4.265 1280.12 3201
13 3.005 902.01 29334
14 2.981 894.68 18417
15 2.960 888.28 3665
16 2.765 829.99 17097
17 2.700 810.46 132716
18 1.717 515.35 22871
19 1.693 508.03 24961
20 1.669 501.01 18745
21 1.644 493.38 801
22 1.419 425.93 12033
23 1.398 419.53 9048
44.38
88.34
195.90
123.07
3.00
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
ppm



Confocal Image of h-BMSC treated with DNA complexes of PEI25 (DNA/polymer ratio of 1:5 (μg: μg)). The cells were analysed 24 hrs post-treatment. Complex internalization was observed with Cy5-labeled gWIZ-GFP (red), nuclei were stained with Hoechst (blue) and GFP expression was observed as green